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AND MULTIDRUG
RESISTANCE
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PREFACE

The concept of multidrug resistance (MDR) in tumors originated from the pioneer work of June Biedler in the early 1970s, who identified a wide profile of cross-resistance in Chinese hamster cells selected for resistance to actinomycin D. At that time, it was not predictable that 40 years later, almost 36,000 publications would be retrieved from PubMed when searching the simple term of MDR! Also remarkable, it should be emphasized that the concept of ABC proteins emerged from studies on MDR: P-glycoprotein, identified and named by Victor Ling in 1976, is only the first of a wide variety of membrane transporters involved in many cellular functions and properties.

MDR is defined as the ability of a living cell to display resistance to a wide spectrum of drugs that are not structurally or functionally related. A huge amount of work has been dedicated to the development of MDR in cancer cells, which represents a major hurdle in the treatment of cancer by chemotherapy. However, MDR should not be restricted to cancer cells and anticancer drugs; it also occurs in many other situations, such as the resistance of bacteria, fungus, and parasites, to antibiotics as well as antifungal and antiparasitic compounds.

This book aims to gather the present knowledge on the involvement of ABC transporters in drug transport and resistance. As mentioned above, this topic has been of great interest in the past decades, when P-glycoprotein was the only identified multidrug transporter and when its responsibility in MDR of cancer cells was established. In the post genomic era, a total of 48 different ABC transporters have been identified in humans, and a much larger number have been identified in bacteria and parasites. An increasing number of compounds transported by ABC proteins have been recognized, and no recent comprehensive review about the role of ABC transporters in drug resistance has been published yet.
An interesting characteristic of ABC drug transporters is that they are “druggable,” not only at the level of the cancer cell, but also at the level of their physiological localizations: the intestinal epithelial cell, the renal tubular cell, the hepatocyte, and the endothelial cell of intracerebral blood vessels. MDR reversal has been the subject of many investigations aimed at developing innovative therapeutic strategies against cancer and infectious diseases. More recently, the blockade of physiological barriers has also become a goal for the development of inhibitors of ABC transporters.

As a consequence, an expanding number of original works have been published and certainly a didactic overview is needed to extract their significance and provide the basic features for students’ guidance. This field is in constant progress and needs regular updates. This book provides an updated overview of the MDR problems in different organisms and presents the state of the art for circumventing the phenomenon. It should be useful to the scientific community, teachers, associations, and students, as well as physicians. It gives an updated overview of MDR problems in different organisms and presents state-of-the-art information for circumventing the phenomenon.

Each chapter has been written by one of the specialists in the covered field, although it was not possible to gather all of the “great names” of MDR and ABC proteins. The involvement of a large number of authors presented the risk of a certain lack of cohesiveness and of some overlap. For example, the same important fact or the same reference may be cited in diverse occurrences. However, rather than detract from the overall unity and consistency of the book, this should allow each field to be entirely covered in an independent review, making the book more useful to every type of reader.

We wish to thank those who kindly offered their time and competence to provide a valuable contribution for the success of this enterprise.

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INTRODUCTION: WHAT IS MULTIDRUG RESISTANCE?

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PREFATORY REMARKS

The ability of cells or organisms to resist treatment by a combination of structurally unrelated drugs is known as multidrug resistance or MDR. It is a pervasive and insidious clinical problem in the treatment of cancer and infectious diseases. The fact that MDR is frequently observed is surprising since it appears to run counter to the fundamental principle that using a combination of unrelated drugs should militate against the emergence of a resistant disease. The Goldie–Coldman hypothesis for effective applied cancer chemotherapy is based on such a premise, that is, using an alternating noncross-reacting combination of drugs to prevent the emergence of a resistant disease (1). The assumption is that a drug-resistant disease arises spontaneously at a given mutation rate, for example, $10^{-7}$ per cell per generation and that resistance to two unrelated drugs in the same cell would require two independent mutational events generated with a frequency dependent on the product of the two rates, for example, $10^{-14}$. It stands to reason therefore that the probability of resistance to three or more independent drugs would be diminishingly small and should not normally be found. This principle appears to apply in the use of antibiotics in the treatment of bacterial diseases. However, mammalian cells
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in culture (2) appear to generate MDR mutants (resistance to many drugs, e.g., four or more) at an astonishing rate of \(10^{-6}\) or higher. Such a high rate for resistance to so many drugs suggests that the mechanism of resistance is not independent for each of the drugs. Rather, a common pathway affecting all the drugs is likely involved.

The specific form of MDR discussed in this book is that of multidrug efflux mediated by ATP-binding cassette (ABC) transporter proteins. This arises from the common necessity of drugs with intracellular targets to cross the membrane bilayer into cells. The bilayer itself, by virtue of being a common part of the pathway for drugs to reach their targets, becomes the locus of resistance, often through increased expression of preexisting transporter proteins at the target membrane.

Nontransport-related MDR can also occur when a common mechanism of cell death occurs as an end point of the action of many drugs, such as topoisomerase-mediated DNA repair (3, 4) or apoptosis (5), where changes in the proteins mediating these repair/cell suicide pathways can reduce the effects of all the drugs that trigger them. Numerous drug detoxification enzymes exist, such as the large family of cytochrome P450 oxidases, which can modify drugs in ways that reduce their toxicity or enhance their efflux. Also, transport proteins other than ABC transporters are capable of multidrug efflux, and some ABC transporter-mediated MDR phenotypes require mutation of the transporter to adjust its rate of activity or substrate specificity. However, these are generally minor modes in a field dominated, at least clinically, by changes in the expression or availability of wild-type transporter proteins on target cell membranes, or in the tissues that act as barriers between sites of drug introduction and drug action.

This book deals with how ABC transporter-related MDR challenges successful chemotherapy in clinical oncology and some infectious diseases. In this introduction, we would like to touch on (i) some key concepts in our understanding of the mechanism of this form of MDR by providing a historical context, and (ii) what is the evolutionary origin of ABC transporter MDR. We hope that such a perspective will allow the reader to gain an appreciation of the breadth of the MDR challenge and some insights on the prospects for its control. It has become apparent that MDR is an ancient mechanism by which all organisms protect themselves from their chemical environment. In this respect, the role of MDR efflux transporters is complementary to the role of the membrane itself and may be nearly as ancient a feature of living cells.

HOW HAS OUR UNDERSTANDING OF MDR EVOLVED?

The discovery of P-glycoprotein (P-gp) and the role it plays in MDR has been reviewed in some detail recently. We refer the interested reader to Gottesman and Ling (6). The first evidence of MDR attributable to a single genetic change leading to an increase in drug efflux came from work on mammalian cancer
HOW HAS OUR UNDERSTANDING OF MDR EVOLVED?

cell lines. Clones of Chinese hamster ovary cells selected for resistance to colchicines were found to be cross-resistant to daunomycin and puromycin and demonstrated reduced uptake of colchicines (7), which was initially attributed to reduced permeability. At the same time, Keld Danø demonstrated a reduction in daunomycin uptake in Ehrlich ascites cells, which was both energy-dependent and could be competed with Vinca alkaloids, to which these cells were also cross-resistant (8), and proposed a carrier-mediated efflux mechanism for MDR.

Two of the key questions that arose early on were what was the genetic basis of MDR and how frequently would it occur. The latter was answered through the use of Luria–Delbrück fluctuation analysis, originally developed to demonstrate selection of randomly occurring mutants in the appearance of bacterial resistance phenotypes (9). This approach was used to confirm that the appearance of MDR cells was due to selection of preexisting mutants at a single locus (2, 7), rather than multiple mutational steps or the regulatory induction of a resistant state following drug exposure.

That single genetic change resulted in an increase in expression of a 170 kDa plasma membrane glycoprotein (10), which came to be named P-gp because of its supposed role in mediating a change in membrane permeability. Experiments in which the MDR-bearing chromosome was transferred into a drug-sensitive cell line showed that the MDR locus remained linked to the original colchicine resistance phenotype (11). The high level of expression of P-gp in some cell lines allowed the study of P-gp function in isolated membrane vesicles (12), the raising of antisera (13), and eventually the purification and reconstitution of pure P-gp into artificial membrane vesicles (14), thus providing final proof of the competence of this single protein to bind and transport multiple drugs across a bilayer.

The permeability interpretation seemed justifiable at first, given that the drug resistance phenotype was reversible when the cells were treated with membrane-fluidizers such as mild detergents and anesthetics (15, 16). Even before the idea that MDR was the product of a single protein had been firmly established, a number of compounds had been identified that reversed drug resistance in vitro, and these led naturally to the idea that, if P-gp were blocked by some inhibitory compound, MDR could be circumvented and better chemotherapy outcomes would result (17). What followed from that was a series of discoveries of novel chemosensitizer drugs, refinement through identification of analogs with improved specificity, and clinical trials (discussed in Part IV of this book). There have been some early successes, particularly combination treatment of retinoblastoma by chemotherapy with the addition of cyclosporin (18) to inhibit P-gp. There have also been some unexpected problems with this approach stemming directly from the role that the target transporters play in normal physiology. It was not immediately appreciated that the normal function of MDR proteins, at least in part, is precisely to provide the organism with resistance to external chemical threats of a diverse and unpredictable nature. This appears to be especially true for P-gp, for which no necessary
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physiological role has yet been identified, but which is widely expressed in the bodies of all animals, most particularly on the epithelium of the gastrointestinal tract; on the organs of detoxification (liver and kidneys) (19); on the endothelium of the blood–brain (20), blood–placental, and blood–testis barriers (21); and on the surfaces of the quiescent stem-cell populations in the blood-forming system (22).

The discovery of other clinically significant mammalian ABC transporters, starting with the cystic fibrosis transmembrane conductance regulator (CFTR) (23) and MRP1 (24), changed the scene dramatically, and with the genomic revolution in DNA sequencing technology, has allowed us to identify all of the ABC-related genes in the genome of any organism (25). What has become clear from study of the resulting menagerie of diverse transporters is that many of these transporters exhibit overlapping specificities. The possibilities for compensatory effects, where the loss or inhibition of one ABC transporter simply opens up a niche for the productive expression of an alternative transporter already available in the same genome and perhaps even normally expressed in the same tissue, offer a huge challenge to our thinking of how to modulate these mechanisms for therapeutic benefits.

This is especially challenging since these transporters are likely playing important physiological roles, such as maintaining the blood–tissue barrier (20) or transporting some endogenous substrate required for at least one particular tissue or process. P-gp and the other constitutive MDR proteins such as MRP1 and breast cancer resistance protein (BCRP) are largely responsible for actively maintaining the background levels of resistance to toxins that characterize every tissue; and in their absence, enhanced and unexpected sites of toxicity may become apparent following drug treatment. Enhancing the toxicity of a chemotherapeutic regime for its target tumor is of little benefit if the patient suffers enhanced toxicity as well. Any broad-spectrum inhibitor capable of circumventing all anticipated sources of MDR would likely cause unacceptable toxicity. It is necessary therefore to seek out more specific inhibitors, although this is complicated because typically, MDR inhibitors are compounds that themselves somewhat resemble MDR substrate drugs, and which therefore tend to interact with multiple MDR proteins, more or less by definition. Some researchers have therefore proposed instead (22) to modulate ABC transporters with the opposite goal of enhancing the chemotherapy resistance of normal tissues whose sensitivity to drugs limits the doses that can be given patients.

It was not initially obvious that the direct model of P-gp action we now accept was correct at all, and it is still not certain that this model is sufficient by itself to explain all of the drug resistance seen in MDR cell lines that express P-gp. Many proposed “indirect” mechanisms of MDR posited that by transporting a single substrate, such as chloride (26) or adenosine triphosphate (ATP) (27), P-gp could alter some cellular parameter that would then affect a large number of drugs simultaneously, for instance, via a change in intracellular pH or membrane protein phosphorylation or membrane fluidity (15). Equally plausible, P-gp could have transported drugs bound to a common
moiety conjugated to multiple drugs by a variety of drug-metabolizing enzymes (28). All of these were plausible mechanisms, often reflecting genuine consequences of P-gp expression in at least some cells, though none have proved to be sufficiently general to explain MDR itself.

What those results really pointed to was the fact that the membrane and the efflux pumps act as a system, with perturbations of the membrane affecting not only the transporter proteins directly, but also the rates at which their substrates partition into, and permeate through, the membrane, a critical physical parameter in determining whether and how a drug interacts with its potential efflux pathway (29).

There was, for a time, a reluctance to accept the biochemical plausibility of a single protein interacting in a stereo-specific manner with such a wide range of compounds. This multifunctionality is not a feature unique to ABC transporters, as other multisspanning membrane transporter superfamilies such as the major facilitator superfamily (MFS) and resistance, nodulation, and cell division (RND) families in prokaryotes do have members capable of conferring MDR phenotypes (30). Beyond transporters, some drug metabolizing enzymes, such as Cyp3A4, that act on multiple drugs (31) overlap with those recognized by P-gp; so the philosophical problem, if there is one, is quite incapable. The answer appears to be that for hydrophobic compounds, the problem of substrate recognition is made much simpler by the presence of the membrane itself, which has the effect of concentrating the substrate at the transporter many hundred-fold, relative to the apparent concentration in the bulk solvent. A computational survey of P-gp substrates (32) and nonsubstrate molecules found that their affinity for P-gp could be predicted simply from the number of suitably spaced pairs or triplets of electron-donor groups on an otherwise planar and hydrophobic backbone. Drugs that are able to bind into a hydrophobic pocket on the enzymes or transporter can apparently interact through a few polar residues strongly enough to trigger catalysis, or a productive transport ATPase cycle, without requiring a precise fit to the van der Waals surface of the enzyme. The concentration and orientation of substrate in the membrane and isolation from competing solvent molecules afforded by the membrane, allow interactions with a relatively high Km to occur with significant frequency and specificity. This imprecision is acceptable in part because, given the necessity of dealing with multiple xenobiotic compounds, the exact form of the reaction product is not crucial so much as achieving an adequate turnover of drug molecules transported or modified.

**EVOLUTIONARY ORIGINS OF MDR**

The advent of genomic DNA sequencing technology now allows the genome complement of ABC transporter genes to be identified (25). When the entire complement of ABC transporters present in the human genome is represented in the form of a phylogenetic tree (left side of Fig. 1), it becomes apparent that the three MDR proteins of present clinical concern (MDR1, MRP1, BCRP)
are not closely related to one another, leading us to conclude that the MDR phenotype has evolved at least three times independently among ABC transporters (in subfamilies B, C, and G). P-gp and MRP1 are both closely related to others (bile salt export protein [BSEP], MRP2) that are capable of transporting some drugs as well as their normal physiological substrates (bile salts and conjugated bilirubin, respectively) (33, 34), which we can consider to be MDR proteins, though of currently uncertain clinical significance. These MDR-capable proteins appear to have repeatedly evolved from more specific transporters, most likely ones transporting peptides or a small range of related hydrophobic compounds. In two subfamilies, B and C, the MDR-capable transporters are most closely related to proteins capable of peptide transport, such as the transporter associated with antigen processing (TAP) transporters for antigenic peptides (35) in subfamily B and MRP6 in subfamily C, which transports at least one peptide involved in regulating calcium metabolism (36). P-gp at least is capable of transporting a number of membrane-active antibiotic peptides (37, 38), while non-MDR subfamily G transporters are mostly involved in sterol transport, such as the exclusion of some dietary plant sterols from absorption in the gut by the ABCG/ABCG8 heterodimer (39).

The ABC transporter family as a whole has been divided into six subfamilies (A–F), distinguishable from one another by sequence divergence as well as structural organization (25). While the functional arrangement of ABC transporters is thought to be invariant, two membrane-spanning domains and two ATPase domains, these may be encoded as a single protein (inset at the upper left of Fig. 1), or as “half-transporters” which then homo-
heterodimerize to form a functional transporter. The evolutionary tree suggests that large, complete, transporters have evolved by serial duplication and fusion of half-transporters at least three separate times (subfamilies A, C, and within subfamily B), and that half-transporters have, in turn, evolved at least twice, in two different orientations (subfamily G and at the origin of the cluster of subfamilies D, C, and B) presumably from precursors in which isolated ATPase domains associated noncovalently with their membrane proteins, the latter structural pattern being the norm among bacterial ABC transporters (40), most of which function as importers for specific nutrients.

Bacterial genomes, in addition to their numerous "permease" importers, contain ABC exporters representative of almost the entire range of diversity present in the eukaryote subfamilies consistent with an ancient origin of the exporter group of ABC proteins (Fig. 1) (41). A number of MDR-capable ABC transporters are found in bacteria (right side of Fig. 1), as well as a number of antibiotic efflux transporters limited to single classes of compounds. MDR phenotypes in bacteria are determined by a greater diversity of transporters than in eukaryotes, with some examples from subfamily B being related to peptide transporters (such as HlyB, Fig. 1), as well as several examples in subfamily F, a group that, in eukaryotes, has evolved entirely into nontransporter regulatory roles in protein translation (42).

As noted above, MDR proteins often transport peptides or other physiological molecules, and some MDR-capable proteins have essential physiological substrates as well, such as bile salt transport by BSEP in humans or lipid A transport by MsbA in Escherichia coli (43), suggesting a mechanistic continuum of substrate recognition capacity between the MDR transporters and ABC proteins dedicated to single substrates. This is particularly evident in the case of the bacterial peptide transporters of subfamily B, which in nature serve only to export single species-specific protein toxins (44), but which are capable of recognizing and transporting completely random artificial sequences so long as they contain a suitable mixture of amphiphilic and charged residues (45). As with the classical MDR substrates themselves, the essential feature of these peptide signals seems to be that electron-donating residues are presented in context of the membrane. Significantly, the number of such peptide-peptide interactions, rather than a precise set, is what is required for recognition, implying that when ABC transporters such as HlyB recognize two entirely differently peptides with equal affinity, as they do, they do so by using different sets of intermolecular contacts (46). Thus, it is not difficult to see the substrate recognition system of molecules like HlyB as analogous, and probably homologous, to P-gp's recognition of suitably spaced electron-donor sites within a planar hydrophobic molecule.

The rather flexible substrate recognition mechanism common to many ABC transporters has the result that many transporters have substrate ranges exceeding those required for their physiological roles and probably overlapping those of other ABC transporters. As a result, if a transporter is lost through mutation, a related transporter with an overlapping function may be
immediately available to compensate for the loss. In knockout animals, ABC transporter mutations are rarely lethal; this is an unusual outcome when one considers that the ABC transporter family is one of the oldest and most widespread of all gene families. By at least one measure of gene family evolution, the frequency of orthologous pairs identifiable between members of the same gene family across phyla, the ABC transporters are unusually poorly conserved for such an ancient gene family. We have argued that this is an example of “dynamic coherence,” where rates of gene duplication and deletion tend to be the same for all members of the gene family at any point in time within each evolving lineage (47). Periods of reduction in ABC transporter diversity are followed by fresh expansion of surviving subfamilies, such that even in phyla with broadly similar numbers of ABC transporters, the members of the family we observe are the products of more recent lineage-specific expansions. This implies a degree of independent assortment of functions among transporters over time. The extent of functional overlap among ABC transporters and the ease with which they evolve new functions force us to begin to view homeostasis as an evolutionary phenomenon. The relaxed selection pressures against loss or modification of ABC transporters have made it impossible for organisms to maintain a rigid mapping of function to gene, and accepting a history where individual transporters can come and go forces organisms to negotiate their constant needs for efflux functions of various kinds against a background of a shifting cast of proteins that are competent, but not uniquely suited, to fill those roles. From a practical bioinformatic point of view, this makes it unusually difficult to predict the function(s) of any particular ABC transporter on the basis of sequence comparison alone.

This practical difficulty in predicting ABC transporter phenotypes presents an increasingly serious problem as MDR in pathogenic organisms becomes an ever more common occurrence. We are faced with a bewildering array of diverse, and often highly divergent, genomes and transporters to sift for potential resistance determinants. The unreliability of simple in silico predictions based on homology means that potential MDR loci must be tested in laboratories for their contribution to resistance or examined in field surveys that correlate therapy failure with pathogen genetic polymorphisms, complicating the search for appropriate inhibitory compounds or alternative drug regimes that can circumvent an entire MDR suite of resistances. But when we have accepted the full scale of problems that known MDR determinants present to us, we should not grow complacent. The MDR phenotypes we encounter in pathogens or cancer therapy, or in getting drugs past the blood–brain barrier, are only those revealed by the drugs we use. New forms of MDR appeared following the failure of earlier modes of therapy in the face of specific resistance mechanisms, for example, the emergence of MDR as a clinical problem in bacteria as increasing levels of β-lactam resistance has encouraged the use of antibiotics aimed at intracellular targets, permitting the preexisting membrane-level defenses of the bacteria to intervene. In the future, exploiting entirely novel drugs will not only reveal that some currently understood MDR
mechanisms are preadapted to counter them, but it will likely uncover new MDR mechanisms, probably involving genes currently regarded as transporters specialized for physiological compounds bearing a passing structural similarity to the new class of drugs.

In some ways, MDR in the clinical setting seems as challenging now as it appeared in the 1970s, when its causes were unknown and presumed to be intractably complex. Since then, we have identified the multiple genes responsible, made explicit their mechanisms, and articulated unifying principles governing MDR. This book encapsulates the scope and direction of scientific work that is now making real progress in dealing with MDR. The work is incremental and requires much attention to detail, but it is leading to a practical mastery of both the difficulties and the opportunities afforded to us by our deepening understanding of this most diverse of biochemical phenomena.

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REFERENCES


PART I

ABC PROTEINS: AN OVERVIEW AND DESCRIPTION OF THE STRUCTURE, GENOME, NORMAL TISSUE EXPRESSION, PHYSIOLOGICAL ASPECT, AND MECHANISM OF ACTION
1

THE P-GLYCOPROTEIN 170: JUST A MULTIDRUG RESISTANCE PROTEIN OR A PROTEAN MOLECULE?

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1.1. INTRODUCTION

The ATP-binding cassette (ABC) proteins represent a highly diversified superfamily in all living kingdoms, with 49 human proteins, 14 of which are associated with various diseases (1, 2). They are found in all animal and plant species from prokaryotes to eukaryotes, and their functional characteristics are extended from ion transport to macromolecule efflux (3, 4). Although differences are observed in their functions, substrate specificities, molecular mechanisms, and in vivo localizations, they share a high degree of sequence and structural homology (5). The best known and best characterized of them is P-glycoprotein (P-gp; subfamily B, member 1: ABCB1), which is encoded by the MDRI (now ABCB1) gene, located on chromosome 7 in humans. It is the first eukaryotic ABC member identified and was discovered by Juliano and Ling (6) because of its implication in multidrug resistance (MDR) of cancer cells to chemotherapy (7). It consists of two halves that share a high degree of similarity. Each homologous half contains six hydrophobic transmembrane domains (TMDs) and a relatively hydrophilic intracellular loop encoding an adenosine triphosphate (ATP) binding site (nucleotide-binding domain [NBD]). By extruding cytotoxic drugs out of the cells before they reach their cellular target, P-gp expression leads to failure of AIDS and cancer chemotherapy (8). It is now recognized that several causes can explain its overexpression, such as gene amplification and gene polymorphisms. The studies on ABCB1 polymorphism and its functional consequences have become a major topic of research (9, 10). In addition, many studies have shown that P-gp is expressed in several normal tissues (e.g., intestinal epithelial cells, blood–brain barrier [BBB], and placenta) and that its primary function is to prevent the uptake of toxic compounds from the gut into the body, to expel them in the bile or urine, and to protect some very sensitive organs, such as the brain, from them (11). P-gp is also involved in other physiologic processes, such as control and regulation of apoptosis, stress, hypoxia, stem-cell differentiation, cellular immune response, or plasma membrane dynamic (12–14).

1.2. P-gp 170: FROM GENE TO PROTEIN

1.2.1. ABCB1 Gene: Structure, Regulation

MDR or ABCB genes constitute a small family in which two genes are closely related in humans (MDR1 and MDR2, now ABCB1 and ABCB4) and in rodents (mdr1, mdr2, and mdr3) (15). Full-length cDNAs for human ABCB1 and rodent mdr1 and mdr3 genes were shown to confer an MDR phenotype to drug-sensitive cells after DNA-mediated transfer. The proteins encoded by the human MDR2 gene and by its mouse counterpart are specifically involved in phosphatidylcholine translocation between plasma membrane leaflets (16). The human MDR genes are adjacent to each other on the long arm of chro-
mosome 7, distant by 330 kpb. \( \text{ABCB1} \) and \( \text{ABCB4} \) coding sequences are 76% identical. \( \text{ABCB1} \) gene has been shown to contain 29 exons and 28 introns (one of them longer than 40 kbp) with a total span greater than 120 kbp (17).

The degree of \( \text{ABCB1} \) gene amplification tightly parallels the expression of the MDR phenotype in cell lines selected for resistance. Chromosomal rearrangements have been observed in several cell lines and clinical samples. For instance, translocation has been observed between chromosomes 4 and 7; the resulting somatic cell hybrids showed an overexpression of \( \text{ABCB1} \), and this translocation provides a model for activation of \( \text{ABCB1} \) (18, 19). Nevertheless, amplified genomic regions are not observed in all resistant cell lines. In the human \( \text{ABCB1} \) promoter (Fig. 1.1), analysis of sequences upstream from \( \text{ABCB1} \)-coding regions has revealed that two distinct transcription start sites can be used, respectively located 136 and 140 bp upstream from the first ATG codon. The proximal site is used in most MDR cell lines and normal tissues (20–22). Two other minor transcription start sites are located about 100 bp downstream from these promoters. The proximal promoter (P1) spans the region from –198 to +43. It is TATA-less and contains two Y-box consensus sequences (–113 to –118), at least two GC boxes, and other GC-rich regions which may bind Sp1 factors. In this downstream promoter, sequences from –6 to +11 (relative to the P1 transcription start site) are sufficient for proper transcriptional initiation. This transcription start site has a strong homology with the initiator (\( \text{Inr} \)) sequence of the murine terminal deoxynucleotidyl

FIGURE 1.1. Schematic illustration of the human \( \text{MDR1} \) gene promoter used in MDR cells. The positive transcription factors YB-1 (Y-box protein 1) and MEF1 (MDR1 promoter-enhancing factor) bind to Y-box elements (or CCAAT-box like). The complex formed with transcription factors NF-kB/c-Fos also negatively regulates these regions. This complex is only detected in sensitive cells. GC boxes are recognized by transcription activators NF-Y and Sp1, activated by signal transduction pathways involving AMPc-dependent kinases (PKC). The \( \text{Inr} \) sequence (–6 at +11) is sufficient to promote transcription in the absence of TATA box promoters.
transferase gene (20). The Inr sequences are located at the transcription start site and can direct transcription from a RNA polymerase II promoter in the absence of a TATA box. Recently, some authors have shown the role of the highly structured 5′ end region of ABCB1 mRNA in P-gp overexpression (23).

The transcriptional regulation of ABCB1 gene expression is highly regulated by complex events and several signaling pathways. For example, ABCB1 gene transcription requires transcriptional factors and coregulators such as p53, c-myc, c-jun, HIF-1, and CtBP1. Altered methylation of the human ABCB1 promoter is sometimes associated with acquired MDR (24–26). Moreover, ABCB1 gene expression can also be regulated by different physiological processes, including differentiation factors (retinoic acid, sodium butyrate), steroid hormones (estradiol), and environmental stress (thermic and osmotic shock, low external pH). Antitumoral agents can also induce ABCB1 gene expression in human and rodent cell lines by transcriptional regulation. Overexpression can also result from spontaneous selection of mutants overexpressing P-gp rather than a direct induction of its expression (27).

Basal transcription of the human ABCB1 gene is controlled by a negative regulation involving a GC-rich region, located from –56 to –45 and from –110 to –103. Moreover, the region containing Y-box and GC elements seems essential for activation of ABCB1 after UV irradiation, suggesting a cooperative interaction between these boxes (28). A CAAT element binds two transcriptional factors, NF-κB and c-Fos (bases –116 to –113) in cells such as MCF7 cells (29). This protein complex is absent in MCF7 doxorubicin-resistant cells; and consequently, it has been suggested that it inhibits ABCB1 gene expression in sensitive parental cells. Conversely, the proximal promoter also contains different sites recognized by transcriptional activators, such as Sp1-activated by AMPc-dependent kinases (29). The transcriptional factor Y-box-binding protein 1 (YB-1) accumulates in the nucleus of MDR cells, where it binds to Y boxes and might also activate ABCB1 transcription. In sensitive counterparts, this factor is only detected in cytoplasm (30, 31). These Y boxes are also involved in the overexpression of ABCB1 gene in HL60 vincristine-resistant cells (32), by the mean of MEF-1 transcriptional factor (MDR1 promoter-enhancing factor); the interaction is also absent in sensitive cells (33). In vivo studies have shown that RAS and RAF oncogenes can regulate human P-gp expression.

The transcription rate of the ABCB1 gene can also be modulated by p53 itself or by p53 family members in response to a large subset of stimuli. For example, in the ABCB1 gene promoter, an Sp1 binding site is present and binds the promoting transcription heteroduplex Sp1-p53, modulating the expression of ABCB1 gene, when cells are treated with pro-apoptotic agents. Several studies suggested that p53 could be a potent repressor of ABCB1 gene transcription when activated by cytotoxic agents. Nevertheless, the repression is dependent on the interaction of p53 with other transcription factors; whereas
the interaction of p53 with an \textit{ABCB1} promoter, via a novel p53 DNA binding site (the HT site), leads to a direct repression of transcription (34). Another study showed that the reintroduction of wild-type p53 in doxorubicin resistant cells confers a sensitive phenotype that is correlated with a decrease in their tumorigenicity (35). On the other hand, p53 can inhibit P-gp function by mediating the inhibition of protein kinase C-alpha (PKC-\(\alpha\)) promoter activity, because PKC-\(\alpha\) can phosphorylate and activate P-gp (36). Other members of the p53 family (namely p63 and p73) can regulate the transcription of the \textit{ABCB1} gene, but a differential regulation can be observed. In fact, p63 and p73 regulate the majority of p53 target genes, but transient transfection assays demonstrated that p63 and p73 activated rather than repressed \textit{ABCB1} transcription. This upregulation is DNA binding-dependent but not through the HT site; p63 and p73 interact with the \textit{ABCB1} promoter via the alternate p63/p73 element, APE (37).

The human \textit{ABCB1} gene promoter presents many regulating sequences that are bound by several different kinds of transcription factors. Analyses point out specific sequences upstream from the \textit{ABCB1} gene such as, the inverted CCAAT sequence, also called the Y box (~82 to ~73), which binds the NF-YA transcription factor to regulate \textit{ABCB1} expression in a positive way (38). This sequence is also involved in the binding of another transcription factor, CCAAT/enhancer binding protein beta (C/EBP\(\beta\)). Cotransfection assays by either C/EBP\(\beta\) or C/EBP\(\beta\)-LIP (a dominant-negative form of C/EBP\(\beta\)) in the breast cancer cell line MCF-7 and its doxorubicin resistant variant MCF-7/ADR have shown that mutations inside the Y box abolished \textit{ABCB1} expression by C/EBP\(\beta\). The binding of C/EBP\(\beta\) to another sequence, AP-1 box (~123 to ~111), negatively regulates the expression of the \textit{ABCB1} gene (39). The mechanisms of \textit{ABCB1} activation by C/EBP\(\beta\) also involve interactions with Y-box-associated proteins and differential sequences binding in a certain cellular biochemical context. Some Y-box-associated proteins, such as the YB-1, also regulate the transcription of genes involved in cell growth, DNA replication, and DNA repair. Finally, a study has identified a cis-regulating element for \textit{ABCB1} gene transcription (40). These authors characterized the invMED1 sequence in the 5′-flanking region of the human \textit{ABCB1} gene; this one interacts with a nuclear protein, LRP130, and stimulates the transcription of \textit{ABCB1} in CEM leukemia cells. Interestingly, the level of LRP130 did not vary with the resistance level, but its binding intensity is variable with the \textit{ABCB1} gene expression. Furthermore, as this invMED1 sequence is also located in promoter regions of other \textit{MDR}-related genes, the invMED1/LRP130 couple could be a potential central regulator of the transcription of these genes. Another protein frequently mutated in cancers, the transcription factor c-myc, is also a strong activator of \textit{ABCB1} transcription. It acts by binding the E-box motif (namely, CACGTG), which is localized within the proximal promoter of the \textit{ABCB1} gene (~272, ~444). In neuroblastoma, a childhood cancer, the overexpression of the neuronal variant N-myc (MYCN) enhances \textit{ABCB1} gene expression and constitutes a marker for poor prognosis.
The expression level and function of \textit{ABCB1} gene also depends on some gene polymorphisms. During the last decade, several single-nucleotide polymorphisms (SNPs) have been identified in the coding region of the gene (15, 43). The first studies carried out in normal human patients showed significant correlations between polymorphisms in exon 26 (C3435T) of \textit{ABCB1} and expression levels and functions of \textit{ABCB1} (44). Some other polymorphisms may be associated with altered \textit{ABCB1} expressions and/or P-gp functions; they can be associated with altered drug metabolisms and/or pharmacokinetics and have an impact on drug efficiency and toxicity. In the context of rheumatoid arthritis, a study showed that the \textit{ABCB1} genotypes 3435CC and 3435TC result in lower probabilities of remission after treatment with methotrexate and glucocorticosteroids, compared with patients with the 3435TT genotype (45). Other authors have shown that the \textit{ABCB1} polymorphisms could be a risk factor for several other diseases such as renal epithelial tumor, bowel diseases, and Parkinson’s disease (46–48). On the contrary, Morita et al. (49) did not observe differences in transcellular transport and intracellular accumulation between cells with polymorphic variants (G2277T/A and C3435T) and cells expressing the wild-type genotype.

Furthermore, the C3435T polymorphism in exon 26 may affect the function of P-gp by influencing its expression level, thus modifying cancer prognosis in breast cancer (due to chemotherapy resistance) (50, 51). It may be one of the risk factors for susceptibility in upper aerodigestive tract cancers, which are associated with tobacco use and alcohol consumption (52). Furthermore, another variation, G1199A, appears to alter the transepithelial permeability and efflux of fluorescent substrates \textit{in vitro}. It confers more resistance to cells selected by cytotoxic agents such as vinblastine and vincristine (53). This feature could be an explanation for the relative interindividual difference in sensitivity to antineoplastic agents and drug resistance. In addition to the numerous SNPs identified, insertions, duplications, or deletions of sequences in the \textit{ABCB1} gene could also play a role in altered P-gp functions (54). For example, an increase of \textit{ABCB1} DNA copy numbers leads to an enhanced P-gp expression, which is characteristic of drug-resistant cell lines in comparison with the drug-sensitive parental cell lines. A study based on the comparison of the SNPs occurring in the entire 200 kb of the \textit{ABCB1} gene in five different populations (Chinese, Malays, Indians, Caucasians, and African-Americans) has shown that a recent positive selection has occurred at the
human ABCB1 gene locus. This positive and population-dependent selection confers a typical haplotype of the ABCB1 locus in a given population and, consequently, a potential population-dependent susceptibility to MDR (55). Numerous correlations were observed between ethnicity-related polymorphisms and haplotypes in the human ABCB1 gene. For example, Kimchi-Sarfaty et al. (56) identified that the 3435C > T occurred in 24.2% of the U.S. population and in 69.3% of the Ashkenazi-Jewish population.

It appeared that genotype analysis of ABCB1 SNPs is becoming increasingly important in identifying genetic variants underlying susceptibility to human disease. Recent results suggested that ABCB1 polymorphisms might influence the intracellular concentration of cyclosporine, a P-gp substrate preventing graft rejection after solid organ transplantation. The ABCB1 1199A carriers presented a 1.8-fold decreased cyclosporine intracellular concentration, whereas the 3435T carriers showed a 1.7-fold increase. In contrast, 61A > G, 1236C > T, and 2677G > T polymorphisms did not modify cyclosporine intracellular and blood concentrations (57). Nevertheless, opposite results appeared to be likely due to differences in cancer types (58, 59). Future research on ABCB1 polymorphism will allow to better understand the factors that contribute to interindividual variability in drug exposure, response, and toxicity (10, 43).

1.2.3. P-gp Structure

The human ABCB1 gene encodes P-gp, a protein of 170 kDa containing ~1280 amino acids (approximate mass of 170–180 kDa). It is organized in two homologous halves, corresponding to duplication of an ancestral gene and/or fusion of two ancestral molecules. A structural model for the glycoprotein was proposed by Jones and George (60). It was obtained from hydropathy plots and computer prediction algorithms (Fig. 1.2).

During the year 2001, the bacterial P-gp homologue (MsbA) of Escherichia coli was the first ABC transporter to be crystallized. Nevertheless, the described structure was controversial, and new structures of bacterial multidrug ABC transporter at high resolutions (3 Å) were proposed (61). Among these is SAV1866, the bacterial P-gp homologue (62). On the other hand, since 1997, Rosenberg et al. (63) studied the structural organization of the P-gp. They have obtained low- to high-resolution three-dimensional (3D) structures for P-gp using cryo-electron microscopy of two-dimensional (2D) crystals. During the year 2005, they obtained the first 3D structure for an intact eukaryotic ABC transporter (64). It contains a wide hydrophilic pore (5 nm for internal diameter and 10 nm for external diameter), closed on the internal cytosolic side, forming an aqueous compartment inside the hydrophobic membrane bilayer. This cup-shaped chamber has been proposed to include an opening allowing a lateral entry of drug substrates to be excluded. The accepted model for
FIGURE 1.2. Predicted membrane topology and three-dimensional structure of P-gp.
(a) Each of both N- and C-terminal halves are composed of 16 transmembrane anti-
parallel β-sheets and 6 cytoplasmic α-helices. The A, B, and C rectangles correspond
to the ATP-binding domains. Adapted from Jones and George (60). (b) The single
polypeptide chain is folded in two halves, each containing six transmembrane α-helices.
The transmembrane α-helices are connected by extracellular or cytosolic loops, fol-
lowed, in cytosol, by large domains containing for each half a Nucleotide Binding
Domain (NBD). Drawing with PyMOL.
human P-gp suggests that the single polypeptide chain is folded in two transmembrane domains (TMDs), each half containing six transmembrane α-helices. There is evidence that the two TMDs together constitute the drug transport pore. The transmembrane α-helices are connected by extracellular or cytosolic loops, followed by a large cytosolic domain containing an ATP-binding site (65) or NBD (NBD1 and NBD2 for the first and the second half, respectively). Each NBD contains nucleotide-binding motifs, including Walker A (P-loop) and Walker B sequences, and the ABC signature motif (LSGGQ). A central sequence connects the two homologous halves of the protein and is called the “linker” region. The two halves share 43% sequence identity and 78% similarity, and TMDs of these proteins display β-sheets rather than α-helices. The linker region also plays an important role in P-gp function. Its flexible secondary structure is sufficient for the coordinate functioning of both halves of P-gp, which are likely required for the proper interaction of the two ATP-binding sites. Both NBDs of P-gp can bind and hydrolyse ATP.

There is great evidence that for efficient ATP hydrolysis, the two NBDs have to interact by forming a sandwich dimer so that the LSGGQ motif of one NBD comes into contact with the loop of the other NBD to form the nucleotide-binding pocket (66). Moreover, it is evident that the other transmembrane segments, such as segment 1 (67) and segment 7 (68), play roles in the drug-binding pocket, whereas a mutation in segment 6 (residue G346) affected drug transport in cells by a reduction in basal ATP hydrolysis, but had no effect on drug binding (69).

1.2.4. Posttranslational Modification

Phosphorylation. The linker region (75 amino acids long: 633–709) contains phosphorylatable serine residues (661, 667, 671, 675, and 683) recognized by different kinases, such as protein kinases C (PKC) and protein kinases A (PKA) (70). PKA inhibition does not influence P-gp expression and function, but P-gp phosphorylation by PKC modulates the activity of the pump (71). The first studies have indeed reported that enhancement of PKC activity by phorbol esters increased the resistance level of cells and reduced drug accumulation (72).

Since phorbol ester treatment increases P-gp phosphorylation, these results suggest that phosphorylation may enhance drug efflux. Ratsaninghe et al. have observed (73) differential expressions and activities of PKC and tyrosine phosphatase in MCF7 MDR cells in comparison to sensitive counterparts. This relationship of P-gp efflux activity with decreased and with increased phosphorylation suggests that its activity may be modulated not only by kinases but also by phosphatases (74). Moreover, PKC inhibitors may directly interact with P-gp. On the contrary, other authors (75) concluded that phosphorylation did not play a significant role in regulating P-gp activity in MCF-7/ADR cells. As a conclusion, the mechanisms of P-gp inhibition by PKC inhibitors and the role of its phosphorylation remain unclear. PKC blockers may affect drug
transport both by (i) direct competition with transported drugs for binding to
P-gp and (ii) indirect inhibition through a pathway involving PKC inhibition,
but independent of P-gp phosphorylation (76).

Specifically, Ser-661, Ser-667, and Ser-671 are, both in vitro and in vivo, the
major sites of phosphorylation, and they all occur within classical PKC con-
sensus motifs. The number and identity of the kinases that phosphorylate P-gp
in MDR cells remained uncertain for a long time (77). It seems most likely
that P-gp is phosphorylated by one or more PKC isoenzymes (78). In the PKC
family, PKC-α phosphorylates and activates P-gp, whereas its inhibition by p53
leads to decreased P-gp phosphorylation (36). Previously, a critical role for the
linker region Ser-661 in the positive regulation of P-gp ATPase activity by
PKC-α was suggested by the demonstration that mutation of this serine to
asparagine abolished the enhancement of drug-stimulated P-gp ATPase activity
by PKC-α in a baculovirus expression system (79). Moreover, results on
proteoliposomes containing P-gp suggest that differential phosphorylation
patterns of the transporter could be linked to environmental molecular
composition (lipids, presence of detergents) and structure (80).

Glycosylation The P-gp apparent molecular weight is reduced from 170 kDa
to 140 kDa after enzymatic treatment with different glycosidases such as
peptide-N-glycosidase F or endo-β-N-acetylglucosaminidase (81). The primary
sequence of P-gp suggests that 10 putative N-glycosylation sites are present.
Nevertheless, only three potential sites of extracellular N-glycosylation
(residues Asn 91, 94, and 99) exist in the first extracellular loop. In fact,
glycosylation may contribute to a precise folding and a correct trafficking of
P-gp to the plasma membrane. It is first synthesized in the endoplasmic
reticulum (ER) as a core-glycosylated intermediate with a molecular mass of
about 150 kDa. The carbohydrates are subsequently modified in the Golgi
apparatus to yield a protein of about 170 kDa that is consequently delivered
to the cell membrane. Using the mutational studies, Loo and Clarke found
that 10% of the point mutations affected the processing of P-gp. These mutants
are retained in the ER as core-glycosylated intermediates associated with the
molecular chaperones calnexin (82) and Hsc70 (83). However, tunicamycin
treatment inhibiting glycosylation of P-gp in MDR cells does not affect drug
sensitivity, although the efficiency in obtaining drug-resistant clones is
drastically reduced (84). Thus, glycosylation seems to be involved in P-gp
processing and/or stability. Transfection of MDR cells with wild-type ubiquitin
or treatment with an N-glycosylation inhibitor increased the ubiquitination of
P-gp and increased its degradation in the proteasome (85). On the other hand,
Gribar et al. (86), using a vaccinia virus-based transient expression system,
obtained HeLa cells expressing several types of P-gp mutants. First, HeLa
expressing “P-gp-N/Q” (91, 94, 99N→Q) showed a 40%–50% lower cell surface
compared to HeLa cells expressing the wild-type protein, although the
substrate specificity of the pump was not affected. The reduced expression was
not due to glutamine substitution but to sugar moiety deprivation; indeed, in
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HeLa cells expressing a P-gp with the substitution 99N→D or with the 99N deletion, the level of cell-surface P-gp remained unchanged. In the same way, mutagenesis of the three sites in the human protein (Asn to Gln, Ala, or Asp) reduced the apparent molecular size to around 140 kDa, but did not modify the ATPase activity of the mutated P-gp, which remained able to confer drug resistance (87). Moreover, the nature and sequence of glycosylated chains are very complex. Recently, Greer and Ivey (88) have described several possible N-glycanic structures of overexpressed human P-gp. One of them contains a high-mannose complex oligosaccharide, while two other structures present terminal sialic acids. The α6 sialyl terminal groups and β1–6 branching glycans are highly expressed in cancers due to the regulation of acetylglucosaminyltransferase V, which could include the glycosylation of P-gp (89).

1.3. TISSULAR, CELLULAR, AND ORGANELLE EXPRESSION OF P-gp 170

1.3.1. Expression in Normal Tissues and Tumors

Several normal tissues express high levels of the ABCB1 gene, such as apical membranes of epithelial cells from kidney proximal tubule, intestine, and lung. ABCB1 gene is also found in brain microvascular endothelia, placenta, adrenal cortex, testis, uterus, lymphocytes, and hematopoietic cells (90–92). In such tissues, P-gp localization and its highly conserved structure during evolution suggest an important role for this protein in protecting mammalian cells against various toxins and/or in transporting endogenous substrates (93, 94).

As a result of this tissue localization, P-gp functions in three main areas (95): (i) P-gp limits drug entry into the body after oral drug or toxin administration as a result of its expression in the luminal (apical) membrane of enterocytes; (ii) once the xenobiotic has reached the blood circulation, P-gp promotes drug elimination into bile and urine as a result of its expression in the canalicular membrane of hepatocytes and in the luminal membrane of proximal tubule cells in the kidneys, respectively; (iii) in addition, once a xenobiotic has reached the systemic blood circulation, P-gp limits drug penetration into sensitive tissues. In particular, in the blood brain barrier (BBB), P-gp is localized in both luminal and abluminal membranes of capillary endothelial cells, pericytes, and astrocytes (96). This localization strongly suggests an important efflux role of P-gp, restricting the penetration of drugs and toxic agents in the central nervous system, thus playing the role of a gatekeeper (97). Studies on knockout mice lacking P-gp have confirmed these ideas since these animals show a disrupted BBB and can be up to 100-fold more sensitive to several neurotoxic drugs (98, 99). Furthermore, the knockout mice studies have clarified that MDR plays a more important role in preventing drug absorption and uptake in gut and brain than in drug excretion in the bile and urine (100).
ABC transporters were often detected in a wide variety of stem cells, including melanoma and hematopoietic stem cells (101, 102). P-gp especially is expressed in primitive stem cells, including human CD34+ cells, which can be identified by their ability to transport fluorescent dyes that are P-gp substrates, such as rhodamine 123 (103). Maturation of these cells was accompanied by a decrease in P-gp expression and functional activity. It was suggested that ABC transporters in human stem cells could act as protectors from genetic damage by naturally occurring xenobiotics (104). However, as initially described by Gottesman and Pastan (105), this constitutes a “double-edged sword” because the conserved expression of P-gp after the stem cells’ malignant transformation in acute myeloid leukemia could decrease sensitivity of leukemia cells to chemotherapy (102). Today, several therapeutic assays have been conducted using retroviral \textit{ABCB1} gene transfer to convert drug-sensitive hematopoietic cells into drug-resistant cells, in order to protect normal cells from intensive cancer chemotherapy (106). The aim of this approach is to combine high-dose chemotherapy with transplantation of \textit{ABCB1}-transduced hematopoietic stem cells; clinical benefits are under investigation.

A recent study (107) also reported expression and function of P-gp in human fetal neural stem/progenitor cells, hNSPCs. Data suggested that P-gp was functionally expressed in cultured hNSPCs and was downregulated during differentiation, indicating that \textit{ABCB1} expression might be important in maintaining hNSPCs in an undifferentiated state. Those data are corroborated by a recent review from Mizutani et al. (108) who reported that high expression of P-gp prevents stem-cell differentiation, leading to the proliferation and amplification of this cell repertoire. Links between \textit{ABCB1} expression and the differentiation stage were also investigated in neoplastic cells treated with all-trans retinoic acid (ATRA), which is used against certain forms of leukemia. Data appeared controversial, as reported by Stromskaya et al. (109). The authors showed that increasing differentiation of leukemic cells (induced by RARα overexpression) induced an increase in \textit{ABCB1} gene expression in cells from solid tumors. Nevertheless, it did not result in elevation of constitutive P-gp functional activity, but it could participate in the control of P-gp induction. Sulová et al. (110) recently reported that combined treatment of P-gp positive cells with verapamil and ATRA induced a depression of P-gp expression and/or transport function whereas ATRA alone did not. Taken together, these data show that interconnections between retinoic acid-mediated differentiation and MDR regulation remain complex and dependent on the cell context.

P-gp is also expressed in the cancer cells that have developed drug resistance (111). It corresponds to the first known function of this protein, described in 1976 by Juliano and Ling (6). Certain tumors originating from tissues with naturally high levels of P-gp expression may be intrinsically drug resistant (e.g., colon, kidney, pancreas, and liver carcinoma) (112, 113). On the other hand, tumors with low basic levels of P-gp expression (such as hematological malig-
nancies) sometimes display a marked increase after chemotherapy (114, 115); this phenomenon is associated with acquired resistance. There is a poor understanding of events leading to overexpression of \textit{ABCB1} in response to chemotherapy. An induction of P-gp by chemotherapeutic agents has been suggested, although the mechanism of this induction remains unclear (12). Upon exposure to both endogenous and exogenous stresses (metabolic modifications, hypoxia, chemotherapy), cancer cells are committed to adaptation. Enhancement of \textit{ABCB1} expression constitutes one part of the response.

1.3.2. Cellular Localization of P-gp

Numerous studies have suggested a different intracellular localization of cytotoxic drugs between sensitive and MDR cells (116–120). Most of the drug accumulates in the nucleus of sensitive cells. In MDR cells overexpressing P-gp, the protein is mainly located in the plasma membranes of the cells, and altered drug distribution has been observed in resistant cells. The drug is largely excluded from the nuclei and is sequestered in perinuclear vesicles that move toward the cell periphery to create punctate cytoplasmic distribution patterns (121). The number of these drug-accumulating vesicles per cell seems to correlate with the level of drug resistance, as observed in an MDR Chinese hamster ovary cell line (116). Vesicle formation displays biphasic kinetics, with an initial rapid increase followed by a plateau where no further increase is observed. It has been suggested that a pH shift in various cytoplasmic organelles might contribute to this intracellular redistribution of anticancer drugs (122). Owing to their positive electric charge at physiologic pH, most anticancer drugs (vinca alkaloids, anthracyclines) are accumulated under their protonated form on the side of a membrane at which the pH is lower. This suggests that cationic molecules become “acid-trapped” in acidic cytoplasmic vesicles (123).

Several studies have tried to identify the drug sequestration compartments associated with P-gp function (124). Ferrao et al. (125) demonstrated the involvement of P-gp in drug compartmentalization in leukemic cell lines and patient samples, suggesting that cytoplasmic localization could be involved in the sequestration of doxorubicin in organelles, preventing it from reaching its nuclear targets. Moreover, it has also been detected in the nuclear membrane, in the cytosol (126), and in several cytoplasmic compartments of different cell lines, such as the Golgi apparatus (127) and the ER. A study suggested that P-gp was first present in ER before moving to the Golgi and finally reaching the plasma membrane. Moreover, drug accumulation was raised when P-gp was localized in ER or in the Golgi rather than on plasma membrane (128). On the other hand, Bennis et al. (129) observed a preferential accumulation of doxorubicin in subcellular components distinct from nuclei in doxorubicin-resistant K562 cells. In cells transfected with the \textit{ABCB1} gene, P-gp was detected in vesicles located around the periphery of the nuclei (130), suggesting a mitochondrial pattern, while Gong et al. (131) have shown that
accumulation of daunorubicin occurred in mitochondria-like organelles in K562-resistant cells. In addition, Munteanu et al. (132), then Solazzo et al. (133), independently demonstrated a mitochondrial P-gp localization by several methods and different specific monoclonal antibodies in K562 cells’ MDR variants, in MDR1 P1(0.5) hepatocarcinoma cells, and in ABCB1-transfected (PNA1)NIH/3T3 cells. The two groups have studied P-gp expression in whole cells by confocal microscopy and in purified isolated mitochondria by western blot. They used functional assays on isolated whole mitochondria by flow cytometry (assays requiring different washing and centrifugations to eliminate debris and contaminations by other membranes such as plasma membranes) to verify that the mitochondrial P-gp was functional. In contrast, Paterson and Gottesman (134) did not observe P-gp in mitochondria of MCF-7 ADR and KB-V1 cells. Thus, the presence of P-gp in mitochondria is dependent on the MDR cell origin.

1.4. P-gp 170: A PROTEIN IMPLICATED IN NUMEROUS FUNCTIONS

1.4.1. Multi-Molecule Transporter

As previously mentioned, P-gp was originally identified in resistant tumor cells as part of the mechanism of MDR; but over the last decade, it has been demonstrated that P-gp is also expressed throughout the body to confer intrinsic resistance to the tissues by exporting unnecessary or toxic exogenous substances or metabolites (135). It is thought that MDR substrates enter the cell through the lipid bilayer by passive diffusion and bind reversibly to P-gp in the bilayer or on the cytoplasmic side of the cell membrane. Subsequently, P-gp utilizes energy from the ATP hydrolysis to transport MDR drugs out of the cell against a concentration gradient (136, 137). P-gp can interact mainly with two classes of compounds: The first one, classically considered as substrates, are generally hydrophobic, positively charged or neutral, and include natural products, chemotherapeutic drugs, or steroids. The second group is constituted by modulators that are able to reverse MDR by blocking P-gp drug efflux without being transported by the pump (see Part IV, Chapter 8).

Drug transport involves two steps. First, there is a catalytic cycle of ATP hydrolysis, which drives transport. This involves low-affinity binding of ATP to both NBDs, which induces the formation of a putative nucleotide sandwich dimer (138). Second, the drug is moved from the cytoplasmic side to the extracellular side of the membrane. The P-gp drug-binding site is constituted by the transmembrane helices and is located within the cytoplasmic membrane leaflet. Three models of P-gp mechanisms of action (Fig. 1.3), not rigorously exclusive of each other, are currently reported: classical pump, vacuum cleaner, and flippase (2).
FIGURE 1.3. Different functional models of P-gp. (a) The pump model according to which P-gp may form a transmembrane pore, through which drugs expelled from MDR cells pass, thanks to the ATP hydrolysis energy. (b) In the vacuum cleaner model, drugs interact with the membrane lipids, then with P-gp, which turns inside the membrane and may also release drugs in the extracellular medium. (c) “Flippase” model: The drug inserted in the inner leaflet of the lipidic bilayer may be translocated (“flipped”) on the external leaflet from which it may slowly diffuse in the extracellular medium.
In the classical-pump model, P-gp forms a pore composed of the clustering of the 12 hydrophobic segments, and actively translocates (in an ATP-dependent manner) polar compounds out of the cell as the ion-translocating pumps (105). Evidence for the direct interaction of many of the substrates or reverting agents with the transporter has been obtained, such as drug-binding studies and photoaffinity labeling experiments. The majority of experimental data strongly supports this drug-pump model (137). Drugs interact in cytoplasm with the transmembrane region of the two halves of P-gp (transmembrane segments 5–6 and 11–12), coming together to form a single, large, and flexible drug-binding pocket, possibly containing several binding sites for the substrate (8). It seems that at least two molecules can simultaneously bind different overlapping regions. Then, P-gp expels the drugs directly into the extracellular medium due to the energy from ATP hydrolysis. Authors have demonstrated that the drug-stimulated ATPase activity was directly correlated to the ability of P-gp to transport these drugs (139, 140). Even if data concerning the stoichiometry of the exchange has remained controversial for a long time, probably because of the high basal ATPase activity (141, 142), it seems that one nucleotide is cleaved per P-gp molecule (143). Thus, the function of P-gp is associated mainly with a reduced accumulation of intracellular drugs by way of an active efflux and/or by an intracellular redistribution of these drugs. No substrate-transporter binding that is able to transfer it to P-gp has been described.

According to the “hydrophobic vacuum cleaner” model, P-gp may detect and eliminate hydrophobic substrates directly from the lipid bilayer (144, 145). As most substrates are hydrophobic, it has been proposed that they first equilibrate between the aqueous internal compartment and the inner membrane leaflet before P-gp meets the substrate. In a second step, nucleotide binding and/or ATP hydrolysis causes conformational changes of the transporter, which subsequently can extract substrates from the inner leaf and pump them directly to the external aqueous medium (146, 147). This model is strengthened by data demonstrating unidirectional transport of fluorescent P-gp substrates from the cytoplasmic leaflet of the plasma membrane to the external aqueous environment (148).

In the “flippase” model, P-gp encounters drugs in the inner leaflet of the plasma membrane and flips them to the outer leaflet from which they diffuse into the extracellular medium (149). This model is based on the analogy between amphipathic drugs and the normal phospholipids of membranes. Whereas the lateral mobility of phospholipids within the membrane is high, the spontaneous rate of flipping between the two leaflets of the membrane is very low because the polar-heads groups of the phospholipids cannot be easily transferred across the hydrophobic internal part of the membrane, which is constituted by the acyl chains of the phospholipids. Although this model was initially only based on theoretical considerations, it received a considerable boost when Smit et al. (150) found that the murine mdr2 P-gp is essential for the normal transport of phosphatidylycholine from the hepatocytes into bile.
According to this model, P-gp may flip drugs from the inner to the outer leaflet of the bilayer where they can partition with the aqueous phase. Recognition and binding of diverse sets of substrates must be associated with a preferred membrane location, determined by molecular properties and lipid interactions (2, 151, 152). It remains that it would be difficult to distinguish experimentally between the hydrophobic vacuum cleaner model and the flippase model.

1.4.2. Lipid Transport

In agreement with this flippase function, a growing number of publications have reported a role of P-gp in phospholipid translocation. P-gp has been reported to regulate the translocation of phosphatidylcholine and phosphatidylethanolamine, as well as sphingomyelin and several other short-chain phospholipid analogs (108, 153). This could explain in part the wide range of substrates recognized by P-gp, due to the different hydrophobic interactions inside lipid bilayers. More specifically, the simple glycosphingolipid (GlcCer) is a P-gp substrate candidate. It is synthesized from ceramide on the cytosolic surface of the Golgi apparatus and enters the outer leaflet of the plasma membrane. Interestingly, GlcCer levels are much lower in cells lacking MDR transporters (154). Nevertheless, it remains unclear whether P-gp translocates natural long chain lipids since \(\text{ABCB1}\) knockout transgenic mice have no detectable abnormality in lipid metabolism (155, 156). P-gp could also be involved in trafficking cholesterol from the plasma membrane to the ER, even if it remains unclear whether the P-gp-facilitated cholesterol trafficking is associated with its conventional drug transport activity (154, 157). Another study conducted by Garrigues et al. (158) suggested a coupling between the basal ATPase activity of P-gp and its intramembrane cholesterol-redistribution function. Data were fully consistent with the possibility that P-gp may actively translocate cholesterol in the membrane. Finally, P-gp-mediated cholesterol redistribution in the cell membrane makes it likely that the protein contributes to stabilizing the cholesterol-rich microdomains, especially rafts, and that it is involved in the regulation of cholesterol trafficking in cells. Thus, P-gp activity is particularly sensitive to its lipid environment. In some cases, P-gp appears to be within specialized raft-like membrane microdomains, where its ATPase activity is five times higher than in crude membranes (159, 160). These observations remain controversial (161). More generally, P-gp retains its function in liquid-ordered cholesterol and sphingolipid model membranes, and P-gp activity requires a microenvironment of raft microdomains or intermediate-density domains (162, 163).

1.4.3. Control and Regulation of Apoptosis

A growing number of publications debate about the role of P-gp in apoptosis (164). Of course, due to its drug efflux function, P-gp exerts a strong down-regulatory effect on drug-induced cell death, but it seems that this prevention
is not limited to this mechanism. Several works reported that P-gp might play a role in regulation of cell death against different stimuli. Robinson et al. (165) showed that P-gp overexpression was associated with resistance to serum starvation-induced apoptosis in Chinese hamster ovary fibroblasts and that the resistance was reversed by verapamil, indicating that P-gp was required for this resistance. Other groups demonstrated that functional P-gp can confer resistance to a wide range of caspase-dependent apoptotic stimuli (death receptor ligation, UV radiation, etc.). Different mechanisms could underlie this function. It has been demonstrated that functional P-gp could inhibit activation of the caspase cascade (especially caspases 8 and 3), downstream FAS ligation without disturbance of death-inducing signaling complex (DISC) formation. The inhibition seemed to be dependent on ATP hydrolysis (166). By contrast, the caspase-independent apoptosis pathway was not affected by P-gp expression, suggesting a caspase-specific role for P-gp. Caspase inhibition could also be explained by an increase in intracellular pH due to expression of functional P-gp, while apoptotic events such as caspase activation need acidic pH (164). In addition, cellular stresses (tumor necrosis factor [TNF], FAS ligation, radiation) are often associated with ceramide generation, which can directly induce mitochondrial cytochrome c release. It has been demonstrated that P-gp might both decrease ceramide production by reducing the availability of sphingomyelin and augmented ceramide glycosylation by translocating glucosylceramide across the Golgi membrane, thus detoxifying and inhibiting their apoptotic functions (167). Recently, it was suggested that downregulation of P-gp consecutive to CIAPIN1 inhibition, a new apoptosis inhibitor, could sensitize leukemia cells to chemotherapeutic drugs by upregulating the pro-apoptotic BAX protein (168). Similar results were obtained with hepatocellular carcinoma cells expressing the MDR phenotype. In this model, apoptosis could be restored by downregulation of P-gp expression (169). These data were corroborated by studies of the association between phosphatidylinositol 3-kinase/AKT pathway and MDR of gastric cancer cells (170). It was shown that inhibition of P-AKT expression significantly upregulates p53 expression, and downregulates P-gp expression and ABCB1 transcription.

### 1.4.4. P-gp Importance in Immune Response

Variable levels of P-gp expression have been reported in lymphocytes, ranging from 20% to 80% in B cells and from 30% to 100% in T cells. Thus, the link between P-gp expression and the function of lymphocytes remains controversial (171). It has been shown that inhibition of P-gp efflux by monoclonal antibodies or pharmacological inhibitors resulted in the reduction of NK and CD8+ cytotoxic activity. P-gp expression was also reported in skin dendritic cells. These cells are key players in the immune system with the capacity to support innate and specific immunity and to initiate primary immune responses. P-gp seems to be involved in dendritic-cell migration toward lymph nodes through afferent lymphatic vessels (172). One can hypothesize that P-gp could
modulate both NK and CD8+ activity and dendritic-cell migration by regulating cytokine transport, since it has been shown that IL-1β, IL-2, IFNγ, and TNF could be transported across the cellular membrane out of activated lymphocytes (164). Nevertheless, the real place and biological relevance of P-gp implication in physiological immune system functions remains to be demonstrated.

Overexpression of P-gp was also found in lymphocytes from various autoimmune diseases such as rheumatoid arthritis or systemic lupus erythematosus. It may be due to a long-term use of drugs inducing P-gp expression (173). Persistence of activated cell compartments characterizing these diseases could induce P-gp expression. Another role for P-gp in autoimmune diseases was suspected with observations performed on ABCB1 knockout mice. The animals have been reported to be more susceptible to inflammatory bowel diseases. It has been suggested that P-gp, in regard to its gut localization, could prevent accumulation of inflammation-inducing bacteria (94).

1.5. CONCLUSION

Finally, since the discovery of P-gp (product of the ABCB1 gene) in 1976 in cancer tissue, several thousands of articles have been published, showing the interest of the knowledge of its gene, its structure, and its role. From this amount of data, a more rational approach to P-gp inhibition should emerge. Recent studies have focused on ABCB1 pharmacogenetics, which is involved in both drug pharmacokinetics and cancer MDR (2, 10). Nevertheless, numerous data about P-gp remain partial and/or unclear. For instance, the mechanism of P-gp-mediated drug transport is not yet completely elucidated, especially the coupling between ATP cleavage and transport; the molecular phenomena leading to ABCB1 overexpression in response to chemotherapy is poorly understood; and the effects of various genotypes and haplotypes on P-gp function remain controversial. Today, we know that P-pg is present in normal cells and in tumor cells, where it plays a role to efflux hydrophobic endogenous and exogenous compounds. P-gp is involved in numerous physiological and pathologic pathways, in normal and cancer tissues; and the implication of P-gp in so many processes has opened several important new topics of investigation.

REFERENCES


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(MRP/ABCC PROTEINS)

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2.1. INTRODUCTION

Subsequent to the discovery of P-glycoprotein (P-gp), investigations of cancer cells displaying the multidrug resistance (MDR) phenotype not associated with ABCB1 expression led to the discovery of ABCC1, the founding member of the ABCC subfamily (1). At present, this subfamily C includes a total of 13 ABCC proteins, including the gene defective in cystic fibrosis (ABCC7) and the two sulfonylurea receptor genes (SUR1, SUR2). Most of these proteins (ABCC1–6 and ABCC10–12) have been identified as active ATP-dependent membrane transporters for organic anions or therapeutic compounds. In contrast to these active transporters, ABCC7, the cystic fibrosis transmembrane conductance regulator (CFTR), is a regulated chloride channel, while ABCC8 (SUR1) and ABCC9 (SUR2) are sulfonylurea receptors and best described as intracellular adenosine triphosphate (ATP) sensors, regulating the permeability of specific K⁺ channels.

Genetic variation in these genes is the cause of or contributes to a wide variety of human disorders with Mendelian and complex inheritance, including Pseudoxanthoma elasticum (PXE) (ABCC6), cystic fibrosis (CFTR/ABCC7), persistent hyperinsulinemic hypoglycemia of infancy (SUR1/ABCC8), and MDR phenotypes (ABCC1). Hereditary deficiency of ABCC2, known as Dubin–Johnson syndrome (DJS) in humans, causes an increased concentration of bilirubin glucuronosides in blood.

Several members of different ATP-binding cassette (ABC) transporter subfamilies are capable of transporting an extraordinarily structurally diverse array of endo- and xenobiotics and their metabolites across cell membranes. Together, these transporters play an important role in the absorption, distribution, and elimination of exogenous (aflatoxin B1 metabolites, pesticides) and endogenous molecules (leukotriene C4 [LTC4], E217βG) in the body.

In this chapter, current knowledge of the biochemical, physiological, and pharmacological properties of nine members of the multidrug resistance protein (MRP)-related ABCC subfamily (ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC6, ABCC10, ABCC11, and ABCC12) are summarized.

2.2. STRUCTURAL SIMILARITIES AMONG ABCC PROTEINS

The identification of the complete set of human abcc genes allows a comprehensive phylogenetic analysis of this subfamily. The proposed nomenclature and topology of “short” and “long” abcc proteins is in excellent agreement with the phylogenetic tree obtained (Fig. 2.1).

At present, there are no high-resolution structural data available on any eukaryotic ABCC transporter, in part due to the difficulty of obtaining crystals with these proteins. Therefore, biochemical experiments (mutagenesis analyses, photolabeling studies) are important to elucidate their membrane topol-
ogy, their transport and catalytic functions, their positions, and the orientations of membrane-spanning segments within the polypeptide chain (2–5). The members of the ABCC subfamily fall into two different subclasses (“short” and “long”) on the basis of the phylogenetic tree, their domain arrangement, and the membrane topology (Fig. 2.2).

The membrane topology model for human ABCC1 was described when the hydrophobicity analysis of the aligned sequences yielded a close matching of the transmembrane segments, thus suggesting a 6 + 6 transmembrane α-helix topology for the reference transporter ABCB1 as well. However, ABCC1 contains an N-terminal extension of about 250 amino acids. This highly hydrophobic amino-terminal segment of ABCC1 was suggested to be membrane embedded with five transmembrane helices as defined TMD0 (transmembrane domain 0) and an extracellular N-terminal amino acid (Fig. 2.2) (2, 6, 7).

ABCC1, 2, 3, ABCC6, and ABCC10 proteins form a subcluster within which each member possesses the N-terminal TMD0 (approximately 250 additional amino acids). The N-terminal TMD0 is absent from ABCC4, 5, 11, and 12. The presence of a TMD0 domain in the long ABCC transporter is unique; The TMD0 of ABCC1 does not play a crucial role in transport activity of the protein, while the presence of the intracellular L0 loop is necessary for ABCC1

**FIGURE 2.1.** Dendrograms based on entire sequences (structural similarities) of the full-length ABCC proteins. Dendrograms are based on ClustalW alignments and were generated using Tree View. Topologies of “short” and “long” ABCC proteins were reported. The transcript and protein size were also indicated for each ABCC proteins.

![Dendrogram of ABCC proteins](image)
TRANSPORT ACTIVITY AND FOR THE PROPER INTRACELLULAR ROUTING OF THE PROTEIN (5, 8). TMD0 HAS BEEN DESCRIBED AS IMPORTANT FOR ABCC1 RETENTION IN, OR RECYCLING TO, THE PLASMA MEMBRANE. TMD0 BECOMES ESSENTIAL FOR TRAFFICKING OF ABCC1 WHEN THE CARBOXYL-TERMINAL REGION OF THE PROTEIN IS MUTATED. THIS MEANS THAT THE TMD0 OF ABCC1 PLAYS A ROLE IN THE INTERNALIZATION OF ABCC1 (5). IN THE CASE OF ABCC1, THE EXTRACELLULAR POSITION OF THE NH₂ TERMINUS IS SUPPORTED BY A LARGE AMOUNT OF EXPERIMENTAL DATA (2, 9). VERY RECENTLY, YANG ET AL. DEMONSTRATED A NEW FUNCTION OF THE TMD0-L0 REGION IN THE DIMERIZATION ABILITY OF ABCC1. THESE RECENT FINDINGS SUGGEST THAT ABCC1 MAY EXIST AND FUNCTION AS A DIMER AND THAT TMD0-L0 LIKELY PLAYS A ROLE IN SOME STRUCTURAL AND REGULATORY FUNCTIONS (10).

2.2.1. Transport Cycle

To achieve export, ABCC transporters require a minimum of two TMDs that form the ligand-binding sites and provide specificity for the transported substrate and two nucleotide-binding domains (NBDs) that bind and hydrolyze ATP to drive the translocation of the bound substrate (Fig. 2.3). Substrate binding is thought to occur through interactions within these transmembrane helices, as suggested by photolabeling experiments and site-directed mutagenesis analyses (11–25). The TMDs are not homologous between proteins of the ABCC subfamilies, explaining how different ABCC transporters can handle...
extremely diverse substrates, including glucuronide metabolites or reduced glutathione (GSH) conjugates (see PART IV).

In contrast to the TMDs, the structural similarities between NBDs imply that they share a common evolutionary origin. NBDs have several characteristic motifs, including the Walker A and B motifs common to many nucleotide-binding proteins and other motifs like the ABC signature (motif C), stacking aromatic D-, H-, and Q-loops, which are unique and highly conserved in the ABCC subfamily. Thus, it is appropriate to consider an ABC transporter (at least in the closed dimer form, Fig. 2.3) as having two ATP-binding pockets with both NBDs contributing to each pocket, rather than each NBD having a separate ATP-binding site. Structural data show that the two ATP-binding pockets are not independent but are located at the interface of an NBD dimer “sandwich” (26–28). This implicates that the two NBDs act in concert as a single step rather than influencing distinct steps in the transport cycle (Fig. 2.4). The minimal ABC transporter has four domains. Two TMDs bind the ligand, while transport is driven by ATP binding and hydrolysis by the two NBDs. The Walker A motif appears to wrap around the phosphate chain of ATP. The Walker B motif contributes an aspartate residue that coordinates and stabilizes a magnesium ion, which is required for ATP hydrolysis (29–31). Similarly, Asp and His in the D- and H-loop, respectively, make contact with water that stabilizes the binding of the nucleotide, while the conserved Gln residue in the Q-loop interacts with the catalytic Mg$^{2+}$ and hydrolyzes a water molecule. The Q-loop glutamine also interacts with the γ-phosphate of ATP via a water molecule. In addition, the position of the Q-loop is important because it is in a position to couple the ligand-binding sites within the TMDs to the ATP-binding sites of the NBDs. These structural data are fully
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reviewed by Deeley et al. (4), Linton and Higgins (32), and Sauna and Ambudkar (33).

The “ATP-switch” model is the product of recent advances in structure determination and of biochemical data from a number of ABC transporters (4, 33, 34). Transport activity is a multistep process involving communication via conformational changes between NBDs and TMDs. The driver for transport is an on-off “switch” between two principal conformations of the NBDs: a “closed dimer” formed by binding two ATP molecules at the dimer interface (step 3) and an “open dimer” resulting from the dissociation of the “closed dimer,” facilitated by ATP hydrolysis and PO$_4$/ADP release. The “switch” from the “open” to the “closed” conformation of the NBD dimer induces conformational changes necessary for transport in the TMDs. The reverse of this switch, from the “closed” dimer to the “open” dimer after ATP hydrolysis (steps 3-4), resets the transporter to be ready for the next transport cycle (Figs. 2.3 and 2.4).

In Fig. 2.4, we describe a model of the hypothetical transport cycle of ABCC1, based on the schematic for export of vinblastine by ABCB1/P-gp...
adapted from Linton and Higgins and fully described in the review, “Structure and Function of ABC Transporters” (4, 32).

Step 1. The cycle is initiated by binding of substrate (such as LTC4, GSH) to its high-affinity site(s), thereby strongly increasing affinity of NBD1 for ATP.

Step 2. This initial binding of ATP by NBD1 facilitates the binding of a second molecule of ATP at NBD2. Consequently, a “closed dimer” formed by binding two ATP molecules at the dimer interface is performed. The conformational changes resulting from ATP binding are transmitted to the TMDs such that the substrate-binding site is exposed extracellularly and its affinity for substrate is reduced. Substrate is released extracellularly.

Steps 3–4. To restore the basal conformation, hydrolysis of ATP may occur sequentially at the NBDs. Sequential release of PO\textsubscript{4}, then ADP, restores the transporter to its basal configuration. The protein maintains a low-affinity state following hydrolysis of ATP at NBD2, as long as NBD1 is occupied by ATP and ADP has not been released by NBD2. These data are strongly supported by experimental data (34). Nevertheless, how the protein resets for another cycle remains to be elucidated and hinges on whether or not NBD1 is catalytically active (Fig. 2.4) (4).

Interestingly, in the ABCC proteins, this catalytic cycle deviates in some cases from the scheme commonly described for other ABC proteins. In ABCC1, the NBDs behave very differently with respect to both ATP binding and hydrolysis. High-affinity binding of azido-ATP to NBD1 is readily demonstrable, while under hydrolytic conditions in the presence of vanadate, ADP is trapped predominantly by NBD2. Furthermore, binding of azido-ATP and the trapping of ADP by NBD2 requires that NBD1 be able to bind and possibly to hydrolyze ATP. In contrast, binding of ATP by NBD1 remains readily detectable when NBD2 is inactivated by mutations that eliminate ATP binding or ATPase activity. Mutation of the Walker A motifs in each NBD also has different effects on transport activity (35, 36). Mutation of the conserved Walker A Lys residue in NBD1 only partially inactivates the protein while the comparable mutation in NBD2 essentially eliminates transport activity by a significant alteration of the tertiary structure of the protein (35–37). In addition, structural analysis of NBD1 revealed that the Walker A Ser685 forms a hydrogen bond with the Walker B Asp792 and interacts with magnesium and the beta-phosphate of the bound ATP. The interaction between the hydroxyl group of 685 residue and the carboxyl group of Asp792 plays a crucial role for the protein folding, and the interactions of the hydroxyl group at 685 with magnesium and the beta-phosphate of the bound ATP play an important role for ATP-binding and ATP-dependent solute transport (38). Recently, the acid glutamate following the Walker B motif in most NBDs of ABC proteins has been described to be critical for cleavage of the β-γ phosphodiester bond of
ATP. Nevertheless, in NBD1 of ABCC1 (and other ABCC, Fig. 2.4), this residue is Asp. The lack of glutamate has a profound effect on the ATP binding and hydrolysis characteristics of the NBD and, as a consequence, on the catalytic cycle of ABCC1 (39). Interconversion of these two residues profoundly affects the ability of the mutated NBDs to bind, hydrolyze, and release nucleotides (39). Therefore, regardless of whether the bound ATP at NBD1 is hydrolyzed or not, the release of the bound nucleotide from NBD1 may bring the protein back to its original conformation and facilitate the protein’s starting of a new cycle of ATP-dependent solute transport (40). Similarly, substitution of H827 of H-loop in NBD1 with residues that prevented formation of these hydrogen bonds had no effect on the ATP-dependent solute transport, whereas corresponding mutations in NBD2 almost abolished the ATP-dependent solute transport completely (41). In contrast, substitutions of H1486 in H-loop of NBD2 with residues that might potentially form these hydrogen bonds exerted either full function or partial function. This suggests that hydrogen-bond formation between the residue at 1486 and the gamma-phosphate of the bound ATP and/or other residues, such as putative catalytic base E1455, together with S769, G771, T1329, and K1333, etc., holds firmly all the components necessary for ATP binding/hydrolysis so that the activated water molecule can efficiently hydrolyze the bound ATP at NBD2.

In recent years, due to crystallographic studies, much progress has been made in the elucidation of the three-dimensional structures of ABC transporters, including novel models for nucleotide hydrolysis and translocation catalysis. However, site-directed mutagenesis as well as the identification of natural mutations is still a major tool to evaluate effects of individual amino acids on the overall function of ABC transporters. These data were fully reported in the review specially dedicated to Frelet’s mutation analysis (3).

2.2.2. Genomic Organization

Adjacent to the abcc6 gene, the abcc1 gene maps to chromosome 16p13.1 and was identified in the small-cell lung carcinoma cell line NCI-H69 (Fig. 2.5) (1). Nine alternative splicings were later described (nine isoforms: P33527-1, P33527-2, P33527-3, P33527-4, P33527-5, P33527-6, P33527-7, P33527-8, P33527-9). The abcc2 (MRP2/canalicular multispecific organic anion transporter [cMOAT]), abcc3 (MRP3), abcc4 (MRP4, MOATB), and abcc5 (MRP5/MOATC) genes map respectively to chromosomes 10q24, 17q21.3, 13q32, and 3q27 (Fig. 2.1). The genomic organization of abcc1, 2, 3, 4, 5, and 6 is fully and clearly described on the following website (http://nutrigene.4t.com/humanabc.htm).

The human abcc10 gene, consisting of 22 exons and 21 introns, greatly differs from other members of the human ABCC subfamily. It is localized on chromosome 6 (6p21). The abcc10 cDNA sequence encodes a 1492 amino acid ABC transporter whose structural architecture resembles that of abcc1 in that its transmembrane helices are arranged in three TMDs (see Fig. 2.1).
STRUCTURAL SIMILARITIES AMONG ABCC PROTEINS

However, in contrast to the latter transporters, a conserved N-linked glycosylation site is not found at the N-terminus of abcc10 (42).

Phylogenetic analysis determined that abcc11 and abcc2 are derived by duplication and are most closely related to the abcc gene. They are tandemly located on human chromosome 16q12.1 (Fig. 2.5) (43, 44). The abcc11 gene produces two major transcripts of 4.5 kb (abundant in breast) and 4.1 kb (abundant in testis). The 4.5-kb transcript has an open reading frame of 1382 amino acids. The 4.5-kb abcc11 transcript consists of 31 exons and is located in a genomic region of over 80.4 kb on chromosome 16q12.1 (43, 45). Similarly, the abcc12 gene also has two major transcripts of 4.5 kb and 1.3 kb. In hormonally regulated tissue such as breast cancer, normal breast, and testis, the abcc2 gene transcript is 4.5 kb in size and encodes a 100-kDa protein. When compared with closely related ABC family members, it lacks transmembrane domains 3, 4, 11, and 12, and the second NBD. In other tissues including brain, skeletal, muscle, and ovary, the transcript size is 1.3 kb. Because of the unusual topology of the two variants of abcc12, the authors speculated that they may have a different function from other family members. The cloned murine Abcc12 cDNA was 4511 bp long, comprising a 4101 bp open reading frame. The deduced peptide consists of 1367 amino acids and exhibits high sequence identity (84.5%) with human abcc12. In addition to the abcc12 transcript, two splicing variants encoding short peptides (775 and 687 amino acid residues)
were detected. In spite of the genes coding for both \textit{abcc}11 and \textit{abcc}12 being tandemly located on human chromosome 16q12.1, no putative mouse orthologous gene corresponding to the human \textit{abcc}11 was detected at the mouse chromosome 8D3 locus (Fig. 2.5).

The \textit{abcc}13 gene appears to have undergone a process of gradual pseudo-genesiation in mammals. On chromosome 21q11.2, spanning 90kb is an \textit{abcc}13 gene-like sequence with the highest similarity to \textit{abcc}2. The open reading frame of this transcript is capable of encoding a polypeptide of only 325 amino acids, compared to the 1500 amino acids of the most-related ABC transporter. The \textit{abcc}13 gene is highly expressed in the fetal liver, bone marrow, and colon. Its expression in peripheral blood leukocytes of adult humans was much lower and no detectable levels were observed in differentiated hematopoietic cells (46). The human locus is now thought to be a pseudogene incapable of encoding a functional ABC protein (47).

2.3. ENDOGENOUS EXPRESSION LEVELS AND PHYSIOLOGICAL FUNCTIONS OF ABCC PROTEINS

ABCC transporters play an important role in mediating the cytoplasmic concentration of endogenous and exogenous substances. They therefore influence the pharmacokinetic profile of a variety of drugs. Numerous ABCC members are associated with a broad spectrum of physiological functions, including detoxification, defense against xenobiotics, and oxidative stress. By virtue of their localization in the plasma membranes in the intestine, liver, blood–brain, and other vital biological barriers, a majority of ABCC drug transporters limit xenobiotic absorption from the gut and xenobiotic entry into the central nervous system and cause drug–drug interactions, decreased drug efficacy, and MDR for chemotherapeutic agents (Figs 2.6 and 2.7). They are also present in liver and kidney, organs important for the excretion of potentially toxic xenobiotics, metabolites, and endogenous waste products. Thus, elucidating which drug entities are substrates for ABCC drug transporters is a crucial step both in drug development and for adequate use of therapeutic compounds. Tissue distribution and ontogeny data are important components of understanding and extrapolating pharmacokinetic data from mice to humans and are crucial to elaborating various knockout models to explore specific physiological functions of ABCCs.

Another interesting observation is that an altered level of ABCC expression due to exposure to hormones or xenobiotics (environmental toxins or drugs) may change the kinetic parameters of ABCC substrates (such as vin-cristine or methotrexate) and may therefore have clinical implications. These specific regulation mechanisms are briefly summarized in this Part. Regulation of ABCC expression by xenobiotics are fully described in the referenced reviews (48–52).
FIGURE 2.6. Molecule transit in the human body. This scheme illustrates absorption and excretion phenomena of substances with the principal pathways by which endo- and exogenous molecules travel within the human body thanks to ABCC transport.

FIGURE 2.7. ABCC protein expression in human tissues. This scheme illustrates the principal tissues where ABCC proteins and transcripts have been described.
2.3.1. ABCC1

Described in 1992 (1), ABCC1 is found ubiquitously expressed in normal tissues, with highest levels in the lung, testis, kidney, and peripheral blood mononuclear cells (15). In polarized cells, ABCC1 is localized to basolateral plasma membranes (53–56). ABCC1 was found to be localized to the luminal side of brain capillary endothelial cells (Figs. 2.7 and 2.8) (57).

ABCC1 functions as a multispecific organic anion transporter, with (oxidized) glutathione (GSSG), cysteinyl leukotrienes, glucuronides, sulfate conjugates of steroid hormones, bile salts, and activated aflatoxin B1 as substrates. It also transports drugs (such as vincristine) and other hydrophobic compounds in the presence of glutathione. Furthermore, the ability of ABCC1 to transport both GSH and GSSG raises the possibility that the protein contributes to maintenance of the cell redox state. The levels of free GSH are decreased in cells overexpressing the protein and are increased in tissues from Abcc1−/− mice that normally express high levels of the protein (58–60). The GSH and GSH-conjugate transport by ABCC1 is fully described in the review by Cole and Deeley (61). Furthermore GSH is released by cells undergoing apoptosis. Several studies strongly supported that ABCC1 is responsible for this GSH release (62, 63). Furthermore, verapamil (the S-isomer but not the R-isomer) triggers apoptosis through stimulation of GSH extrusion mediated

**FIGURE 2.8.** ABCC protein localization in polarized cells. Proteins are symbolized by their number (1 = ABCC1, 2 = ABCC2, and so on…).
by ABCC1. Induction of apoptosis of ABCC1 cells may represent a novel approach in anticancer treatment (64, 65).

Like Mdr1a−/−/1b−/− and Bcrp1/Abcg2−/− knockout mice, Abcc1−/− knockout mice were found to be viable, healthy, and fertile (58, 66). These models allowed the characterization of the crucial functions of ABCC1. Particular data of Abcc1−/− knockout mice strongly supported a role for ABCC1 in xenobiotic defense by transporting various substrates, including toxic molecules present in tobacco smoke, which is the principal risk factor for chronic obstructive pulmonary disease. Very recently, van der Deen et al. (67) demonstrated that using mice lacking both Abcc1 and Mdr1a/1b genes (called triple knockout [TKO] mice), Abcc1−/− mice display elevated glutathione levels in tissues that normally have a high Abcc1 expression, for example, in the lungs possibly as a compensation mechanism for the elevated pulmonary oxidative stress (58, 67). A decrease of the inflammatory response to cigarette smoke exposure in the lungs of Abcc1/Mdr1a/1b TKO mice was observed compared to wild-type mice. A possible explanation for the lower inflammatory response in TKO mice is an impaired transport of the pro-inflammatory mediator LTC4 (66), an important physiological high-affinity substrate for ABCC1. Nevertheless, during inflammation, other cytokines can be produced such as interleukin-6 (IL-6). The first evidence for a putative role of IL-6 in the regulation of Abcc1 expression in HepG2 cells and in liver was described (68, 69). Recently, a positive regulation of Abcc1, 3, 4 expression levels has been reported (70) in normal human epidermal keratinocytes and in primary human dermal fibroblasts exposed to IL-6 in combination with its soluble α-receptor or oncostatin. Since ABCC1 and ABCC4 have been shown to function as a prostaglandin derivate efflux transporter (71–73) and IL-6-type cytokines are known to induce the expression of COX-2 in certain cell types (74, 75), it is tempting to speculate that increased prostaglandin efflux might be one additional means by which IL-6 can contribute to inflammation. Mice lacking Abcc4 exhibited a decrease in plasma prostaglandin metabolites and an increase in inflammatory pain threshold compared to wild-type mice, which confirms ABCC4 involvement in inflammatory situations (73).

Other interesting observations have shown the crucial importance of ABCC1 expression in the migration of dendritic cells from peripheral tissues to the lymph nodes. Dendritic cells are antigen-presenting cells involved in T-cell activation. They acquire antigens in peripheral tissues and migrate to lymph nodes where they localize to the T-cell-rich paracortex (76). Migration of dendritic cells was reduced in Abcc1−/− mice and restored to the levels found in wild-type animals when exogenous cysteinyl leukotrienes (LTC4 and LTD4) were administered. Thus, LTC4 efflux from these cells by ABCC1 is critical for migration.

To study the age influence on ABCC-related activity in cells from the immune system, ABCC-related activity was studied in murine bone marrow
cells and thymocytes of young (3–4 weeks old), adult (2–3 months old), and old (18 months old) mice. Although all thymocytes expressed the ABCC1 molecule in an active form and aging did not affect this pattern, the authors observed a modification of ABCC-related activity in the bone marrow according to age. This alteration of ABCC transport activity may be related to other protein isoforms of this subfamily (77).

After severe injury, the liver can undergo partial regeneration. This process depends in part on proliferation and differentiation of hepatic progenitor cells. After treatment of rats with 2-acetylaminofluorene (2-AAF) followed by partial hepatectomy, treated animals showed increased hepatic mRNA levels of the genes encoding Mdr1b, Abcc1, and Abcc3 in periportal progenitor cells and of the Mdr1b protein in periportal hepatocytes. These functional upregulations of Abcc1 and Abcc3 proteins may have a cytoprotective role in conditions of severe hepatotoxicity (78). By contrast, Abcc2 protein levels did not change during liver regeneration following partial hepatectomy in rats, suggesting that ABCC2-mediated secretion is well-preserved in the growing liver (79). But a switch in expression from the apical Abbc2 to the basolateral Abcc1 protein has also been reported in cultured rat hepatocytes entering the cell cycle (80). Consequently, as in human proliferating hepatocytes and in periportal progenitor cells Abcc1 levels are strongly increased, this up-regulation of Abcc1 may be associated with cell proliferation. Furthermore, in mouse hematopoietic cells, Abcc1 is expressed, and 50% of precursor cells showed Abcc activity (Fluo-3 transport activity) (77). Taken altogether, this indicates that Abcc1 plays a physiological function in immature cells.

2.3.2. ABCC2

Abcc2 has been cloned from various species, including humans, dogs, mice, rats, and rabbits and is physiologically expressed predominantly at the hepatocyte canalicular membrane (81–86). It is also present at the brush-border membrane of renal proximal tubule cells (87, 88), at the absorptive enterocytes (89, 90), and at the apical membrane of gallbladder epithelial cells (Figs. 2.7 and 2.8) (91). Its location in the brain is a matter of debate. Most previous studies failed to determine Abcc2 mRNA or protein in the brain or cell preparations from the brain of different species, including humans (92). Taipalensuu et al. focused on the human jejunum and found a transporter expression with the following ranking: ABCC2 > ABCB1 ∼ ABCC3 > ABCC5 and ABCC1 > ABCC4 (93). In addition, Abcc2 mRNA transcripts have been detected in low levels in other tissues in the rat, including the lung and the stomach (94), and in peripheral blood cells, especially in CD4+ lymphocytes (95). ABCC2 colocalizes with ABCB1 and ABCG2 (96). Expression of ABCC2 in nuclear membranes in human tissues is specific for poorly differentiated cells, including stem cells (97).

During development in the rat, ABCC2 levels are low in the fetal liver, and they increase neonatally after birth to reach adult levels at 4 weeks (98). Rat
liver progenitor cells also minimally express ABCC2 (99). During pregnancy, ABCC2 levels decreased in the liver but remained preserved in the small intestine (100, 101). In human placenta, ABCC2 has been detected on apical syncytiotrophoblast membranes (102). The placenta serves, in part, as a barrier to exclude noxious substances from the fetus. In humans, a single-layered syncytiotrophoblast of polarized trophoblast cells and the fetal capillary endothelium separate the maternal and fetal circulations. Immunofluorescence and immunoblotting studies demonstrated that ABCC2 was localized in the apical syncytiotrophoblast membrane while ABCC1 and ABCC3 were predominantly expressed in blood vessel endothelia. Given the cellular distribution of these transporters, ABCC isoforms were suggested to serve to protect fetal blood from entry of organic anions and to promote the excretion of glutathione/glucuronide metabolites in the maternal circulation.

Genetic variation in the \textit{abcc2} gene results in the DJS, a disease characterized by conjugated hyperbilirubinemia. Mutations leading to DJS are either associated with complete absence of immunohistochemically detectable ABCC2 in affected patients or impaired protein maturation and sorting (103–113). Acquired or hereditary deficiency of ABCC2 causes an increased concentration of bilirubin glucuronides in blood because of their efflux from hepatocytes via the basolateral ABCC3, which compensates for the deficiency in ABCC2-mediated apical efflux (114). The natural Abcc2 mutant TR-90 (transporter deficient rats) and Eisai hyperbilirubinemic (EHBR) rats have significantly contributed to the understanding of human ABCC2 transport characteristics. Mutant rats lacking the cMOAT, designated further as ABCC2, have a hyperbilirubinemic phenotype and impaired secretion of bilirubin glucuronides, sulphated bile salts, glutathione-conjugates, and GSH into the bile (115). \textit{Abcc2}−/− mice are healthy and exhibit biochemical abnormalities (116–118). These rats and mice serve as models for Dubin–Johnson disease as this disorder is caused by mutations in the \textit{abcc2} gene.

Abcc2 expression is reported as markedly altered in various rat models of cholestasis such as bile duct ligation and endotoxin- or phalloidin-induced cholestasis (119–122). All three cholestatic models resulted in a marked decrease in Abcc2 protein and its tissue localization at the canalicular membrane, except in ethinylestradiol-treated rats. After endotoxin treatment, the normally sharply delineated canalicular staining of Abcc2 had changed to a fuzzy pattern, suggesting an abnormal localization in a subapical compartment. Simultaneously, an upregulation of Abcc1 and mdr1b is observed and may confer resistance to hepatocytes against cytokine-induced metabolic stress and also compensate the canalicular excretion deficiency of Abcc2 (122).

ABCC2 likely plays a major role in the pharmacokinetics of many drugs through the regulation of their intestinal absorption and their biliary or renal elimination. Consequently, an alteration of its expression by exposure to hormones or xenobiotics and also of its activity may change the kinetic parameters of drugs. Indeed, biliary secretion of endogenous substrates such as bilirubin conjugates may be impaired. Recently, it has been shown that ABCC2
expression levels are regulated by hormones. For example, ethynylestradiol strongly reduces Abcc2 expression in rats (123). Similarly, sexual steroids decrease Abcc2 expression at a posttranscriptional level in pregnancy (124). In contrast, dexamethasone (DEX) has been described to increase ABCC2 protein expression in various tissues (125) and liver (126, 127). RU486 and the antiglucocorticoid Pregnenolone 16 α-carbonitrile (PCN) were also found to increase Abcc2 transcripts in primary hepatocytes. Using Pregnane X receptor (PXR) −/− mice, Kast et al. (128) fully demonstrated that DEX and PCN regulated Abcc2 expression by activation of PXR signalling pathway. This was supported by the fact that PXR has been shown to bind with the ER-8 motif found in abcc2 promoter as a heterodimer with the 9-cis retinoic acid receptor (94, 128). Other PXR agonists such as the peptide mimetic HIV protease inhibitor ritonavir also induced ABCC2 mRNA levels in primary human hepatocytes (129). Furthermore, in vivo administration of rifampicin to humans also increased ABCC2 mRNA and protein in the duodenum (130). Interestingly, induction of ABCC2 by PXR ligands is associated with up-regulation of other PXR-controlled detoxifying proteins such as cytochrome P450 3A4 and ABCB1 (129), thus outlining their coordinated expression in response to certain xenobiotics. Complementary data about regulation of ABCC2 expression by xenobiotics are reported in the reviews from Haimeur et al. (48), Payen et al. (49), and Catania et al. (50). (48–50).

2.3.3. ABCC3

Cloned in human, rat, and mouse (94, 131–135) and detected in liver, intestine, and adrenal gland, ABCC3 is found on basolateral membranes of intrahepatic bile-duct epithelial cells (cholangiocytes), hepatocytes surrounding the portal tracts, intestinal epithelial cells (90, 136, 137), and polarized Madin–Darby canine kidney (MDCK) cells transfected with an ABCC3 construct (Figs. 2.7 and 2.8) (114, 138, 139). ABCC3 expression is higher in the colon than in the ileum (140). Furthermore, ABCC3 appeared to be the most abundantly expressed transporter in investigated parts of the human intestine, except for the terminal ileum, where ABCB1 showed the highest expression. All transporters showed alterations in their expression levels from the duodenum to the sigmoid colon. The ranking of transporter gene expression in the duodenum was ABCC3 >> ABCB1 > ABCC2 > ABCC5 > ABCC4 > ABCC1. In the terminal ileum, the ranking order was as follows: ABCB1 > ABCC3 >> ABCC1 ~ ABCC5 ~ ABCC4 > ABCC2. In all segments of the colon (ascending, transverse, descending, and sigmoid), the transporter gene expression showed the following order: ABCC3 >> ABCB1 > ABCC4 ~ ABCC5 > ABCC1 >> ABC C2 (141).

Under normal conditions, only low levels of ABCC3 are found in the liver; however, strong ABCC3 staining was observed in hepatocytes from ABCC2-deficient Dubin–Johnson patients (DJS) (114). This has been demonstrated in rodent models of DJS (e.g., the EHBR rat), in which Abcc3 mRNA and protein expression in the liver and the kidney are increased significantly (142).
Furthermore, induction of Abcc3 also occurs in cholestatic rat (143) and human livers (114, 138), which further supports up-regulation of Abcc3 as a protective mechanism (i.e., bilirubin and metabolite removal) when ABCC2 is either absent or nonfunctional. In fact, ABCC2 and ABCC3 expression was inversely correlated under conditions of reduced ABCC2 expression. ABCC3 is a highly inducible retrograde transporter that can efflux organic anions from hepatocytes into blood for eventual excretion into urine (144, 145). Furthermore, its localization on basolateral membranes of cholangiocytes and enterocytes suggests that ABCC3 may also be involved in the recirculation of bile salts from these cells into the blood (enterohepatic circulation). Nevertheless, two recent reports have presented controversial data, showing that bile acid homeostasis is not perturbed in Abcc3−/− mice (146, 147).

2.3.4. ABCC4

ABCC4 is expressed at low levels in a variety of human tissues with high levels occurring in pituitary cells (148), lung, prostate, and proximal tubules in the kidney (149–157). ABCC4 was clearly localized by confocal laser scanning microscopy to the luminal side of brain capillary endothelial cells. The ABCC4 protein was also detected in astrocytes of the subcortical white matter (57). ABCC4 can also release prostaglandins from cells, thus suggesting its involvement in inflammatory processes (72).

The accumulation of adefovir and tenofovir in kidney was significantly greater in Abcc4 knockout mice, suggesting that ABCC4 is involved in the luminal efflux of both adefovir and tenofovir (158). ABCC4 is relatively highly expressed at the blood–brain barrier (BBB), suggesting that ABCC4 may play a role in nucleotide penetration or retention in the brain (159, 160). ABCC4, 5, and 11 are efflux transporters of cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP). Thus, these transporters may play roles in the regulation of intracellular levels of cyclic nucleotides, which are important as “second messengers” of intracellular signalling. The membrane localization of ABCC4 in polarized cells remains unresolved. Although ABCC4 was found in the basolateral membrane of tubuloacinar cells of the prostate (161) and in the sinusoidal membrane of human, rat, and mouse hepatocytes (162, 163), ABCC4 was localized in the apical membrane in kidney proximal tubule epithelia (159, 160). It has also been reported at low expression in the liver, suggesting that the role of ABCC4 in hepatic transport is minor under basal conditions.

Furthermore, using Abcc4−/− mice as a model system, and the nucleotide analogue (9-(2-phosphonyl-methoxyethyl)adenine; PMEA) as a probe, Kruh et al. (164) demonstrated the ability of ABCC4 to function in vivo as an endogenous resistance factor. Abcc4-null mice treated with PMEA exhibit increased lethality associated with marked toxicity in bone marrow, spleen, thymus, and gastrointestinal tract. An increase of PMEA penetration into the brain was also observed in Abcc4−/− mice, suggesting that the pump may be a component of the BBB for nucleoside-based analogues (164).
2.3.5. ABCC5

ABCC5 is expressed in almost every tissue of the human body, with highest expression in skeletal muscle followed by the brain (Figs. 2.7 and 2.8) (149, 165). Abcc5 in mice is predominantly expressed in the brain with significant expression in gonads, placenta, lung, and stomach. ABCC5 is preferentially localized in the basolateral membrane of syncytiotrophoblasts and in and around fetal vessels. Transcripts of ABCC5 decrease with gestational age (166). ABCC5 was clearly located at the luminal side of brain capillary endothelial cells and was also detected in astrocytes of the subcortical white matter and in pyramidal neurons (57).

The tissue expression of mouse Abcc5 is reported to be similar to that in humans and rats (135, 167, 168). Abcc5 knockout mice are healthy and fertile and do not show any observable physiological dysfunctions. Even if the substrate pattern of ABCC5 is much narrower than that of ABCC4, ABCC5 serves as a transporter for organic anions and drugs, including vincristine, LTC4, etoposide, PMEA, or daunorubicin (156, 157, 169–171). ABCC5 is routed to the basolateral membrane in polarized cells, including ABCC5-transfected MDCKII cells.

2.3.6. ABCC6

Although expression levels of ABCC6 are consistently and constitutively high in the liver of humans, mice, and rats, the functional significance of this expression still remains poorly understood. In the liver, ABCC6 is localized to both the basolateral (high level) and canalicular (low level) plasma membranes of hepatocytes (172–175).

Abcc6 was initially cloned from rat liver (143) and has been subsequently cloned in humans and mice (172, 174, 176). The mouse and rat ABCC6 orthologs show greater than 78 % amino-acid identity with human ABCC6. Mutations in the abcc6 gene have been implicated in the etiology of PXE, a hereditary connective tissue disorder characterized by loss of tissue elasticity (affecting skin, retina, and blood vessels) (177–180). The phenotype of the Abcc6/- mouse shares calcification of elastic fibers with the human PXE disease, which makes this model a useful tool to further investigate the etiology of PXE (181). Abcc6/1(-/-) and Abcc6/3(-/-) double knockout mice exhibited connective tissue mineralization similar to that observed in Abcc6(-/-) mice (182), suggesting that Abcc1 and Abcc3 do not modulate the effects of Abcc6 in this mouse model.

The physiological importance of ABCC6 expression in the liver remains to be further elucidated. The only ABCC6 substrate identified thus far is BQ123, a cyclopentapeptide antagonist of the endothelin receptor (179). Because of its constitutive expression in the liver and its capacity to transport BQ123, ABCC6 was suggested to function as a transporter for small peptides involved in cellular signaling and/or autocrine or paracrine regulation of hepatocellular
functions (172, 179). Chinese hamster ovary cells transfected with ABCC6 cDNA show increased resistance to a variety of anticancer agents, including etoposide, doxorubicin, daunorubicin, and cisplatin, but not to vincristine or vinblastine (179).

2.3.7. ABCC10

Information on the ABCC10 protein expression pattern in tissues has not yet been reported. Using reverse transcriptase polymerase chain reaction, ABCC10 transcripts were detected in most tissues with relatively higher levels reported in the pancreas, testis, colon, spinal cord, tonsils, lung, trachea, and skin (42), with significant intestinal expression and moderate expression in the liver, ovary, uterus, and placenta (42, 183). Two Abcc10 genes have also been identified in mice (Mrp7A and Mrp7B). They show more than 80% amino-acid similarity with their human counterparts (184). Murine Abcc10 is 84% identical to abcc10. Its transcript is expressed in many tissues with highest levels in the testis, ovary, gut, kidney, and lung (42, 135, 185). Rat Abcc10 transcripts were reported to have been expressed in the liver, kidney, and ileum (186).

ABCC10 transports anticancer compounds, estrogen-glucuronides, and leukotrienes (42, 183, 187). Fluorescence in situ hybridization indicated that abcc10 gene maps to chromosome 6p12–21 in proximity to several genes associated with glutathione conjugation and synthesis, suggesting its involvement in phase III (cellular extrusion) of detoxification. ABCC10 transports estradiol-17β-glucuronide that was susceptible to competitive inhibition by LTC4, glycolithocholate 3-sulfate, MK571, and lipophilic agents (cyclosporine A). Of the inhibitors tested, LTC4 was the most potent, in agreement with the possibility that it is a substrate of the pump (183). Drug resistance to docetaxel and, to a lesser degree, paclixetaxel, vincristine, and vinblastine was reported (188). ABCC10 does not transport methotrexate, dinitrophenyl-S-glutathione (DNP-SG), monovalent bile salts (glycocholic acid and taurocholate), or cyclic nucleotides (cAMP and cGMP) (183). Further studies examining substrate specificity, subcellular localization, and physiological function are needed to clarify the role, if any, that ABCC10 may play in the development of the MDR phenotype in salivary gland adenocarcinoma cell lines (189).

2.3.8. ABCC11

In spite of contradictory data about ABCC11 transcript expression in tissues (Figs. 2.7 and 2.8), ABCC11 levels are expressed in a variety of human tissues, including normal breast, ovary, lung, testis, kidney, liver, colon, and brain (43–45). In transfected MDCKII and HepG2 cells, ABCC11 is localized at the apical pole (190). Despite extensive searches within the mouse genome, a murine ABCC11 ortholog was not found (191).
ABCC11 mediates transport of estradiol-17β-glucuronide, dehydroepiandrosterone 3-sulfate, as well as LTC4 and the monoanionic bile acids taurocholate and glycocholate, but not prostaglandin E1 or E2 (190, 192). Because ABCC11-transfected cells show no resistance to vincristine, doxorubicin, etoposide, or taxol, it is unlikely these compounds are its substrates. Taken altogether, the resistance profiles of ABCC4, ABCC5, and ABCC11 are similar but not completely identical. In ABCC11-transfected porcine kidney LLC-PK1 cells, ABCC11 is responsible for resistance to the pyrimidine analogs 5′-fluoro-5′-deoxyuridine, 5′-fluorouracil, and 5′-fluoro-2′-deoxyuridine. As 5-fluorouracil is an essential component in the treatment of breast, colon, and head-and-neck tumors, expression of ABCC11 in those types of cancers would be of particular interest. It is worth mentioning that breast cancer is a hormonally responsive tumor and ABCC11 is able to transport steroid sulphates. Recently, the notion that expression of ABCC11 in estrogen receptor α-positive breast cancers may contribute to decreased sensitivity to chemotherapy combinations that include 5-fluorouracil was reported (193). Consequently, ABCC11 may be a potential predictive tool in the choice of anticancer therapies in estrogen receptor positive breast cancers. Additional studies, including protein expression studies in tumors, should be carried out to characterize the potential contribution of ABCC11 to the chemosensitivity of these cancers.

In the study of eight families, the chromosomal localization of the paroxysmal kinesigenic choreoathetosis (PKC) critical region is 16p11.2-q12, a section where the abcc11 gene is localized. PKC, the most frequently described type of paroxysmal dyskinesia, is characterized by recurrent, brief attacks of involuntary movements induced by sudden voluntary movements. This disease was linked to abcc11 mutations. Consequently, several studies suggested that specific mutations of abcc11 could be related to this hereditary disorder. However, recently, it has been demonstrated that mutations of Abcc11 can be ruled out as the cause of PKC (194).

A surprising physiological function of ABCC11 has been found by two interesting genetic analyses. For the first time, genetic evidence was observed for an association between the degree of apocrine colostrum secretion and human earwax type. Earwax (cerumen) is secreted by ceruminous apocrine glands. Human earwax consists of wet and dry types. Dry earwax, which lacks cerumen, is frequent in East Asians, whereas wet earwax is common in other populations. Earwax type is a Mendelian trait, with wet earwax dominant to the dry type. Yoshiura et al. determined that a single nucleotide polymorphism at nucleotide 538 (538G→A; 180 Gly→Arg) of the abcc11 gene (rs17822931) is responsible for determination of earwax type (195). The AA genotype corresponds to dry earwax, and GA and GG to the wet type. In membrane vesicle assays from transfected cells with ABCC11 180 Gly (wild-type) or ABCC11 180 Arg (variant), cells with allele Arg show a lower excretory activity for cGMP than those with allele Gly. Furthermore, frequency of women without colostrum among dry-type women was significantly higher than that among wet-type women (p < 0.0002) (196). Consequently, ABCC11 may be crucial in
the lactation process. Furthermore, similarly to ABCC3 and ABCC5 transporters, ABCC11 expression can be down-regulated in vitro by estrogen in mammary cells (193, 197). This specific ABCC11 regulation likely involved estrogen receptor α (193). These ABCC regulations by estrogen in estrogen receptors α-positive cells may suggest that the hormonal environment may have an impact on mammary gland function.

2.3.9. ABCC12

Little is known about the ABCC12 protein. The gene has two major transcripts of 4.5 and 1.3 kb. In breast cancer, normal breast, and testis, the abcc12 gene transcript is 4.5 kb in size and encodes a 100-kDa protein. In other tissues including the brain, skeletal muscle, and ovary, the transcript size is 1.3 kb. This smaller transcript encodes a nucleotide-binding protein of approximately 25 kDa in size (44, 45, 198). Because the 4.5-kb RNA is highly expressed in breast cancer and not expressed at detectable levels in essential normal tissues, ABCC12 could be a useful target for the immunotherapy of breast cancer. The unusual topology of the two variants of ABCC12 suggests that they may have a different function from other family members. In testis, the transcript has been localized to Sertoli cells of the seminiferous tubules, with lower levels in Leydig cells. ABCC12 mRNA is also widely expressed in fetal tissues such as liver, spleen, kidney, and lung (44). The mouse Abcc12 gene was expressed at high levels exclusively in the seminiferous tubules in the testis (191).

Transport activity of ABCC12 as well as localization in polarized cells remain to be examined. With the recent identification of the murine ortholog, the generation of a knockout mouse would be useful regarding the physiological significance of ABCC12 (191).

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ABC2: A NEW CHALLENGE IN CANCER DRUG RESISTANCE

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3.1. INTRODUCTION

The ability of cancer cells to acquire resistance to multiple chemotherapy drugs, termed multidrug resistance, remains a significant impediment to cancer therapy. While several mechanisms can mediate cellular resistance to chemotherapeutic agents, energy-dependent outward transport of drugs mediated by ATP-binding cassette (ABC) transporters is one that has garnered considerable study. Over 30 years ago, Dano first described drug-resistant Ehrlich ascites tumor cells that exhibited active efflux of daunomycin inhibited by vinblastine or vincristine (1). The resistance was found to be mediated by overexpression of P-glycoprotein (P-gp), encoded by the MDR1/ABCB1 gene, by far the best-characterized drug transporter to date (2, 3). P-gp has been shown to confer resistance to an ever-expanding list of chemotherapy drugs, including the anthracyclines, taxanes and vinca alkaloids (2). While cementing the role of P-gp in drug resistance in solid tumors has been problematic, a large body of evidence seems to strongly associate P-gp expression with drug resistance in acute myelogenous leukemia (AML) (4, 5). Reversing resistance due to P-gp has proven problematic as combination studies with early-identified and second-generation inhibitors yielded disappointing results (6). However, some studies have suggested that the addition of a P-gp inhibitor can improve clinical outcome (7). Recently developed, highly specific P-gp inhibitors offer the hope that the contribution of P-gp to clinical drug resistance can finally be determined (8–10).

Following the discovery of P-gp, another ABC transporter was cloned by Cole et al. (11). Termed the multidrug resistance-associated protein, or MRP (later MRPI, ABCC1), this transporter confers resistance to a somewhat smaller range of drugs comprising the vincas, anthracyclines, and epipodophyllotoxin and may have a role in transporting irinotecan and its active metabolite SN-38 (12). A role for MRPI in drug resistance in chronic myelogenous leukemia (CML) has been postulated (13). MRPI is also expressed in about 80% of small-cell lung cancer samples and 100% of nonsmall-cell lung cancer samples (14). MRPI, however, has not been the subject of as intense a study as P-gp has in terms of clinical drug resistance.

Subsequent to the discovery of MRPI, various groups began reporting non-P-gp-, non-MRPI-mediated drug resistance in cell lines selected with mitoxantrone (15–19). In addition to high levels of mitoxantrone resistance, these mitoxantrone-selected cells often displayed resistance to doxorubicin and etoposide, but not to vinblastine or cisplatin (17, 19). Doyle and colleagues were the first to clone the transporter, calling it BCRP for breast cancer resistance protein, since it was cloned from the human breast cancer MCF-7/AdVp subline (19, 20). Simultaneously, with the discovery of Doyle et al., Allikmets et al. searched expressed sequence tag databases and described a nearly identical transporter, termed ABCP for ABC transporter highly expressed in placenta (21). Finally, our laboratory cloned a cDNA from a mitoxantrone-selected human colon carcinoma cell line, S1-M1-80, and called the gene MXR, for
mitoxantrone resistance gene (22). The Human Genome Nomenclature Committee assigned the name ABCG2, since it is the second member of the “G” subfamily of ABC transporters. This terminology will be used throughout this chapter, which will summarize the spectrum of drugs that ABCG2 transports, examine inhibitors of ABCG2-mediated resistance, and discuss the mounting evidence for its role in drug resistance as well as in pharmacology.

3.2. CHROMOSOMAL LOCALIZATION AND MECHANISMS CONTROLLING EXPRESSION

The ABCG2 gene spans over 66 kb and is made up of 16 exons and 15 introns. The resulting protein is a half-transporter composed of 655 amino acids that runs as a 72 kDa protein (23). Comparing the amino acid sequence of ABCG2 to other known transporters suggests that it is distantly related to ABCB1 (P-gp) and ABCC1 (MRP1), while closely related to ABCG1, a lipid transporter in human cells (20). Fluorescence in situ hybridization studies with a bacterial artificial chromosome probe containing ABCG2 localized the gene to 4q21-4q22 in cells with a normal chromosome 4 (24). In cell lines with high levels of ABCG2 expression, we found multiple rearrangements involving chromosome 4. In the MCF-7 MX and MCF-7 AdVp 30 cell lines, amplification of the ABCG2 gene was observed, while in the S1-M1-80 cell line, only a balanced chromosome 4 and 17 translocation was noticed (24). In a series of mitoxantrone-selected SF295 glioblastoma cell lines, ABCG2 was amplified in double minute chromosomes (25).

Little is known about the molecular mechanisms regulating ABCG2 expression. Like most TATA-less gene promoters, the ABCG2 promoter contains numerous Sp1, AP1, AP2 sites and a CCAAT box. ABCG2 also has a putative CpG island located upstream of the gene. Initial characterization of the ABCG2 promoter identified a sequence 312 bp directly upstream from the transcriptional start site as the basal promoter (23). To date, most studies examining the regulation of ABCG2 under physiological conditions are focused at the transcriptional level. Two functional cis elements in the ABCG2 promoter, namely the estrogen (26) and hypoxia (27) response elements, and a peroxisome proliferator-activated receptor γ response element upstream of the ABCG2 gene (28) have been identified and validated by electrophoretic mobility shift analysis. Ebert and colleagues proposed that aryl hydrocarbon receptor upregulates ABCG2 expression, but the responsive element was not identified (29). Conflicting data have been reported for the effect of the sex hormones estrogen, progesterone, and testosterone on ABCG2 expression (26, 30-32). In particular, the effect of estradiol on ABCG2 expression appears cell-dependent and controversial (26, 30). Moreover, ABCG2 expression is upregulated in the mammary gland during lactation (33). We and another group have independently found that DNA methylation can repress ABCG2 expression in human renal carcinoma (34) and multiple myeloma cell lines (35).
Overexpression of the ABCG2 gene is frequently observed in cancer cell lines selected with a number of chemotherapeutic drugs (20, 22, 36–38). In some of these resistant cell lines, gene amplification and chromosome translocation have been shown to play roles in the increased expression of ABCG2 (24). The use of alternative 5′ promoters at the ABCG2 gene due to differential expression of splice variants at the 5′-untranslated region (5′UTR) of ABCG2 mRNA in drug-selected cells may offer another novel mechanism of ABCG2 upregulation (39), a finding similar to observations in MDR1 where rearrangement of the 5′ region resulted in capture of the MDR1 gene by another promoter (28, 40).

Recently, we have reported that overexpression of ABCG2 in resistant cells was correlated with an increase in a set of permissive histone modification marks and a decrease in a repressive histone mark at the proximal ABCG2 promoter. Also observed were increased binding of RNA polymerase II and the chromatin remodeling factor Brg-1 but decreased association of class I histone deacetylase (HDAC) and Sp1 (41). Interestingly, this pattern of histone modifications and multiprotein complex binding at the ABCG2 promoter also correlates well with the activation of ABCG2 upon romidepsin (an HDAC inhibitor currently in clinical trials) treatment in selected cell types. In cell lines that did not respond to romidepsin with ABCG2 upregulation in spite of clear upregulation of other HDAC-regulated genes, the repressive histone mark, HDACs, and Sp1 were retained at the ABCG2 promoter even when increased histone acetylation was observed at the promoter due to inhibition of HDACs. A proposed model illustrating the distinct pattern of various histone modifications and other nuclear factors associated with the ABCG2 promoter at the permissive and repressive states is shown in Fig. 3.1 (41). ABCG2 could be utilized as a model for studying the cell-type specific gene regulation by HDAC inhibitors and also their mechanism(s) of antitumor activity.

In studies aimed at determining the mechanism underlying upregulation of ABCG2 in resistant cancer cells, we observed increased mRNA stability in drug-selected, ABCG2-overexpressing sublines compared to that in a number of sensitive parental cancer cell lines. Interestingly, the 3′-untranslated region (3′UTR) of the ABCG2 mRNA was found to be longer in the parental cells than in the resistant cells. Using the miRBase TARGETS prediction algorithm, a putative microRNA (miRNA) binding site was found only in the longer ABCG2 3′UTR in the parental cell lines but not in its shorter counterpart in the resistant cell lines. We hypothesized that the binding of the putative miRNA at the 3′UTR of ABCG2 suppresses the expression of ABCG2 in the parental cell lines by promoting mRNA degradation and/or inhibiting protein translation. In the resistant cell lines, the miRNA cannot bind to ABCG2 mRNA because of the shorter 3′UTR, thus allowing the overexpression of ABCG2. The hypothesis was rigorously tested by reporter gene assays, mutational analysis at the miRNA binding sites, and forced expression of miRNA.
**FIGURE 3.1.** A model for the upregulation of ABCG2 in multidrug-resistant cells by permissive histone modifications and recruitment of chromatin remodeling factor. In untreated parental cells, prominent association of the repressive histone mark, Me$\varepsilon$-K9 H3, with the ABCG2 promoter locks the ABCG2 expression at low levels. In resistant cells, the repressive mark is replaced by permissive histone marks, Ac-K9 H3, Me$\varepsilon$-K4 H3, and P-S10 H3, followed by the recruitment of a chromatin remodeling factor (Brg-1) and RNA polymerase II (Pol II), and the release of class I HDACs from the ABCG2 promoter. The switch to an open chromatin configuration also occurs in a limited number of cell lines upon treatment with romidepsin, upregulating the expression of ABCG2. However, in other cell lines where ABCG2 is not affected by romidepsin treatment, the repressive histone mark Me$\varepsilon$-K9 H3 is retained, although more acetylated histone H3 (Lys 9,14) are found binding to the promoter. HDACs and likely other corepressors remain bound to the promoter, suppressing the ABCG2 expression.

Co-rep = co-repressors; Co-act = co-activators; HAT = histone acetyltransferase; HDAC = histone deacetylase; TF = transcription factor.

inhibitors or mimics. Figure 3.2 shows a working model indicating how the putative miRNA affects ABCG2 differentially in parental and resistant cells. Apparently, ABCG2 can be regulated at multiple levels. Further studies are warranted to accurately characterize the mechanisms controlling ABCG2 expression.
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FIGURE 3.2. Proposed model for the involvement of miRNA in the regulation of \textit{ABCG2} at the 3'UTR. A putative miRNA binds to the 3'UTR of ABCG2 mRNA and suppresses the expression of ABCG2 in the parental cell lines. In the resistant cell lines, the miRNA cannot bind to ABCG2 mRNA because of the shorter 3'UTR and thus, mRNA degradation and/or repression on protein translation are relieved, allowing the overexpression of ABCG2.

3.3. TISSUE LOCALIZATION

With the discovery of ABCG2 came lines of inquiry to determine the location, expression, and possible physiological role of ABCG2. By Northern blot analysis, Doyle et al. reported high levels of ABCG2 expression in the placenta, as well as lower levels in the brain, prostate, small intestine, testis, ovary, and liver (20). ABCG2 expression was absent in the heart, lung, skeletal muscle, kidney, pancreas, spleen, thymus, and peripheral blood leukocytes (20). We also found high levels of ABCG2 in the central nervous system, liver, adrenal gland, placenta, prostate, testis, and uterus as well as lower levels in the small and large intestine, stomach, lung, kidney, and pancreas as determined by Northern blot (42).

Maliepaard et al. examined ABCG2 expression by immunohistochemistry using the monoclonal BXP-21 and BXP-34 antibodies and reported high levels in the placenta, specifically in the syncytiotrophoblasts (43). High expression was also observed in the colon, small intestine, biliary canaliculi, breast, venous endothelium, and capillaries (43). Using a polyclonal rabbit anti-ABCG2 antibody as well as the monoclonal 5D3 antibody, we also found high expression
in the syncytiotrophoblasts of the placenta, alveolar pneumocytes of the lung, sebaceous glands, small and large intestine, bile canaliculi, and blood vessels, as well as the endothelium of the nervous system (42). The location and level of expression of ABCG2 lend clues to determining its likely role in normal physiology. Further research has served to confirm these likely roles.

3.3.1. Placenta

Given its high expression in the placenta in the syncytiotrophoblasts of the chorionic villus, ABCG2 is believed to protect the developing fetus from the possible transmission of toxins as well as to remove toxins from the fetal space. Jonker et al. were the first to demonstrate the ability of ABCG2 to protect the fetus (44). When Abcb1/2-deficient pregnant mice were administered topotecan along with the ABCG2 and P-gp inhibitor elacridar (GF120918), fetal plasma levels of topotecan were twice as high as those measured in maternal plasma, supporting the theory that ABCG2 protects the developing fetus from possible toxins in the maternal space (44). In placental perfusion studies, Staud et al. demonstrated transport of cimetidine from the fetal to the maternal space, against a concentration gradient, suggesting that ABCG2 serves to remove potential toxins from the fetal space (45). Similarly, Zhang et al. found that, when pregnant wild-type or Abcg2-/- mice were administered the ABCG2 substrate nitrofurantoin, the fetal plasma area under the concentration-time curve was five times higher in Abcg2-/- mice compared to wild-type mice (46), clearly supporting that ABCG2 limits fetal penetration of the drug.

3.3.2. Mammary Gland

In an apparent deviation from its role in tissue protection, ABCG2 in the mammary gland serves to concentrate toxins into breast milk. Jonker et al. reported that ABCG2 expression was upregulated in the lactating mammary glands of mice, cows, and humans, and reported higher levels of the ABCG2 substrates topotecan and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in the milk of lactating mice compared to Abcg2-deficient mice (33). Similarly, ABCG2 has been shown to concentrate dietary carcinogens (47) as well as the antibiotics nitrofurantoin (48), ciprofloxacin (49), and enrofloxacin (50) in milk. Subsequently, van Herwaarden et al. shed some light on the possible normal physiological role of ABCG2 in the lactating breast, demonstrating that ABCG2 secretes riboflavin into milk (51). Interestingly, ABCG2 has been linked to milk production and composition in dairy cattle (52).

3.3.3. Testis

ABCG2 may serve to protect germinal stem cells from genotoxic mutagens, as high levels of ABCG2 have been reported in the interstitial cells of the
normal testis as well as in Sertoli–Leydig cells (42). Bart and colleagues have also reported high ABCG2 expression levels in myoid cells and cells of the luminal capillary endothelial wall of the normal testis (53). ABCG2 has also been posited to form part of the blood-testis barrier, as the tissue-to-plasma concentration ratios for ABCG2 substrates such as PhIP and prazosin in the testis were found to be significantly increased in Abcg2-deficient mice compared to wild-type mice (54).

3.3.4. Blood–Brain Barrier

ABC transporters, such as P-gp, have previously been shown to form part of the BBB (55), leading to studies seeking to determine the contribution of ABCG2 in limiting brain penetration of substrate compounds (see Part V, Chapter 13). Cooray et al. reported ABCG2 to be localized to the microvessel endothelium of the brain (56). Immunohistochemical analysis revealed that rat ABCG2 is expressed on the luminal side of rat brain capillaries, suggestive of brain-to-blood transport (57). Additionally, ABCG2 was found to be more highly expressed in porcine brain endothelium than ABCB1 or ABCC1 based on mRNA analysis (58, 59). This was also found to be true for human brain vessels (59). Similarly, Cistermino et al. reported an almost 700-fold higher level of Abcg2 mRNA in wild-type mouse brain capillaries compared to the cortex (60). They also noted a threefold higher level of Abcg2 mRNA in the capillaries of Abcb1/2-deficient mice compared to wild-type mice, suggesting that Abcg2 might compensate for the loss of P-gp at the BBB.

Mounting evidence has confirmed that ABCG2 restricts brain penetration of substrate compounds. Breedveld et al. demonstrated that inhibition of both P-gp and ABCG2 resulted in the highest brain penetration of imatinib in mice (61). Bihorel et al. (62) showed that brain uptake of the imatinib metabolite, CGP74588, was 1.5 times higher in Abcg2-/- mouse brain compared to wild-type mice and that coadministration of the transport inhibitor elacridar with imatinib increased brain penetration of imatinib in Abcb1/2-deficient mice (62). Perhaps the strongest evidence to date stems from the research of Enokizono et al. who showed a 9.2-fold increase in brain penetration of genistein in Abcg2-/- mice compared to wild-type mice (54).

3.3.5. Gastrointestinal Tract (GI)

Initial studies of ABCG2 expression pointed to high expression of the transporter in the intestine (see Part V, Chapter 14) (44). Specific examination of ABCG2 expression along the human GI tract showed that levels of ABCG2 were highest in the duodenum, and then decreased from the terminal ileum, ascending colon, transverse colon, descending colon, and sigmoid colon to the rectum, where the lowest levels of ABCG2 were found (63). The expression of ABCG2 in the GI tract suggested that it may play a role in limiting the oral absorption of substrates.
Jonker et al. were the first to confirm this role, noting more than sixfold higher plasma levels of topotecan in Abcb1/2-deficient mice when topotecan was administered in the presence of elacridar compared to a drug administered in the absence of the inhibitor (45). Similarly, oral administration of topotecan to Abcb1/2-deficient mice in the presence of the inhibitor Ko143 resulted in a four- to sixfold increase in plasma topotecan levels compared to levels in the absence of Ko143 (64). Intestinal uptake of dietary carcinogens such as PhIP and aflatoxin B1 (48, 65), antibiotics (49, 50, 66), quercetin (66), the glycoprotein IIb/IIIa antagonist ME3277 (67), the CDK inhibitor JNJ-7706621 (68), and sulfasalazine (69) were increased in Abcg2-/− mice compared to wild-type mice, providing strong evidence for the role of ABCG2 in oral drug absorption.

3.3.6. Kidney

In the kidney, we localized ABCG2 to the proximal tubule (43), and subsequent studies have reported expression at the proximal tubule brush border membrane (70), suggesting the potential involvement of ABCG2 in renal drug excretion. In support of this theory, impaired renal excretion of 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole sulphate (71) and edaravone sulphate (72) was noted in Abcg2-/− mice compared to wild-type mice.

3.3.7. Liver and Biliary Tract

High levels of ABCG2 expression in the liver and biliary apparatus imply a potential role for the protein in substrate efflux from the liver. Jonker et al. were among the first to show that ABCG2 plays a role in the biliary excretion of substrates, reporting that hepatobiliary excretion of topotecan was decreased in Abcb1a/b-deficient mice with a cannulated gall bladder that were given the ABCG2 inhibitor GF120918 before intravenous administration of topotecan (45). In mice with a cannulated gall bladder, hepatobiliary excretion of PhIP was found to be significantly decreased in Abcg2-/− mice compared to wild-type mice, suggesting that ABCG2 plays a role in its excretion (65). Similarly, Merino et al. found that hepatobiliary excretion of the antibiotic nitrofurantoin was nearly completely abolished in Abcg2-deficient mice compared to wild-type mice (49).

3.3.8. Stem Cells

ABCG2 has also been shown to be the molecular determinant of the “side population” or SP phenotype observed in hematopoietic stem cells. Hematopoietic stem cells are usually characterized by their ability to transport the fluorescent compound Hoechst 33342. SP cells extrude the Hoechst dye and have surface antigen expression profiles that correspond to a stem-cell
phenotype (73). Zhou et al. were the first to report that Abcg2 was responsible for the SP phenotype in mice (74), and their observations were subsequently confirmed in humans (75). While ABCG2 may serve as part of the stem-cell phenotype, it is neither necessary nor sufficient for stem-cell development and cellular differentiation, given that Abcg2 knockout mice are viable and free of major developmental abnormalities (76, 77). Since the initial report of Zhou and colleagues, ABCG2 has been identified as a potential phenotypic marker for stem cells in multiple normal tissues (78–81).

3.4. SUBSTRATES AND INHIBITORS OF ABCG2-MEDIATED TRANSPORT

Overexpression of ABCG2 was first identified in drug-selected cancer cell lines. Selection with mitoxantrone usually results in overexpression of ABCG2, and resistance to mitoxantrone is the most distinctive feature of the phenotype conferred by ABCG2 (82). Selection with the camptothecins, topotecan, or SN-38 also results in ABCG2 overexpression, and cross resistance to other camptothecin analogs such as irinotecan, homocamptothecin, diflomotecan, DX-8951f, and BNPI350 has also been observed in ABCG2-overexpressing cells (83). Resistance to the parent compound, camptothecin, however, is not conferred by ABCG2 to any significant extent. We have demonstrated ABCG2 overexpression in breast cancer cells selected for resistance to flavopiridol (37). Antifolates such as methotrexate are also transported, as are indolocarbazole topoisomerase inhibitors, photosensitizers, and tyrosine kinase inhibitors such as gefitinib and imatinib (83). The list of chemotherapeutic agents that are transported by ABCG2 continues to grow. In Table 3.1 chemotherapeutic and diagnostic substrates for ABCG2 are listed. It is likely that an important role in the pharmacology of many will be demonstrated, whether or not ABCG2 will confer clinical drug resistance to these substrates.

In early studies with the MCF-7/AdVp cell line, we reported adenosine triphosphate (ATP)-dependent transport of rhodamine 123 (84), and Rabindran et al. found rhodamine 123 transport in the mitoxantrone-selected S1-M1-3.2 line (85). However, rhodamine transport was not found in all cells expressing ABCG2 (86). When sequencing the gene in a series of parental and ABCG2-overexpressing cells, we found that cells that were able to transport rhodamine had a glycine or threonine at position 482 in the ABCG2 protein, while cells that expressed wild-type ABCG2 with an arginine at this site could not transport them (87). Allen et al. reported that in mouse fibroblast cell lines lacking functional P-gp or MRP1, selection with mitoxantrone, topotecan, or doxorubicin resulted in ABCG2 overexpression. In the case of cells selected with doxorubicin, all cells acquired a mutation at amino acid 482, changing the wild-type arginine to a methionine or serine (88). This suggested amino acid 482 to be a “hot spot” for mutation; but, to date, such mutations have not been found in clinical samples and do not appear to have clinical relevance. However,
TABLE 3.1. Selected Chemotherapeutic Substrates and Inhibitors of ABCG2

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Inhibitors (References)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitoxantrone (82)</td>
<td>Fumitremorgin C (85, 89)</td>
</tr>
<tr>
<td>Daunorubicin* (20, 88, 125, 176)</td>
<td>Ko143 (64)</td>
</tr>
<tr>
<td>Doxorubicin* (20, 85, 88, 125, 176)</td>
<td>Cyclosporin A (84, 93, 94)</td>
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<tr>
<td>Epirubicin* (125)</td>
<td>Gefitinib (97–99, 201)</td>
</tr>
<tr>
<td>Bisantrene* (85, 125)</td>
<td>Imatinib (99, 100)</td>
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<tr>
<td>Flavopiridol (37, 177)</td>
<td>Elacridar (GF120918) (92, 183)</td>
</tr>
<tr>
<td>Etoposide (18–20, 178)</td>
<td>Tariquidar (XR9576) (95)</td>
</tr>
<tr>
<td>Teniposide (178)</td>
<td>Biricodar (VX-710) (202)</td>
</tr>
<tr>
<td>9-aminocamptothecin (179–183)</td>
<td>Chrysín (105)</td>
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<tr>
<td>Topotecan (38, 179, 181, 183–186)</td>
<td>6-prenylchrysín (108)</td>
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<tr>
<td>Irinotecan (179, 183, 187, 188)</td>
<td>Tectochrysin (108)</td>
</tr>
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<td>SN-38 (179, 180, 183, 189–191)</td>
<td>Naringenín (106)</td>
</tr>
<tr>
<td>Diflomotecan* (192)</td>
<td>Quercetin (103, 191)</td>
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<tr>
<td>Homocamptothecin* (192)</td>
<td>Acacetin (106)</td>
</tr>
<tr>
<td>DX-8951f (187, 193)</td>
<td>Silymarín (103)</td>
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<td>BNP1350 (194)</td>
<td>Genistein (106)</td>
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<td>J-107088 (195)</td>
<td>17β-estradiol (203)</td>
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<td>NB-506 (195)</td>
<td>Estrone (203)</td>
</tr>
<tr>
<td>UCN-01 (95)</td>
<td>Tamoxifén (204)</td>
</tr>
<tr>
<td>Methotrexate,* methotrexate di-* and triglutamate* (39, 126, 196–199)</td>
<td>Ortataxel (205)</td>
</tr>
<tr>
<td>GW1843* (197)</td>
<td>Novobiocin (206, 207)</td>
</tr>
<tr>
<td>Tomudex* (197)</td>
<td>Reserpin (74)</td>
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<tr>
<td>Imatinib (102)</td>
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<td>Gefitinib (97, 101)</td>
<td></td>
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<tr>
<td>CI1033 (96)</td>
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<tr>
<td>Pheophorbide a (77, 95)</td>
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<tr>
<td>Pyropheophorbide a methyl ester (200)</td>
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<tr>
<td>Chlorin e6 (200)</td>
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<tr>
<td>Protoporphyrin IX (28, 200)</td>
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* Designates compounds affected by the amino acid at position 482.

studies of the mutant proteins may aid in the definition of the drug-binding site and in the development of more effective ABCG2 inhibitors.

The first inhibitor of ABCG2, fumitremorgin C (FTC), was reported before the ABCG2 gene had been cloned (85). FTC, isolated from the fermentation broth of Aspergillus fumigatus, was shown to inhibit resistance in the mitoxantrone-selected S1-M1-3.2 colon cancer cell line (85). After ABCG2 was cloned, FTC was shown to inhibit ABCG2-mediated transport of chemotherapy agents in stably transfected cell lines (89). Clinical use of FTC was prevented by its neurotoxicity, leading to the development of the FTC analog Ko143 (64). Subsequently, other diketopiperazine inhibitors, including the indolyl diketopiperazines (90) and tryprostatin A (91), have been described.
P-gp inhibitors were among the first compounds to be examined for their ability to inhibit ABCG2. We reported that the potent P-gp inhibitor elacridar (GF120918) was also an ABCG2 inhibitor (92). Reserpine has also been shown to inhibit ABCG2-mediated Hoechst 33342 transport in SP cells (74). We also demonstrated inhibition of rhodamine transport in MCF-7/AdVp cells by cyclosporin A (84), a finding that has been subsequently confirmed in ABCG2-expressing cell lines by several groups (93, 94). The specific P-gp inhibitor tariquidar (XR9576) has also been shown to inhibit ABCG2-mediated drug efflux (95).

Several tyrosine kinase inhibitors have been shown to act as ABCG2 inhibitors. First, CI1033 was shown to reverse ABCG2-mediated resistance to SN-38 and topotecan in transfected cells (96). Gefitinib has also been shown to inhibit ABCG2-mediated drug resistance as have imatinib and EKI-785 (97–100). Most likely, tyrosine kinase inhibitors act as competitive inhibitors, since ABCG2 has been shown either to directly transport or to confer resistance to CI1033, gefitinib, and imatinib (96, 97, 101, 102).

Flavonoids constitute a growing class of ABCG2 inhibitors. Silymarin, hesperetin, quercetin, and daidzein, as well as the stilbene resveratrol, were shown to increase intracellular accumulation of mitoxantrone and borondipyrromethene (BODIPY)-prazosin in ABCG2-expressing cells (103). Similarly, Zhang et al. reported chrysin and biochanin A to be potent inhibitors of ABCG2 (104, 105). Imai et al. reported that genestein, naringenin, acacetin, and kaempferol reversed resistance to SN-38 and mitoxantrone in ABCG2-transduced K562 cells (106). Structure activity studies have also identified novel ABCG2 inhibitors, among them 6-prenylchrysin and tectochrysin (107, 108). Although the flavonoids quercetin and genestein more than likely function as competitive inhibitors, it has also been postulated that flavonoids inhibit ABCG2 via interaction with its nucleotide-binding domain (NBD) (107, 109). A more complete listing of reported ABCG2 inhibitors is given in Table 3.1.

3.5. STRUCTURE OF THE ABCG2 PROTEIN

It is generally accepted that for an ABC transporter to be functional at least two NBDs and a minimum of 12 transmembrane segments (TM) are required. This principle is underscored by the recently obtained crystal structures of bacterial ABC transporters where the Walker A motif of one NBD forms the nucleotide sandwich with the C signature motif of the other NBD (110). P-gp is a typical example of a full transporter, with more than 1200 amino acids, 12 membrane-spanning segments, and 2 NBDs encoded in the same molecule, whereas the 655-amino acid ABCG2 with its one NBD and only six TMs is considered a half-transporter. The order of the domains in ABCG2 is reversed when compared to P-gp with the TM domain following the NBD located on the N-terminus. The relevance of this reversed orientation is not yet known. ABC half-transporters either homo- or heterodimerize in order to transport
substrates. The ABCG subfamily members ABCG5 and ABCG8 are regarded as obligate heterodimers. Heterodimerization of ABCG5 and ABCG8 is thought to occur in the endoplasmic reticulum (ER) and is required for expression on the cell surface (111). On the other hand, ABCG1 and ABCG4 may function as either homo- or heterodimers (112).

The minimal functional unit of ABCG2 is at least a homodimer, although the presence of higher order oligomers has been reported. Based on cryonegative stain electron microscopy of purified ABCG2, McDevitt et al. suggested that a tetramer of dimers is formed (113). Xu et al. reported the main form of ABCG2 to be a tetramer and also found higher order oligomers, such as dodecamers, when extracting the protein from an ABCG2-overexpressing cell line using nondenaturing detergents (114).

Homodimerization of ABCG2 is supported by transfections in heterologous systems, for example, S9 insect cells (115) and Lactococcus lactis (116), where the presence of a heterodimeric partner is highly unlikely. Chimeric dimers of two ABCG2 molecules with or without a linker peptide have also been shown to be functional as further proof of homodimerization (117). In addition, coimmunoprecipitation experiments of ABCG2 monomers with two different tags have also been reported, providing evidence for the formation of homodimers (118, 119).

The TM domain of ABCG2 starts at amino acid 361 and consists of six putative transmembrane segments, although some TM-prediction programs predict five or seven alpha helices. The protein has a short intracellular C-terminal segment, and a longer extracellular loop is predicted between TMs 5 and 6. This extracellular loop contains cysteine 603, which has been shown to contribute to a disulfide bond in the ABCG2 homodimer (120). Mutating cysteine 603 to either glycine or alanine results in a functional ABCG2 molecule that is localized to the cell surface, suggesting that the disulfide bond is not essential for function and trafficking and that this bond is by far not the sole force keeping the monomers together. On the other hand, mutants lacking the disulfide bond between cysteine 603 were shown to undergo proteasome-mediated degradation, while the wild-type protein was suggested to be degraded in lysosomes (121). Two other cysteines were also found in the extracellular loop of ABCG2; these were proposed to form an intramolecular disulfide bond, although publications describing mutations to these cysteines are somewhat conflicting (117, 120, 122).

ABCG2 has three canonical N-linked glycosylation sites of which only asparagine 596 was shown to be modified (123). This residue also localizes to the above-mentioned loop between TMs 5 and 6, providing further evidence for the extracellular localization of this part of ABCG2. Several putative phosphorylation sites are also found in ABCG2, of which threonine 362 was recently shown to be phosphorylated by Pim-1 in prostate cancer cells (124). In the same study, phosphorylation of ABCG2 was suggested to be required for optimal efflux activity, oligomerization, and proper localization.
Information regarding the drug-binding site or sites of ABCG2 is also quite limited. Arginine 482 has been suggested to be involved in drug binding or recognition, given that mutations at this residue significantly alter the spectrum of transported drugs. This residue is predicted to localize to the cytoplasmic end of TM3. Interestingly, substituting arginine with any nonpositively charged amino acid results in wider substrate specificity, adding drugs like rhodamine 123 and doxorubicin to the list of ABCG2 substrates (125). So far, methotrexate is the only drug identified to be solely transported by the wild-type protein (R482) (126). On the other hand, two recent publications have suggested that it is not the interaction between the substrates and the particular residue at 482 that leads to the observed alterations in substrate specificity. Both have found that mutation at this position did not alter the binding of the studied substrates or the substrate-analog [125]IAAP (127, 128). These investigators suggested that residue 482 does not play a direct role in drug binding; rather, it might be involved in energy coupling. Against this though is the fact that inhibitors are also impacted by mutations at residue 482. Increased energy coupling leading to increased transport of substrates should make inhibitors have less impact, but instead we see an impact on inhibitors that suggests a more direct role.

Our group has reported mutational analysis of two regions of the ABCG2 protein potentially involved in dimerization. One of these is a GXXXG motif found in TM1 of the transporter (129). This frequently occurring sequence motif has been associated with the dimerization of other membrane proteins, the most extensively studied example of which is glycophorin A (130). In the case of ABCG2, single and double mutation of the GXXXG motif to leucine proved its importance in the proper function and folding of the transporter, with a suggested but not yet confirmed role in dimerization. The GXXXG motif in ABCG2 could also be important in either the formation of higher-order oligomers or in intramolecular TM packing. We have also reported mutational studies of glycine 553 in TM5 of ABCG2 that suggest a role for this well-conserved residue in homodimerization (131). When expressed in the human embryonic kidney cells, mutations at this position resulted in significantly reduced protein expression levels, altered glycosylation, and retention in the ER, even when coexpressed with the wild-type protein. Yet, the mutants can be chemically cross-linked. In insect cells, the mutants localize to the cell surface and do not hydrolyze ATP; and cotransfection with the wild-type protein results in approximately a 35% decrease in transport activity compared to the wild-type, suggesting an interaction between the mutant and the wild-type in this expression system. Both regions of the protein mentioned above require further studies to unequivocally determine their role in ABCG2 dimerization. However, these studies have evolved our understanding of dimer formation from that of two single, completely folded molecules coming together at the cell surface (as for epidermal growth factor receptor [EGFR]) or in the cytoplasm (as for the glucocorticoid receptor) to that of two molecules folding together during protein synthesis in the endoplasmic reticulum (132).
3.6. SINGLE NUCLEOTIDE POLYMORPHISMS

An important aspect of ABCG2 research is determining what impact single nucleotide polymorphisms (SNP) in the \( ABCG2 \) gene have on protein expression and function and the subsequent effect on the oral bioavailability of drugs and, possibly, on treatment response. A number of SNPs have been identified in coding exon regions of the gene; examples of nonsynonymous SNPs are replacing valine with a methionine at amino acid 12 (V12M; exon 2); replacing a glutamine with a lysine at aa 141 (Q141K, exon 5); replacing an isoleucine with a leucine at aa 206 (I206L, exon 6); and replacing an asparagine with a tyrosine at aa 590 (N590Y, exon 15). The V12M, I206L, and N590Y SNPs have not yet been found to confer any alteration in protein expression or function (133). The Q141K SNP, on the other hand, has been shown to have functional significance. This SNP has an especially high incidence rate in the Asian population (~30%) and is also frequently found in Caucasians (~10%) (134). Various researchers have found that the Q141K SNP can lead to lower plasma membrane expression (133–136), reduced drug efflux (133, 137), and reduced ATPase activity (133, 137). This loss-of-function polymorphism leads to lower IC\( _{50} \) levels in cell lines exposed to cytotoxic agents that are ABCG2 substrates, including mitoxantrone, irinotecan, and SN-38 (133, 137). Plasma levels of drugs, including sulfasalazine, rosuvastatin, gefitinib, topotecan, and diflomotecan, have been demonstrated to increase in patients with the Q141K SNP (138–142). In one study, a higher incidence of diarrhea in patients carrying the Q141K SNP was observed following treatment with gefitinib (143). The higher drug levels may result in exquisite sensitivity to certain orally administered chemotherapy drugs, but this may also have a positive effect, resulting in tumors being exposed to higher drug levels, thus eliciting a greater response to treatment. However, the above-mentioned studies have yet to be confirmed, and most of them reported data on a relatively small group of subjects.

3.7. EXPRESSION OF ABCG2 IN CANCER AND ITS PUTATIVE ROLE IN DRUG RESISTANCE

Since functional efflux assays suggested the presence of transporters other than P-gp and MRP1 in leukemia samples (144), the discovery of the \( ABCG2 \) gene led many researchers to examine its expression in leukemia. Ross et al. were the first to report on ABCG2 expression in this disease. They examined 20 AML samples and one acute lymphocytic leukemia (ALL) sample, noting relatively high levels in seven samples and a 1000-fold variation in expression (145). Studying the contribution of ABCG2 to resistance in AML has not led to a conclusive determination. Results vary, with relatively high ABCG2 expression in AML reported by some groups (146) and low levels reported by others (147–149). Similarly, some groups reported that ABCG2 expression was associated with response (150, 151) while others found no correlation (148,
ABCG2 expression was predictive of a decrease in complete remission, shorter 4-year disease-free and 4-year overall survival in patients receiving daunorubicin or mitoxantrone (150). Also, P-gp/ABCG2 coexpression was associated with a lower complete response rate and with worse event-free survival and overall survival in a prospective study of 154 newly diagnosed AML patients (153).

A putative role for ABCG2 in drug resistance in ALL is also being suggested. Steinbach et al. reported a correlation between ABCG2 expression and prognosis (154). Similarly, Stam et al. reported ABCG2 expression correlated with resistance to Ara-C despite the fact that the drug is not considered an ABCG2 substrate (155). In contrast, Sauerbrey et al. did not find a correlation between ABCG2 expression and response (156). To clearly delineate the role of ABCG2 in drug resistance in leukemia, larger studies of ABCG2 expression in clinical samples using validated methods are necessary. One problem in comparing these studies is that different methods were used by the various investigators.

Recently, reports of ABCG2 expression in solid tumors have begun to appear in the literature. The study of Diestra et al., examining ABCG2 expression in 150 paraffin-embedded, untreated tumor samples from various origins, is by far the most complete to date. It describes frequent expression of ABCG2, especially in tumors from the digestive tract, endometrium, and lung, as well as in melanoma, using the BXP-21 monoclonal anti-ABCG2 antibody (157). In breast carcinoma, two studies noted low ABCG2 expression and no correlation with response or progression-free survival (158, 159). While one study reported that ABCG2 and ABCB1 expression correlated with response, but not progression-free survival, in patients treated with an anthracycline-based therapy (160). In a study of 45 oral squamous cell carcinomas, Friedrich et al. found increased ABCG2 expression correlated with loss of differentiation and shorter survival (161). In contrast, Zurita et al. and Diestra et al. found no correlation between ABCG2 expression and response in testicular germ cell cancers or advanced bladder tumors, respectively (162, 163). We have developed the 405 rabbit polyclonal antibody for detecting ABCG2 in clinical samples; two examples are shown in Fig. 3.3 (43). However, the above-mentioned studies aimed at finding ABCG2 in patient samples are still in their infancy. Validated assays and longitudinal studies with clinical correlation are needed to definitively assess the contribution of ABCG2 to cancer drug resistance.

### 3.8. ABCG2 Expression and the Cancer Stem Cell

The cancer stem cell hypothesis is based on the idea that only a small fraction of the malignant cells is capable of repopulating a tumor, similar to the idea of the stem cells found in bone marrow from which all blood cells differentiate. Bone marrow stem cells have long been separated from the marrow using the fluorescent DNA dye Hoechst 33342 based on their ability to transport the
FIGURE 3.3. Immunohistochemical detection of ABCG2 in tumor samples. Formalin-fixed, paraffin-embedded tissue sections stained with the rabbit anti-ABCG2 antibody 405 (1:2500). (a) Choriocarcinoma (200×). (b) Yolk sac tumor (400×).

compound, resulting in a Hoechst 33342-dim SP phenotype (164). Not only has the SP phenotype been used to identify bone marrow stem cells, but it has also been used to identify stem cells in normal breast, lung, and liver tissue (78–80) as well as in corneal stroma (81). SP cells have been found in a number of established cell lines and in tumor samples, and it has been suggested that these cells have stem cell–like qualities, overexpress ABCG2, and confer drug resistance (165–168).
Although it is tempting to consider ABCG2 to be a stem cell marker in light of some reports, care must be taken when trying to link stem cell characteristics with ABCG2 expression. First, ABCG2 expression is not a requirement for stem cells. Abcg2-deficient mice are viable and demonstrate no defect in steady-state hematopoiesis, although Abcg2-deficient mice do lack an SP population (76). Second, the SP fraction is known not to be a pure stem cell population, and the method used for generating SP cells greatly affects the cells included in it (169). Further, since Hoechst 33342 is a substrate for P-gp as well as for ABCG2, P-gp-overexpressing cells can also be included in the SP population. In some cases, ABCG2 expression is part of the normal phenotype; A549 cells have been shown to express ABCG2 and transport Hoechst 33342 (75), but it is not considered a “stem cell” line. Finally, drug-resistant cells that overexpress ABCG2 in response to selection pressure are not necessarily believed to be stem cells.

3.9. AVOIDING THE MISTAKES OF THE PAST IN THE DEVELOPMENT OF ABCG2 INHIBITORS

As detailed in this chapter, a large number of compounds that have the capacity to block ABCG2 in vitro have been identified. Yet, to our knowledge, clinical trials have not yet been undertaken with the intention of reversing drug resistance mediated by ABCG2. The reasons for this are several-fold and quite sound. Whether we have learned from our mistakes or are simply wary of fracture, the delay in entering trials attempting to reverse drug resistance has to be counted as something positive. Reversing multidrug resistance mediated by P-gp can be considered an early version of today’s targeted therapies. Since the P-gp trials were conducted, we have learned to give trastuzumab to women whose breast cancers overexpress Her2 rather than to all patients with breast cancer, imatinib to patients with CML rather than to patients with any form of leukemia, and gettinib and erlotinib principally to patients whose lung cancers bear mutated epidermal growth factor receptor. We have learned that targeted therapy must be administered to patients who have tumors in which the target is critical to maintenance or growth of the tumor. This lesson was not well understood when the early P-gp trials were launched. Rather, the thinking was to determine whether the target was present and to determine its importance based on the responses observed in the clinical trials, that is, to let the clinical trial results validate the hypothesis. Instead, the results were not convincing, and we were left not knowing why. Was P-gp even present in patient tumors? In which tumor type was P-gp important? Was the drug able to achieve sufficient quantities in patient tumors to reverse drug resistance? Were the drugs, verapamil, quinidine, cyclosporine, valsodar, biricodar even able to inhibit P-gp in patients? Numerous negative trials in the face of few answers to these questions have left few investigators with the will to try again.
The first principle has not been met for ABCG2. While there are some leads in the literature, much work remains to identify a tumor type with ABCG2 overexpression as part of its resistance phenotype. The major drug substrates, mitoxantrone, irinotecan, and topotecan do not provide leads to the cancers that ought to be studied. The recent addition of imatinib to the list of substrates opens the possibility that CML could be a tumor type in which an ABCG2 inhibitor could be of benefit. Studies that show expression of ABCG2 in the tumor before or after chemotherapy need to be carried out. Ideally, longitudinal correlative studies would show that levels of ABCG2 increase after chemotherapy and that increased levels are associated with a poorer outcome.

The second principle has also not been met for ABCG2. Surrogate assays have not been developed to allow detection of target inhibition. While it is a simple matter to demonstrate inhibition of ABCG2 in vitro, clinical studies with ABCG2 inhibitors in patients would not be able to confirm at the present time that ABCG2 was inhibited in vivo. 99mTc-sestamibi is a radionuclide imaging agent that has been shown to be a substrate for P-gp. Studies with P-gp inhibitors show retention of sestamibi in P-gp-bearing normal tissues after administration of a potent inhibitor such as valspodar or tariquidar to a patient (9, 170, 171). Further, ex vivo assays show that P-gp-mediated dye efflux from circulating mononuclear cells is dramatically reduced when obtained from a patient after administration of a P-gp inhibitor (172, 173). The development of these assays confirmed that the P-gp inhibitors were able to block P-gp in normal tissues and in tumors. Such assays will be needed for ABCG2 before moving inhibitors to the clinic.

The third principle is missing for ABCG2 and is still painfully missing for P-gp. Even if we knew what tumor type to take to clinical trial, we do not know when to treat the target. Should we try to prevent ABCG2 or P-gp emergence or wait until it is documented as a mechanism of resistance? Early in development, it was thought that all patients and all stages of tumors would be suitable candidates for P-gp inhibition. We now know that polymorphisms exist in P-gp and ABCG2 that may reduce levels of expression (133, 174). Patients with low natural levels of P-gp expression may never develop P-gp as a mechanism of resistance in tumors and may experience toxicity when treated with P-gp inhibitors. Such a possibility is implicated in the results of an acute leukemia trial reported by Baer et al. (7). Patients with high levels of P-gp in leukemic cells had an improved outcome compared to patients with low levels of P-gp when an inhibitor was added, yet the entire population suffered increased toxicity in the presence of the inhibitor. One way to reconcile this disparate observation is to suppose that patients with high natural levels of P-gp would have higher levels in bone marrow stem cells and therefore tolerate P-gp inhibition better than patients with low natural levels. This hypothesis requires validation for P-gp; for ABCG2, such ideas can be considered before the start of clinical trials.
A new twist in the development of a transport inhibitor is the recognition that ABCG2 is responsible for the side population phenotype by which stem cells are identified (175). The notion that drug-resistant cancer stem cells exist as a unique subset of cells within the malignant population offers a separate paradigm for the development of ABCG2 inhibitors. In this paradigm, drugs would be developed to target stem cell pathways that might lead to long-term self-renewal and refractoriness to chemotherapy. These drugs, if substrates for ABCG2, would need to be coupled with inhibitors to achieve therapeutic concentrations in the stem cells. Much work needs to be done to confirm the existence of cancer stem cells and their unique signaling pathways.

The challenges of translating the discovery of ABCG2 to a clinical reality exist on multiple levels. However, having learned from the lessons of the past, until we know how to properly design a trial, how to monitor the inhibitor, and which tumors (or stem cell subset) to target, we have scant hope of creating effective therapy.

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PART II

ABC PROTEINS AND ONCOLOGY: EXPRESSION, DETECTION, AND IMPLICATION OF ABC PROTEINS IN HEMATOLOGICAL MALIGNANCIES AND SOLID TUMORS
4

EXPRESSION, DETECTION, AND IMPLICATION OF ABC PROTEINS IN ACUTE MYELOBLASTIC LEUKEMIA

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4.1. INTRODUCTION

In 1976, Juliano and Ling (1) described a protein that was associated with reduced drug permeability in Chinese hamster ovary cells. The protein was named permeability glycoprotein (P-gp/MDR1/ABCB1). Six years later it was shown that a similar protein exists and confers multidrug resistance (MDR)
in human cells (2). In 1986, Chen et al. (3) provided the first evidence that P-gp is an energy-dependent efflux pump. Marie et al. (4) reported that the expression of P-gp was associated with a poor outcome in patients with acute myeloid leukemia (AML). These findings initiated a great hype. It was hoped that P-gp would be the major cause of drug resistance and that this finding would lead the way to new therapeutic strategies in malignant diseases. Today we know that P-gp is one of 49 different human ATP-binding cassette (ABC) transporters (5). ABC transporters facilitate adenosine triphosphate (ATP)-dependent transport of various substrates via extra- or intracellular membranes. All ABC transporters that are able to transport cytostatic drugs might cause drug resistance in the same way as P-gp.

These transmembrane proteins are specialized in energy-dependent cellular transport. The encoding MDR genes are highly conserved between species, from bacteria to man (6, 7). The role of these proteins is mainly protection against xenobiotics, including many hydrophobic cytostatics of natural origin such as anthracyclins, vinca alkaloids, epipodophyllotoxin, and taxanes (8). The minimum structure of the protein is an ABC unit of 200–250 amino acids, consisting of consensus Walker A and B motifs and the ABC signature, located between the two Walker domains for ATP binding and six transmembrane domains. The more common structure consists of two ABC and 12 transmembrane domains (P-gp/ABCB1) and a few members have 5 more transmembrane domains, with an external N-termination (multidrug resistance associated protein [MRP1]/ABCC1, MRP2/ABCC2, and MRP3/ABCC3) (9). A new classification of this family with eight subgroups is now adopted. The members responsible for drug efflux belong to several classes, that is, ABCB1 (P-gp), ABCC (MRP family), and ABCG2 (breast cancer resistance protein [BCRP]), the evidence for this being that the transfection of their cDNA in a sensitive cell line gives rise to the MDR phenotype (10). These proteins share resistance to anthracyclines but have a variable spectrum of resistance to other natural compounds (vinca alkaloids, epipodophyllotoxins, taxanes). Recently, it was shown that the presence of single-nucleotide polymorphisms in ABCB1 (C345T) or in ABCG2 (T/G482A) results in modification of the drug efflux (11). At present, the exact mechanism of drug binding to ABC pumps is unknown. In ABCB1, mutagenesis showed that transmembrane domains 5, 6, 11, and 12 are crucial for drug efflux. It is suggested that a drug-induced conformational change creates a hydrophobic pocket and is able to bind and transport the substrate out of the cell. The ABC proteins are expressed in tissues that are highly exposed to xenobiotics, for example, the gastrointestinal tract and the lung mucosa, and in organs involved in secretion processes like the liver and kidneys (12). The presence of the three main ABC proteins involved in drug transport, ABCB1, ABCC1, and ABCG2 on endothelial cells, deserves attention. A blockade of these pumps would cause increased uptake of drugs from the bloodstream, decreasing the efficiency of the blood–brain barrier (BBB).
There are two main lines of evidence for clinical relevance of an ABC transporter. First, in vitro studies show that the transporter can efflux cytostatic drugs and confer resistance to these drugs in cell cultures. Second, the expression of the transporter in patient samples is associated with poor response to therapy.

More recently, other mechanisms of resistance to a broad spectrum of drugs have been described: increase in DNA repair and defects of drug-induced apoptosis, either due to strong survival signals delivered to the tumoral cells by microenvironment or because of a defect in the apoptosis pathway. It is reasonable to assume that these mechanisms, parallel to ABC transporters, protect the malignant cells.

This review focuses on in vitro and clinical data on those ABC transporters that seem to be associated with poor response to therapy in acute leukemia and new transporters that seem to be associated with chemoresistance in this disease.

4.2. P-GLYCOPROTEIN (P-gp) ABCB1

The best-characterized ABC transporter is the P-gp, which is encoded by the MDR gene 1 (MDR1/ABCB1). In vitro it confers resistance to many drugs that are used in the treatment of acute leukemia, such as doxorubicin, daunorubicin, idarubicin, mitoxantrone, etoposide (13). After several years of contradictory results concerning the frequency and value of ABCB1 expression in tumors, several workshops (14–16) proposed a consensus on technical recommendations for measuring ABCB1. Protein detection and, more importantly, functional tests by flow cytometry, are recommended for leukemic samples. Recognizing an external epitope, several monoclonal antibodies are available for protein expression; and several fluorescent probes could be used, with and without “specific” inhibitors, for measuring the drug efflux.

The main recommendations are:

1. The tumor cell population in the sample must be as pure as possible, especially if bulk techniques are used (reverse transcription–polymerase chain reaction [RT-PCR]).
2. Two different techniques should be used for validation, preferably with single-cell detection methods. Protein detection and functional testing by flow cytometry are recommended for leukemic samples.
3. Calibration controls (all assays must be positive, including one cell line with a low level of positivity, comparable to clinical leukemic samples) and negative controls must be used. The same control cell lines have to be used by all centers working together.
4. A major confounding factor is the use of an arbitrary minimal cutoff for classifying samples as “positive” or “negative.” It is recommended that the data be reported as continuous variable, expressed as a ratio of Mab/control for protein and as probe+ inhibitor/probe alone for function.
Numerous studies have confirmed that the expression of ABCB1 and functionality are adverse prognostic factors for complete remission (CR) and survival in adults AML (4, 17–19). Several large studies could show that the prognostic impact of ABCB1 is more pronounced in elderly patients with AML than in young adults with AML (20). In pediatric patients, it does not have a prognostic impact at all (21, 22).

Since the first observation in vitro modulation of MDR phenotype by verapamil in 1981, it was recognized that several drugs already in use in the clinic inhibit ABCB1 efflux and reverse MDR phenotype in experimental systems (23, 24). This perspective to overcome clinical MDR has led to the strategy of concomitant administration of chemotherapy and acts mainly as MDR modulator. These compounds, competitive or noncompetitive inhibitors with the drug for the binding site, or by binding to other ABCB1 sites, cause allosteric changes of the molecule, resulting in a decrease of cytotoxic drug binding (25).

Several phase I and II trials were conducted in leukemia, multiple myeloma, lymphoma, and solid tumors, using verapamil, amiodarone, quinine, or cyclosporine A together with an anthracycline, an etoposide, or a taxane (26–32). These trials were not restricted to MDR(+) tumors, and it was difficult to know the benefit of these associations to the patients. The pharmacokinetics performed during these trials suggested evidence that verapamil and amiodarone had a minimum toxic dose below the required dose for in vivo ABCB1 inhibition and that quinine and cyclosporine A could be used as ABCB1 modulators with acceptable toxicity. Another interesting finding was the dramatic decrease of anticancer drug clearance when cyclosporine A was coadministered. This pharmacokinetic interaction led to a prolonged terminal half-life, an increased area under the concentration curve (AUC) of the cytostatic, and therefore an increased toxicity. This interaction is less pronounced for quinine, probably because of the absence of inhibition of P450-mediated metabolism, observed when cyclosporine A is administered.

To confirm the interest of such association, several randomized phase III trials, with or without these modulators, were tested. In the trials published so far (Table 4.1), the chemotherapy used as the reference (with or without modulator) included cytosine arabinoside (Ara-C), a non-ABCB1 substrate, at a high dose, together with daunorubicin or idarubicin, or mitoxantrone (33–39). The use of these high doses of Ara-C were able to eradicate positive ABCB1 cells and reduced the chance of observing a difference between the two regimens. When quinine (30mg/kg/day, given by continuous intravenous infusion) was added to anthracycline and Ara-C, either in poor-prognosis adult leukemia cases (relapsing/refractory/secondary) or in untreated de novo AML, global results (rate of CR, overall survival) did not show any difference from the “control” arm (same chemotherapy without quinine). It was noted that in poor-prognosis patients (i) clinical drug resistance was higher in the control group and (ii) toxic death rate was higher in the quinine group, counteracting the potential benefit of MDR reversal. In de novo untreated
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<th>Studies Analyzing Functionality of ABCB1</th>
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<tr>
<td>(33)</td>
<td>315 ALL</td>
<td>MIT: 12mg/m²/d × 4 Ara-C: 1g/m²/12h × 10 Quinine: 30mg/kg/d</td>
<td>CR, DFS, OS: No difference. Refractory to treatment 28% with quinine versus 40% in other arm ($p = 0.04$). Death in aplasia: 20% with quinine versus 7% in other arm ($p = 0.02$)</td>
<td>ND</td>
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<tr>
<td></td>
<td>140 AML</td>
<td>MIT: 12mg/m²/d × 4 Ara-C: 1g/m²/12h × 10 Quinine: 30mg/kg/d</td>
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<tr>
<td></td>
<td>36 refractory or relapsed ALL</td>
<td>MIT: 12mg/m²/d × 4 Ara-C: 1g/m²/12h × 10 Quinine: 30mg/kg/d</td>
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<td></td>
<td>74 acutised MDS</td>
<td>MIT: 12mg/m²/d × 4 Ara-C: 1g/m²/12h × 10 Quinine: 30mg/kg/d</td>
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<td></td>
<td>43 acutised MPD</td>
<td>MIT: 12mg/m²/d × 4 Ara-C: 1g/m²/12h × 10 Quinine: 30mg/kg/d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(34)</td>
<td>131 ≤65 years old MDS graves</td>
<td>MIT: 12mg/m²/d × 4 Ara-C: 1g/m²/12h × 10 Quinine: 30mg/kg/d</td>
<td>Without differences in the whole population or in ABCB1– patients</td>
<td>90 analyzed patients 42 ABCB1+: 52% de CR in quinine group versus 18% in other group. OS: 13 months versus 8 months ($p = 0.01$)</td>
</tr>
<tr>
<td></td>
<td>213 4–75 years old Refractory or relapsed AML</td>
<td>DNR: 50mg/m²/d × 3 Ara-C: 200mg/m²/d × 10 VP16: 100mg/m²/d × 5 CsA: 5 then 10mg/kg/d</td>
<td>Without difference in CR, DFS, and OS but weak dose of CsA</td>
<td>ND</td>
</tr>
<tr>
<td>(35)</td>
<td>120 ≥60 years old de novo AML</td>
<td>DNR: 60mg/m²/d Ara-C: 100mg/m²/d VP16: 100mg/m²/d PSC833: 10mg/kg/d</td>
<td>In whole population, patients treated in ADEP arm have a poorer prognosis than patients in ADE arm. Increased mortality in ADEP arm.</td>
<td>33 analyzed patients (22ABCB1+/11ABCB1–) ABCB1– have a better prognosis than ABCB1+ in ADE arm. Identical prognosis in ADEP arm. In ABCB1+ patients, median DFS and OS are better in ADEP arm than in ADE arm.</td>
</tr>
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<td>Reference</td>
<td>Patients</td>
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<td>Results</td>
<td>Studies Analyzing Functionality of ABCB1</td>
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<tr>
<td>(37)</td>
<td>425</td>
<td>15–60 years old de novo AML</td>
<td>IDA: 8 mg/m²/d × 3, Ara-C: 200 mg/m²/d</td>
<td>No difference in the whole population</td>
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<td>(38)</td>
<td>226</td>
<td>18–70 years old Relapse and refractory AML, and secondary AML or poor prognosis MDS</td>
<td>DNR: 45 mg/m²/d during 24 h × 3, Ara-C: 3 g/m²/12 h × 10</td>
<td>In whole population, in CsA arm the % of resistant patients decreased, and achievement of CR, DFS, and OS increased</td>
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<td>(39)</td>
<td>129</td>
<td>15–70 years old Refractory or relapsed AML and secondary AML and poor prognosis MDS</td>
<td>MIT: 8 mg/m²/d × 5, Ara-C: 1 g/m²/d × 5, VP16: 100 mg/m²/d × 5</td>
<td>No difference: CR, DFS, and OS</td>
</tr>
</tbody>
</table>

AML = acute myeloid leukemia; ALL = acute lymphoid leukemia; DNR = daunorubicin; MIT = mitoxantrone; IDA = idarubicin; Ara-C = cytarabine; VP16 = etoposide; CsA = cyclosporine A; CR = complete remission; DFS = disease-free survival; OS = overall survival; MDS = myelodysplasia syndrome; MPD = myeloproliferative disorder; ND = not done; ADEP = AraC, DNR, etoposide, PSC833; ADE = AraC, DNR, etoposide.
The addition of cyclosporine A at high doses (16 mg/kg/day) during the 3 days of continuous infusion of daunorubicin and high doses of Ara-C in relapsing/refractory AML patients significantly reduced the frequency of resistance and increased the relapse-free survival and the overall survival of the patients. The effect of cyclosporine A on survival was greater in the patients with P-gp (+) leukemic cells than in the others. This interesting result could not be reproduced, with the same schedule, in patients experiencing a blast crisis of chronic myeloid leukemia (CML). Quinine and cyclosporine A, molecules already on the market for a long time, were chosen for their ability to inhibit in vivo ABCB1 efflux. This was demonstrated by the potency of the serum from patients treated with high doses of these drugs to circumvent ABCB1 efflux in cell lines exposed to this serum.

Several drugs were developed for the unique purpose of MDR reversal. The most tested ABCB1 modulator is PSC833, an analog of cyclosporine D, 10-fold more potent than cyclosporine A, without any renal or immunosuppressive toxicity. Extensive dose-finding trials of coadministered cytotoxic drugs (anthracyclines, VP16, and taxol in solid tumors) were conducted with this modulator used at the fixed dose of 10 mg/kg/day, with continuous intravenous infusion, after a loading dose of 2 mg/kg over 2 h. These phase I studies concluded that it was necessary to reduce the daily dose of mitoxantrone by 30%–50%, the dose of daunorubicin by one-third, and the dose of VP16 by 40%–60%, depending on the associations tested. When such adaptations of dose are used, the AUCs of these drugs are similar in both arms, and toxicities related to the cytostatics are also equivalent. The limiting toxicity of PSC833 alone was reversible ataxia and dysmetria, suggestive of a cerebellar dysfunction possibly due to the inhibition of the blood-brain barrier (BBB). Several randomized phase III trials in AML (in elderly untreated patients and in relapsed/refractory younger patients) compared chemotherapy alone with chemotherapy (with lowered doses of anthracyclines and VP16) and PSC833, given intravenously by 24-h infusion during the administration of the anthracyclines and VP16. A higher rate of early mortality (despite the cytostatic dose reduction) was observed in the elderly patients treated with the addition of PSC833 (Table 4.1), raising the question of the consequences of the ABCB1 inhibition in normal tissues. The trial was stopped prematurely, but it is interesting to see that in ABCB1 patients, the increased number of toxic deaths due to the addition of PSC833 was compensated for by a particularly low proportion of resistant disease (19% vs. 41% without PSC833). Several large multicenter phase III trials testing PSC833 have been completed in relapsed and de novo AML, with the same poor results. Concerning the CR rate, no striking differences were observed between the two arms, tempering enthusiasm concerning the concept of ABCB1 inhibitors in drug-resistant leukemia. If higher toxicity is excluded, the failure of PSC833 to
increase the CR rate in the whole population of AML patients could be explained by the dramatic reduction of cytotoxic drugs, leading to a reduction of the maximum and/or steady-state concentration and detrimental to anti-cancer efficacy. The redundant role of the three major ABC pumps, often present together on the leukemic cell surface, could also be explained. The other drugs engineered for ABCB1 inhibition are less advanced, still in phase I, II, and III in solid tumors and leukemias. Zosuquidar is a promising quinoline derivative specific to ABCB1 and delivered orally with a low effect on the pharmacokinetics of coadministered drugs (40–42).

However, it is too early to conclude that such strategy is not feasible. As we have seen above, it is difficult to find the right inhibitor with a weak interaction with a drug. Zosuquidar is perhaps a promising modulator. In addition, in all studies, the patients were included irrespective of their ABCB1 status. It is difficult to show a statistically significant impact of ABCB1 inhibition in patients under these conditions. Therefore, future clinical trials should use such inhibitors, and they should be restricted only to adult patients who express a functional ABCB1 protein.

However, today we know that ABCB1 is one of 49 different human ABC transporters (5). ABC transporters facilitate ATP-dependent transport of various substrates via extra- or intracellular membranes. All ABC transporters that are able to transport cytostatic drugs might cause drug resistance in the same way as ABCB1, even if this later plays an important function in drug resistance in acute leukemia.

4.3. BREAST CANCER RESISTANCE PROTEIN (BCRP) ABCG2

BCRP/ABCG2 is one of the more recently discovered ABC transporters. It was isolated from MDR MCF-7/AdrVp breast cancer cells (43). BCRP is encoded by the ABCG2 gene, which was mapped to chromosome 4 (44). It has only one ABC and six putative transmembrane domains, suggesting that BCRP is a half-transporter (45). High expression of BCRP has been described in drug-resistant ovary, breast, colon, and gastric cancer and fibrosarcoma cell lines (46). In vitro, it confers resistance to a wide range of drugs that are used in the treatment of acute leukemia such as daunorubicin, etoposide, methotrexate, and mitoxantrone (13).

So far, several studies have been published analyzing the association between expression of BCRP and response to therapy in AML (Table 4.2). Each study pointed toward a prognostic impact of BCRP. Benderra et al. (47), Steinbach et al. (48), and Uggla et al. (49) analyzed BCRP mRNA; Damiani et al. (50) analyzed BCRP protein expression, and in a second study, Benderra et al. (51) analyzed protein expression as well as protein function. In each study, higher levels of BCRP expression or function were associated with a
### TABLE 4.2. Studies Analyzing ABCG2 in AML and ALL

<table>
<thead>
<tr>
<th>Disease</th>
<th>Number of Patients</th>
<th>Techniques Used</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML and ALL (adults)</td>
<td>20 AML and 1 ALL; 14 <em>de novo</em> and 7 relapsed</td>
<td>RT-PCR</td>
<td>33% expressed mRNA of BCRP fivefold above the median value of all samples</td>
<td>(55)</td>
</tr>
<tr>
<td>AML (adults)</td>
<td>20 AML, 12 relapsed and 8 <em>de novo</em></td>
<td>Immunocytochemistry (antibody BXP-34)</td>
<td>BCRP expression: no difference between <em>de novo</em> and relapse AML; BCRP+ patients have an increased <em>in vitro</em> resistance to daunorubicine compared to BCRP− patients</td>
<td>(56)</td>
</tr>
<tr>
<td>AML (adults)</td>
<td>40 <em>de novo</em> AML</td>
<td>RT-PCR</td>
<td>7% expressed BCRP as a transfected cell line and 78% expressed BCRP above normal hematopoietic cells</td>
<td>(57)</td>
</tr>
<tr>
<td>AML (adults)</td>
<td>20 pairs of samples (at diagnosis and at relapse)</td>
<td>RT-PCR</td>
<td>BCRP expression increased at relapse</td>
<td>(58)</td>
</tr>
<tr>
<td>AML (adults)</td>
<td>20 pairs of samples (at diagnosis and at relapse)</td>
<td>Protein expression (BXP-34 and 21), and functionality (mitoxantrone ± fumitremorgine C) in flow cytometry</td>
<td>Correlation between BCRP expression and immature phenotype of blast cells; no difference of expression between <em>de novo</em> and relapse AML</td>
<td>(59)</td>
</tr>
<tr>
<td>Disease</td>
<td>Number of Patients</td>
<td>Techniques Used</td>
<td>Results</td>
<td>References</td>
</tr>
<tr>
<td>-------------------------</td>
<td>--------------------</td>
<td>---------------------------------------------------------</td>
<td>-------------------------------------------------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>ALL (children)</td>
<td>67</td>
<td>RT-PCR</td>
<td>No prognostic factor</td>
<td>(53)</td>
</tr>
<tr>
<td>AML (children)</td>
<td>59</td>
<td>RT-PCR</td>
<td>BCRP associated with lower CR and worse OS</td>
<td>(48)</td>
</tr>
<tr>
<td>AML (adults)</td>
<td>149</td>
<td>Flow cytometry; expression (BXP-34) and function</td>
<td>BCRP activity influences CR and OS</td>
<td>(47)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(mitoxantrone ± fumitremorgine C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AML (adults)</td>
<td>40</td>
<td>RT-PCR</td>
<td></td>
<td>(49)</td>
</tr>
<tr>
<td>AML (adults)</td>
<td>85</td>
<td>Flow cytometry (expression and functionality)</td>
<td>ABCG2 (BCRP) activity influences CR, DFS, and OS</td>
<td>(51)</td>
</tr>
<tr>
<td>AML (adults)</td>
<td>73 de novo AML with</td>
<td>Flow cytometry (BXP-34 antibody)</td>
<td>ABCG2 did not influence CR but affected the duration of CR</td>
<td>(50)</td>
</tr>
<tr>
<td></td>
<td>normal karyotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AML (adults &gt;60 years</td>
<td>154 de novo AML</td>
<td>RT-PCR</td>
<td>ABCB1/ABCG2 coexpression was associated with lower CR and worse EFS and OS</td>
<td>(60)</td>
</tr>
</tbody>
</table>

AML = acute myeloid leukemia; ALL = acute lymphoid leukemia; CR = complete remission; DFS = disease-free survival; EFS = event-free survival; OS = overall survival.
lower chance of survival. Three studies also described significantly higher levels of BCRP in patients who failed to achieve remission (47, 48, 51). In summary, there is little doubt that BCRP correlates with poor prognosis in adult AML (47, 49–51) and probably also in childhood AML (48).

Little is known about the role of BCRP in acute lymphoid leukemia (ALL). The expression is lower in T-ALL than in precursor B-ALL (52, 53); it is also lower in infant ALL than in older patients (54). One small study found a trend for worse outcome in adult patients with higher levels of BCRP (55), and in another such study in pediatric patients no such association was found (53).

4.4. MULTIDRUG RESISTANCE ASSOCIATED PROTEIN (MRP1) ABCC1

The ABCC subfamily of ABC transporters encodes the MRP. The first member of this subfamily, ABCC1, was described in 1992 by Cole et al. (61). It is overexpressed in many multidrug-resistant human cancer cell lines, and transfection experiments with different eukaryotic expression vectors containing full-length complementary DNAs of ABCC1 gene have shown that ABCC1 confers, just like ABCB1, resistance to a wide range of drugs used in the treatment of acute leukemia, such as doxorubicin, daunorubicin, vincristine, etoposide, and methotrexate (13).

Many studies have analyzed the clinical relevance of ABCC1 in AML. The results are very controversial. Many studies reported a correlation between ABCC1 and poor prognosis (62–65). An equally large number found no such correlation (18, 66–68). There are a number of possible explanations for these findings. Many different methods were used to study ABCC1 expression such as PCR, flow cytometry, western blot, immunocytochemistry, or functional assays. This is particularly relevant since many of these methods were established before the other members of the ABCC subfamily were discovered (69). As a consequence, some of the analyses were probably not as specific for ABCC1 as they were meant to be. Even if one tries to compare studies that used similar methods for the detection of ABCC1, the results are still inconsistent. This might be due to different treatment regimens or patient characteristics. For example, the results are controversial in patients treated with anthracyclines and cytarabine, but two studies confirmed the role of ABCC1 overexpression in resistance to treatment with gemtuzumab ozogamicin (70, 71). In conclusion, there seems to be some degree of correlation between ABCC1 and poor prognosis. However, if ABCC1 was a major determinant of drug resistance in AML, this correlation should be found more consistently (Table 4.3).

In ALL, the data are even less suggestive of a relevant prognostic impact of ABCC1. Most studies found no correlation between ABCC1 and poor prognosis (21, 77, 78).
### TABLE 4.3. ABCC1 as Prognostic Factor in AML

<table>
<thead>
<tr>
<th>References</th>
<th>Techniques</th>
<th>Number of Patients</th>
<th>Patients</th>
<th>CR Achievement</th>
<th>DFS</th>
<th>Overall Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>(72)</td>
<td>RT-PCR</td>
<td>(45 patients)</td>
<td>de novo AML</td>
<td>$p = 0.07$</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>(73)</td>
<td>RNase protection assay</td>
<td>(35 patients)</td>
<td>de novo AML</td>
<td>NS</td>
<td>ND</td>
<td>NS</td>
</tr>
<tr>
<td>(74)</td>
<td>RT-PCR</td>
<td>(43 patients)</td>
<td>de novo and relapsed AML</td>
<td>NS</td>
<td>NS</td>
<td>ND</td>
</tr>
<tr>
<td>(75)</td>
<td>RT-PCR</td>
<td>(52 patients)</td>
<td>de novo and relapsed AML</td>
<td>$p = 0.005$</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>(68)</td>
<td>Protein expression Immunocytochemistry</td>
<td>(80 patients)</td>
<td>de novo AML</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>(76)</td>
<td>RT-PCR</td>
<td>(57 patients)</td>
<td>de novo AML</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>(18)</td>
<td>Protein expression (flow cytometry)</td>
<td>(352 patients)</td>
<td>de novo AML</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>(62)</td>
<td>RT-PCR</td>
<td>Protein expression functionality (flow cytometry)</td>
<td>(53 patients)</td>
<td>de novo AML</td>
<td>NS, $p = 0.03$</td>
<td>ND</td>
</tr>
<tr>
<td>(66)</td>
<td>Protein (flow cytometry)</td>
<td>(91 patients)</td>
<td>de novo AML</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>(65)</td>
<td>Functionality (flow cytometry)</td>
<td>(calcine ± probencid)</td>
<td>(80 patients)</td>
<td>de novo AML</td>
<td>$p = 0.008$, $p = 0.01$, $p = 0.02$</td>
<td>ND</td>
</tr>
<tr>
<td>(67)</td>
<td>Functionality (flow cytometry)</td>
<td>CFDA ± MK571</td>
<td>(104 patients)</td>
<td>de novo AML</td>
<td>Patients with the poorest prognosis have a simultaneous activity of both P-gp and MRP1</td>
<td>ND</td>
</tr>
<tr>
<td>(64)</td>
<td>Functionality (flow cytometry)</td>
<td>CFDA ± probencid</td>
<td>(44 patients)</td>
<td>de novo AML</td>
<td>$p = 0.001$</td>
<td>ND</td>
</tr>
<tr>
<td>(63)</td>
<td>RT-PCR</td>
<td>(331 patients)</td>
<td>de novo and secondary AML</td>
<td>NS</td>
<td>$p = 0.01$</td>
<td>NS</td>
</tr>
</tbody>
</table>

AML = acute myeloid leukemia; DFS = disease-free survival; OS = overall survival; NS = not significant; ND = not done.
4.5. MULTIDRUG RESISTANCE ASSOCIATED PROTEIN (MRP1) ABCC3

Besides ABCC1, there exist 11 other members of the ABCC subfamily of ABC transporters (7). Many of them were shown to be able to efflux cytostatic drugs. Only a few studies have analyzed the prognostic relevance of these proteins in leukemia, but each study found that ABCC3 is associated with a lower chance of survival. This was the case for children with AML (79), adults with AML (51), children with ALL (80, 81), and adults with ALL (81). This finding was independent of the method used for the detection of ABCC3. Benderra et al. (51) analyzed protein expression as well as protein activity. Steinbach et al. (79, 80) and Plasschaert et al. (81) analyzed gene expression. Based on this limited number of studies it seems that ABCC3 is the only ABC transporter, which correlates with poor prognosis in AML and in ALL independent of the patient age.

However, in vitro data are less suggestive of a major role of ABCC3 in drug resistance. ABCC3 was shown to cause resistance against etoposide, teniposide, and methotrexate (82, 83). So far, it seems that other important leukemia drugs are no substrates for ABCC3 (13).

4.6. ABCA3

The latest ABC transporter that was correlated with response to chemotherapy in acute leukemia, is ABCA3. Yasui et al. (84) found that a number of drug-resistant cancer cell lines showed higher copy numbers of the ABCA3 gene and a stronger expression of the gene compared to the drug-sensitive parental cell lines. Hirschmann-Jax et al. (85) showed that ABCA3 and ABCG2 are expressed in various malignant stem cells and that the two genes together might be involved in the resistance of these cells against mitoxantrone.

Norwood et al. (86) described the expression of ABCA3 in an in vivo propagated human AML cell line. The same group could show that the protein ABCA3 was expressed in a panel of AML samples (87). Using a low-density microarray for ABC transporters, it could be shown that ABCA3 is overexpressed in pediatric AML compared to healthy bone marrow and that its expression is associated with a poor response to remission induction therapy (88). The prognostic impact of ABCA3 in pediatric AML was confirmed by Chapuy et al. (89).

ABCA3 is located at intracellular membranes (90). Its does not confer a classical drug efflux but rather seems to be involved in the intracellular sequestration and the vesicular transport of its physiological substrates as well as chemotherapeutic agents such as daunorubicin (87, 90).

ABCA3 is also expressed in ALL (91). Its association with response to therapy in this form of leukemia has not yet been examined.
4.7. FUTURE PERSPECTIVES

The highly conserved homology between different ABC transporters predicts that additional members may be involved in the extrusion of xenobiotic and chemotherapeutical compounds (92, 93). Indeed, an increasing number of ABC transporters have been demonstrated to cause resistance to cancer chemotherapeutical agents (Table 4.4) (5, 94). In a recent gene expression profiling study revealing the expression profile of all ABC transporters in human CD34+/CD38− hematopoietic stem cells, all of these transporters were found to be expressed in human CD34+38− hematopoietic cells, most of which in a differential manner in comparison to committed CD34+38+ cells (94). Additionally, recent studies examining ABC transporters’ gene expression in drug-selected cancer cell lines demonstrated overexpression of a number of transporters not previously recognized as associated with drug resistance (ABCA4, ABCA7, ABCB2, ABCB6, ABCB8, ABCB9, and ABCG1) (94). With the exception of ABCC4, all of these transporters are expressed in human CD34+38− hematopoietic cells, again many differentially in comparison to committed progenitors. Together, these data outline the emerging picture that ABC transporter-mediated protection against xenobiotics and other toxic substrates may be conferred by simultaneous activity of many redundant family members in addition to ABCB1, ABCC1, and ABCG2.

<table>
<thead>
<tr>
<th>ABC Transporters</th>
<th>Drug Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA2</td>
<td>Estramustine</td>
</tr>
<tr>
<td>ABCA3</td>
<td>Daunorubicin</td>
</tr>
<tr>
<td>ABCB1 (P-gp)</td>
<td>Anthracyclins, etoposide, imatinib, taxanes, mitoxantrone, vinca alkaloids</td>
</tr>
<tr>
<td>ABCB4</td>
<td>Paclitaxel, vinblastine</td>
</tr>
<tr>
<td>ABCB5</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>ABCB11</td>
<td>Paclitaxel</td>
</tr>
<tr>
<td>ABCC1 (MRP1)</td>
<td>Anthracyclins, etoposide, methotrexate</td>
</tr>
<tr>
<td>ABCC2 (MRP2)</td>
<td>Cisplatin, doxorubicin, etoposide, methotrexate, mitoxantrone, vinca alkaloids</td>
</tr>
<tr>
<td>ABCC3 (MRP3)</td>
<td>Cisplatin, doxorubicin, etoposide, methotrexate, vinca alkaloids</td>
</tr>
<tr>
<td>ABCC4 (MRP4)</td>
<td>Methotrexate, thiopurines</td>
</tr>
<tr>
<td>ABCC5 (MRP5)</td>
<td>Thiopurines</td>
</tr>
<tr>
<td>ABCC6 (MRP6)</td>
<td>Anthracyclins, etoposide, teniposide</td>
</tr>
<tr>
<td>ABCC10</td>
<td>Paclitaxel, vinca alkaloids</td>
</tr>
<tr>
<td>ABCC11</td>
<td>Nucleotide analogs</td>
</tr>
<tr>
<td>ABCG2 (BCRP)</td>
<td>Mitoxantrone, methotrexate, topotecan, imatinib, anthracyclins</td>
</tr>
</tbody>
</table>
definitive role of these transporters in the protection of leukemic cells remains to be determined.

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42. Lancet JE, Gotlib J, Wetzler M, Lugie S, Cripe LD, Tallman MS et al. 2007. Phase I/II study of the P-Glycoprotein (Pgp) inhibitor zosuquidar administered by continuous infusion (CIV) with daunorubicin (DNR) and cytarabine (ARA-C) as primary therapy in older patients with Pgp-positive acute myeloid leukemia (AML). *Blood (ASH Annual Meeting Abstracts)* 110 (November): 299.


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ABC PROTEINS AND ONCOLOGY: EXPRESSION, DETECTION, AND IMPLICATION OF ABC PROTEINS IN SOLID TUMORS

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5.1. INTRODUCTION

As one of the former heads of our hospital’s radiotherapy department used to say, “malignant cells are not only malignant, they are also malicious,” a pun on the French term “cellules malignes,” as these cells are able to escape from various anticancer treatments. Throughout the course of evolution, eukaryotic cells have developed a wide range of mechanisms to protect themselves from toxic compounds derived from the cellular environment, and cancer cells have learned to take advantage of cellular detoxifying mechanisms to resist the various drugs used in cancer chemotherapy.

The clinical problem of drug resistance was first recognized in the 1940s, soon after the introduction of the first anticancer drug, nitrogen mustard. Since that time, elucidation of the mechanisms by which cancer cells escape the effects of cytotoxic drugs, either from the onset of chemotherapy (intrinsic resistance) or after an initial clinical response (acquired resistance), has been a major field of research. Tumor response to chemotherapy or drug resistance may involve multiple mechanisms in relation to the expression of various genes in cancer cells or to the interactions of these cells with their environment, such as the stromal or immune cells as well as the components of the extracellular matrix. Resistant cancer cells are frequently simultaneously resistant to several drugs, which differ by their chemical structure and/or mechanism of action, which is at the origin of the term “multidrug resistance” (MDR). Host factors and cancer cell factors, such as restricted drug access to tumor cells, increased DNA repair, and enhanced detoxification through increased expression of multidrug transporters, can all contribute to MDR phenotypes (1). The MDR phenotype was initially attributed to the expression of a 170 kDa transmembrane protein, P-glycoprotein (P-gp), functioning as an efflux pump at the cell membrane level, cloned as the MDRI gene, identified as an ATP-binding cassette (ABC) transporter, and now known as ABCB1. However, as summarized in Table 5.1, different types of MDR can be distinguished (2), the classical type involving ABC transporters, and the other types related, for instance, to the blockage of drug effector pathways such as the apoptotic or senescent pathway due to mutations in TP53 (3). However, the acronym MDR will be used in the rest of this chapter to indicate the classical MDR phenomenon.
As already described in this book, the ABC superfamily is a large and ancient protein family, present in prokaryotes as in eukaryotes. ABC transporters are believed to date back more than 3 billion years and are involved in a wide variety of physiological processes, from bacteria to fungi and to humans. This superfamily plays a fundamental role in drug resistance (4). The human genome contains nearly 50 genes coding for ABC proteins, most of which still have unknown function. About 15 of these ABC proteins are involved in efflux pumping of exogenous toxins to protect the integrity of the cell and/or the body, suggesting for some authors that these ABC transporters participate in “a chemokinx immunity defense system” (5). These ABC transporters involved in physiological processes are also used by the body and cells to control the uptake and distribution of therapeutic drugs that are considered “xenobiotics” by cells. Cancer cells are not an exception to the rule and use these efflux pumps as safety equipment to detoxify their content from cytotoxic drugs; and, therefore, different ABC transporters are actively involved in cancer cell chemoresistance.

As emphasized by Higgins and Linton (6) in the introduction to ABC Transporters, From Bacteria to Man: “ABC transporters only became sexy in recent years following the realization that they are of considerable medical, industrial, and economic importance. Tens of thousands of cancer deaths each year are a consequence of overexpression of ABC transporters which confer resistance to drugs used for chemotherapy.”

This chapter will successively examine the following specific items: (a) the anticancer drugs which are substrates of ABC transporters and (b) the detection of MDR-related ABC transporters in solid tumor samples.

<table>
<thead>
<tr>
<th>Resistance to</th>
<th>Classical MDR</th>
<th>Defective Drug Effector Pathways</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural product drugs</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alkylating agents</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>X rays</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>ABC transporters/drug pumps</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Defective apoptosis or senescence</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Induction in all types of cancer cells</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

* Schmidt 2003.
5.2. ANTICANCER DRUGS THAT ARE SUBSTRATES OF ABC TRANSPORTERS

Among their various physiological functions, many ABC transporters have been shown to consider as substrates a wide range of drugs used in anticaner chemotherapy, at least on cell lines (Tables 5.2 and 5.3). It was originally shown more than 30 years ago that in vitro – induced resistance of cancer cells to one of these drugs rendered these cells resistant to other unrelated agents. To make a long story short, experimental models of MDR were first proposed at the end of the 1960s in models of mouse leukemia, and in 1973 Danø discovered that resistance to daunorubicin and vinblastine was due to active efflux transport of these drugs in Ehrlich ascites tumor cells (7). At that time, he concluded: “Together with previous findings of reciprocal cross-resistance between daunomycin and the vinca alkaloids, and a decreased accumulation of daunomycin in cells selected for resistance to vincristine and vinblastine, this effect suggests that these drugs are transported by the same extrusion mechanism as

<p>| TABLE 5.2. Drugs Currently Used in Solid Tumor Chemotherapy Interacting with P-gp/MDR1/ABCB1, MRPI/ABCC1, and BCRP/ABCG2 Transporters (According to Shirom [8] and Modified According to Lemos et al. [12]) |</p>
<table>
<thead>
<tr>
<th>P-glycoprotein (MDR1, ABCB1)</th>
<th>MRP1, ABCC1</th>
<th>BCRP, MXR (ABCG2)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anticancer drugs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vinca alkaloids (vincristine, vinblastine)</td>
<td>Vinca alkaloids (vincristine, vinblastine)</td>
<td>Anthracenediones (mitoxantrone, bisantrene)</td>
</tr>
<tr>
<td>Anthracyclines (doxorubicin, daunorubicin)</td>
<td>Anthracyclines (doxorubicin, daunorubicin)</td>
<td>Epidophyllotoxins (etoposide, teniposide)</td>
</tr>
<tr>
<td>Taxanes (paclitaxel, docetaxel)</td>
<td>Epidophyllotoxins (etoposide, teniposide)</td>
<td>Camptothecins (topotecan)</td>
</tr>
<tr>
<td>Epidophyllotoxins (etoposide, teniposide)</td>
<td>Camptothecins (topotecan, irinotecan)</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>Camptothecins (topotecan)</td>
<td>Anthracyclines (daunorubicin, doxorubicin)</td>
<td></td>
</tr>
<tr>
<td>Anthracene diones (bisantrene, mitoxantrone)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tyrosine kinase inhibitors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imatinib</td>
<td>Imatinib</td>
<td>Imatinib</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>Gefitinib</td>
<td>Gefitinib</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nilotinib</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Erlotinib</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Canertinib</td>
</tr>
</tbody>
</table>

Tyrosine kinase inhibitors

Imatinib
Gefitinib
Imatinib
Gefitinib
Nilotinib
Erlotinib
Canertinib
TABLE 5.3. Other ABC Transporters Potentially Involved in Chemotherapy Resistance Evaluated on Cell Lines (9)

<table>
<thead>
<tr>
<th>ABC Family</th>
<th>ABC Transporter</th>
<th>Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA</td>
<td>ABCA2</td>
<td>Estramustine, Mitoxantrone</td>
</tr>
<tr>
<td>ABCB</td>
<td>ABCB1/MDR1</td>
<td>Doxorubicin, daunorubicin, Paclitaxel, vinca alkaloids, Etoposide, mitoxantrone</td>
</tr>
<tr>
<td>ABCB4/MDR3</td>
<td></td>
<td>See Table 5.2</td>
</tr>
<tr>
<td>ABCB5</td>
<td></td>
<td>Doxorubicin, Camptothecin, 10-hydroxy camptothecin, 5-Fluorouracil</td>
</tr>
<tr>
<td>ABCB11/BSEP</td>
<td></td>
<td>Paclitaxel</td>
</tr>
<tr>
<td>ABCC</td>
<td>ABCC1/MRP1</td>
<td>See Table 5.2</td>
</tr>
<tr>
<td>ABCC2/MRP2/cMOAT</td>
<td></td>
<td>Vinca alkaloids, cisplatin, Etoposide, Doxorubicin, epirubicin, Taxanes (paclitaxel, docetaxel), Methotrexate, Topotecan, Mitoxantrone</td>
</tr>
<tr>
<td>ABCC3/MRP3/cMOAT2</td>
<td></td>
<td>Epidophyllotoxins (etoposide, teniposide), Methotrexate</td>
</tr>
<tr>
<td>ABCC4/MRP4/MOAT-B</td>
<td></td>
<td>6-mercaptopurine, 6-thioguanine, irinotecan, topotecan, methotrexate</td>
</tr>
<tr>
<td>ABCC5/MRP5/MOAT-C</td>
<td></td>
<td>6-mercaptopurine, 6-thioguanine, 5-FU, Cisplatin, methotrexate</td>
</tr>
<tr>
<td>ABCC6/MRP6/MOAT-E/PXE</td>
<td></td>
<td>Etoposide, teniposide, Doxorubicin, daunorubicin, epirubicin, Dactinomycin, cisplatin</td>
</tr>
<tr>
<td>ABCC10/MRP7</td>
<td></td>
<td>Taxanes, vinca alkaloids</td>
</tr>
<tr>
<td>ABCC11/MRP8</td>
<td></td>
<td>6-mercaptopurine, 5-Fluorouracil</td>
</tr>
<tr>
<td>ABCG</td>
<td>ABCG2/BCRP</td>
<td>See Table 5.2</td>
</tr>
</tbody>
</table>
daunomycin.” MDR was thus defined as the resistance developed against one
drug, and protecting the cell not only against this drug but also against a range
of structurally and functionally unrelated toxic agents. A few years later (1976),
P-gp was identified as the transmembrane efflux pump responsible for this
resistance, and its gene was cloned 10 years later as the \textit{MDR} gene (1986) (see
Part I, Chapter 1). The \textit{MDR1} gene was in fact the first member of the ABC
transporter superfamily. In 1992, another gene, \textit{MRP1} (MDR-related protein)
was discovered by S. Cole (see Part I, Chapter 2) and was shown to be also
involved in the MDR phenotype. It is now known as \textit{ABCC1}. Other ABC
transporters have subsequently been discovered and cloned.

A total of 15 ABC transporters have been hypothesized to be involved in
drug resistance, but as underlined by Sharom (8) “three ABC proteins appear
to account for most observed MDR in humans and rodents: P-glycoprotein
(P-gp/MDR1/ABCB1), MDR-associated protein (MRP1/ABCC1) and breast
cancer resistance protein ABCG2 (variably known as BCRP, ABCP or
MXR).” The functioning of these three ABC transporters and their substrates
has been described in other chapters of this book. Briefly, P-gp and MRP1
are 170–190 kDa polypeptides, while BCRP is a 72 kDa half transporter.
P-gp and BCRP can export essentially unconjugated drugs, whereas MRP1
exports glutathione (and other) drug conjugates and unconjugated drugs
which are, in that case, cotransported with glutathione (5, 8, 9). The drugs
currently used in the chemotherapy of solid tumors and interacting with these
three ABC transporters are listed in Table 5.2. As a general rule, the drug
substrates of the ABC transporters are, or derive from, natural products (alka-
loids, bacterial antibiotics). No cross-resistance with alkylating agents, plati-
num compounds, or antimetabolites can be observed in cells overexpressing
P-gp, MRP1, or BCRP. However, for some members of the ABCC family,
transport of nucleosides, nucleotides, or antifolates (antimetabolites) was
shown \textit{in vitro}, as well as cisplatin transport. The same anticancer drug may
be the substrate of several ABC transporters, which indicates that a redunda-
dancy has developed throughout the process of evolution. It has been hypoth-
esized that only the top of the iceberg had been discovered and that new
substrates will be discovered for the already identified ABC transporters.
This is especially the case for ABCG2, emphasizing the importance of this
transporter (8, 10).

\textbf{5.2.1. A Special Feature: ABC Transporters and Tyrosine Kinase Inhibitors}

Studies in mice and cell lines have recently demonstrated that P-gp, MRP1,
and BCRP transporters interact (11, 12) with TKIs recently introduced as
treatments for solid tumors (13, 14). Interactions of TKIs with ABC transport-
ners became evident in the last years and were demonstrated to be dual: (a)
TKIs are potential substrates of certain ABC transporters and (b) TKIs are
modulators of ABC transporters (Table 5.4). BCRP has been hypothesized
to be one of the determinants of gefitinib resistance in epithelial growth factor
TABLE 5.4. Interactions of TKIs with BCRP (According to Lemos et al.) (12)

<table>
<thead>
<tr>
<th>TKI</th>
<th>Molecular Target</th>
<th>Substrate of ABC transporter</th>
<th>Modulator of ABC Transporter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canertinib (CI-1033)</td>
<td>HER family</td>
<td>ABCG2</td>
<td>Sensitization to SN-38 and topotecan</td>
</tr>
<tr>
<td>Imatinib (Gleevec)</td>
<td>BCR-ABL</td>
<td>ABCG2 (concentration dependent)</td>
<td>P-gp/MDR (143)</td>
</tr>
<tr>
<td>Gefitinib (Iressa)</td>
<td>EGFR</td>
<td>ABCG2</td>
<td>Modulators of P-gp and ABCG2</td>
</tr>
<tr>
<td>Erlotinib (Tarceva)</td>
<td>EGFR</td>
<td>ABCG2</td>
<td>Modulator of ABCG2</td>
</tr>
<tr>
<td>Nilotinib</td>
<td>BCR-ABL</td>
<td>ABCG2</td>
<td>Inhibitor of ABCG2</td>
</tr>
</tbody>
</table>

receptor (EGFR)-expressing cells (15). Imatinib has also been shown to be transported by P-gp in rat glioma cells (16).

TKIs have also been demonstrated to be modulators/inhibitors of BCRP, P-gp, and MRP1 (11, 12). For instance, canertinib, an ERBB family TKI, may modulate the drug efflux associated with BCRP expression, sensitizing cells to SN-38 and topotecan (12). Imatinib has been shown to be a potent BCRP inhibitor, which, in some cell lines, could attenuate resistance to imatinib by inhibition of BCRP (17). Nilotinib, a novel BCR-ABL TKI, which is more potent and more selective than imatinib, has been shown to be a more potent inhibitor of BCRP than imatinib (12). Gefitinib, another substrate of BCRP but only at low concentrations, is also a potent inhibitor of P-gp and BCRP. Erlotinib significantly potentiates the sensitivity of P-gp or BCRP substrates and increases the accumulation of paclitaxel or mitoxantrone in P-gp or BCRP overexpressing cells and was shown to stimulate the ATPase activity of both P-gp and BCRP (18). Gefitinib reverses BCRP-mediated drug resistance to topoisomerase I inhibitors via direct inhibition (19). All these findings were obtained on experimental models using cell lines. Their relevance to clinical practice needs to be confirmed, but they could possibly be useful for cancer chemotherapy in combination with TKIs as potential inhibitors of ABC transporters.

5.3. DETECTION OF MDR-RELATED ABC TRANSPORTERS IN SOLID TUMOR SAMPLES

Various methods have been used to detect and evaluate the function of ABC transporters. For solid tumors, except for cell lines which will not be considered
in this chapter, most studies of ABC transporter expression have been based on mRNA detection and/or immunohistochemistry (IHC), and functional studies are not available except in some reports (20). However, with the development of radiolabeled ABC transporter substrates, in vivo functional imaging assays will hopefully be available in the near future to help guide treatment of individual patients.

The recommendations of two consensus conferences, one in the United States (21) and the other in France (22), published in 1996 and 1997, respectively, concerning the detection of P-gp in patients’ tumors, are still valid and can be applied to the various ABC transporters studied in human tumors. Before the clinical relevance of ABC transporter expression can be properly evaluated, standard techniques for their measurement must be validated. Several multicenter trials on P-gp have shown large discrepancies in the levels of expression in the same clinical samples evaluated in different centers, and the most marked discrepancies were observed in samples expressing low levels of P-gp (23). At that time, it was concluded that “although standardized procedures have dramatically increased the interlaboratory reproducibility of flow cytometry and polymerase chain reaction assays, data from immunocytochemistry remain difficult to interpret” (23), which is still valid.

In summary, quantitative and qualitative evaluation of ABC proteins in solid tumor samples can be achieved either at the transcriptional (mRNA) or translational (protein) levels, as described below.

5.3.1. mRNA Detection ([RT-PCR] and Microarrays) of ABC Transporters in Tumor Samples

Tissue gene expression has been conventionally studied by northern blot analysis, dot blot analysis, end point-PCR, RNase protection assay, and in situ hybridization. Although these methods are widely accepted and reliable, they require large amounts of RNA as starting material and are usually not amenable to analyzing multiple genes and large numbers of samples. RT-PCR allows real-time measurement of accumulating PCR products using various fluorescence-based technical methodologies monitoring the intensity of the fluorescent signal during the exponential phase of the PCR reaction. Measurement of the fluorescent signal intensity is proportional to the amount of input target DNA and is found to be exquisitely sensitive, exhibiting a satisfactory linearity over a wide range of mRNA concentrations. It allows accurate quantitative evaluation of a large number of samples by analyzing for each sample a total RNA amount as small as 25 ng per 50 µL of reaction mixture. By comparison, most of the other methods mentioned above require 5–20 µg of total RNA per experiment. RT-PCR has therefore been proven to be particularly valuable in studies in which very small amounts of target mRNA are available, such as studies on biopsy material. For this reason, since the mid-1990s, RT-PCR is considered to be the gold standard for mRNA detection, particularly in clinical situations.
5.3.2. Immunohistochemistry

In contrast to mRNA evaluation, IHC has the advantage of discriminating between the various components of the tumor and can be used to evaluate stromal and tumor cell expression as well as to detect heterogeneous expression in different areas. Large panels of monoclonal antibodies (mAbs) are now available to detect a particularly wide set of ABC transporters in tissues or cell suspensions. Some of these mAbs recognize intracellular epitopes, while others recognize cell surface epitopes; some are used on tissue sections after paraffin embedding, while others are restricted to cells or frozen sections. For example, a very thorough study by Scheffer et al. (24) devoted to immunodetection of ABCB1, ABCC1, ABCC2, ABCC3, ABCC5, and ABCB4 products, illustrates the various steps before validation of the use of these antibodies. Immunodetection of ABCB1, ABCB4, ABCC1, ABCC2, ABCC3, ABCC, and ABCG2 products was recently reviewed by Gillet et al. (9).

However, some recommendations must be emphasized. A so-called specific antibody may have overlapping cross-reactivity for different ABC transporters, which must be taken into account: (a) the specificity of commercial antibodies should therefore be checked on western blots/immunoblots on control cell lines and (b) more than one antibody should be used for each protein detected. It is absolutely essential to clearly define positive and negative expressions, compared to well-defined positive and negative controls. Moreover tissue sections should be screened and scored in a blinded fashion by more than one pathologist using criteria established before the analysis (25). However, although IHC is particularly efficient for localization, it is much less reliable for quantitative evaluation.

IHC has been used for detection of MDR-related ABC transporters in a wide set of solid tumors, for example, in breast cancer, but has provided heterogeneous results, as reported in Table 5.5. As indicated previously, the discrepancy between IHC and PCR may be due to the higher sensitivity of PCR methods. These heterogeneous results are also illustrated in ovarian tumors, where P-gp expression was observed in 15%–47% of samples, and MRP1 expression was observed in 22.5%–68% (26) of samples at diagnosis.

IHC results can be compared to those of normal structures, as illustrated in nonsmall-cell lung carcinoma (NSCLC). Berger et al. (27) used IHC to analyze specimens from 126 patients and found no difference in P-gp expression, except in patients treated with neoadjuvant chemotherapy, as observed for lung resistance protein (LRP), compared to normal epithelial cells. In contrast, levels of MRP1 expression were significantly higher, which raises the hypothesis of a role for MRP1 in intrinsic resistance to treatment of NSCLC, although this has not yet been confirmed (27).

5.3.3. Flow Cytometry

Apart from functional studies, which are not useful for routine assessment of ABC transporters in solid tumors in contrast with hematopoietic or lymphoid
TABLE 5.5. Expression of P-gp/MDR1 in breast cancer (Adapted from Bates et al.) (26)

<table>
<thead>
<tr>
<th>References</th>
<th>Method</th>
<th>N Tumors</th>
<th>Antibody</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>(56)</td>
<td>IHC</td>
<td>52</td>
<td>C494</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>46</td>
<td></td>
<td>47</td>
</tr>
<tr>
<td>(142)</td>
<td>IHC</td>
<td>140</td>
<td>JSB1</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>30</td>
<td></td>
<td>39</td>
</tr>
<tr>
<td>(143)</td>
<td>IHC</td>
<td>106</td>
<td>JSB-1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>33</td>
<td></td>
<td>39</td>
</tr>
<tr>
<td>(144)</td>
<td>IHC</td>
<td>61</td>
<td>MRK16</td>
<td>35 (strong)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>UIC2</td>
<td>43 (strong)</td>
</tr>
<tr>
<td>(145)</td>
<td>IHC</td>
<td>31</td>
<td>JSB1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>33</td>
<td>UIC2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Idem normal tissues</td>
<td></td>
</tr>
<tr>
<td>(146)</td>
<td>IHC</td>
<td>48</td>
<td>JSB1</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>52</td>
<td>4E3</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C494</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>84</td>
</tr>
<tr>
<td>(147)</td>
<td>IHC</td>
<td>63</td>
<td>C219</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>134</td>
<td></td>
<td>60</td>
</tr>
<tr>
<td>(148)</td>
<td>IHC</td>
<td>63</td>
<td>JSB-1</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>33</td>
<td></td>
<td>43</td>
</tr>
<tr>
<td>(149)</td>
<td>IHC</td>
<td>359</td>
<td>JSB-1</td>
<td>11</td>
</tr>
<tr>
<td>(150)</td>
<td>IHC</td>
<td>30</td>
<td>JSB-1</td>
<td>67</td>
</tr>
<tr>
<td>(151)</td>
<td>IHC</td>
<td>40</td>
<td>JSB-1</td>
<td>64</td>
</tr>
<tr>
<td>(152)</td>
<td>IHC</td>
<td>15</td>
<td>JSB-1</td>
<td>33</td>
</tr>
<tr>
<td>(153)</td>
<td>IHC</td>
<td>30</td>
<td>MRK16</td>
<td>33</td>
</tr>
<tr>
<td>(154)</td>
<td>IHC</td>
<td>34</td>
<td>JSB-1</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C219</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C494</td>
<td>13</td>
</tr>
<tr>
<td>(59)</td>
<td>RT-PCR</td>
<td>74</td>
<td></td>
<td>3 (high)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>19 (moderate)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>61 (low)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17 (negative)</td>
</tr>
<tr>
<td>(155)</td>
<td>RT-PCR</td>
<td>75</td>
<td></td>
<td>92</td>
</tr>
<tr>
<td>(156)</td>
<td>RT-PCR</td>
<td>40</td>
<td></td>
<td>92</td>
</tr>
<tr>
<td>(60)</td>
<td>RT-PCR</td>
<td>165</td>
<td></td>
<td>44 (high)</td>
</tr>
</tbody>
</table>

malignancies, few reports have concerned the use of flow cytometry in solid tumors. As mRNA evaluation cannot take into account tumor heterogeneity and as IHC is not really reliable for quantitative evaluation, we have developed evaluation of ABC transporters or other MDR-related proteins in solid tumors by using flow cytometry (28, 29). To illustrate such a method, tumor NSCLC samples were dissociated by mechanical scraping, and the cells
FIGURE 5.1. Example of a semiquantitative analysis by cytometry of MRP1 expression from tumor samples dissociated by mechanical scraping after labeling with an anti-MRP1 mAb. The semi quantification is obtained by the ratio (channel of fluorescence for MRP1/channel of fluorescence for the isotypic control). (a) Results obtained for diploid nonsmall cell lung cancer tumor cells. (b) Results obtained for aneuploid nonsmall cell lung cancer tumor cells. X axis: channels of fluorescence; Y axis: number of cells.

obtained were labeled with anti-ABC transporter or anti-LRP mAbs, anticytokeratin mAb, and propidium iodide in order to restrict the study to cancer cells, to obtain semiquantitative evaluation with reference to the isotypic control, and to detect various cell clones according to DNA ploidy, as illustrated in Fig. 5.1. The results of this method of evaluation of MRP1 in NSCLC (28) showed higher rates of expression than those previously reported by IHC (30, 31), suggesting a higher sensitivity of flow cytometry analysis. We therefore consider this method to be complementary to mRNA evaluation or standard IHC; it provides reproducible and accurate results.

5.3.4. In Vivo Imaging of Multidrug Resistance in Patients

Because of the important clinical impact of MDR mediated by overexpression of ABC transporters, a noninvasive in vivo evaluation of the activity of these transporters would be clinically useful to guide treatment decisions. Nuclear medicine is a molecular imaging modality that allows noninvasive in vivo imaging in patients at a molecular level (10⁻⁹–10⁻¹² mol/kg injected, reaching a concentration of one molecule per cell) (32, 33). It allows radionuclide labeling
of small organic medicinal compounds and metal complexes that have been characterized as transport substrates for P-gp and other transporters (34). The radionuclides used for this type of labeling allow easy imaging with a SPECT (single photon computed tomography) gamma camera, a PET (positron emission tomography), or a PET/CT (positron emission tomography/X-ray computed tomography) hybrid camera used in routine clinical practice in cancer patients. This functional approach is particularly interesting, as the expression of transporters detected at the level of messenger RNA or protein, by PCR or IHC, does not always accurately reflect their activity or the in vivo cell resistance status.

5.3.4.1. SPECT Agents for Assessment of P-gp/MDR1-Mediated Transport Activity. The clinically approved agent \(\text{\(^{99m} Tc\)}\)sestamibi (Hexakis[2-methoxyisobutylisonitrile]-technetium-99m), originally developed as a radiopharmaceutical for myocardial perfusion imaging and then for parathyroid and tumor imaging, was the first metal complex shown to be a P-gp transport substrate (35). It possesses a cationic charge and modest hydrophobicity similar to that of many chemotherapeutic agents substrates of the MDR phenotype transporters. In the absence of P-gp expression, this \(\text{\(^{99m} Tc\)}\)-isonitrile complex accumulates in the intracellular compartment in response to the physiologically negative mitochondrial and plasma membrane potentials maintained within the cells (35). However, in P-gp-expressing multidrug resistant tumor cells, net cellular accumulation levels of \(\text{\(^{99m} Tc\)}\)sestamibi are inversely proportional to the level of P-gp expression (35–37). Furthermore, complete reversal of the P-gp-mediated exclusion of \(\text{\(^{99m} Tc\)}\)sestamibi has been induced by treatment with conventional P-gp inhibitors such as verapamil, cyclosporin, and quinidine or newer more potent reversal agents such as PSC833, GF120918, LY335979, or VX710 (34).

Many other \(\text{\(^{99m} Tc\)}\)-labeled conjugates have been developed and evaluated, such as \(\text{\(^{99m} Tc\)}\)tetrofosmin, \(\text{\(^{99m} Tc\)}\)furisfosmin, \(\text{\(^{99m} Tc\)}\)D-58, and \(\text{\(^{99m} Tc\)}\)COMIBI (38, 39). They have been validated as transport substrates for P-gp, but none of these radiolabeled complexes is considered to be more accurate than \(\text{\(^{99m} Tc\)}\)sestamibi in terms of their P-gp-targeting properties (34). Moreover, \(\text{\(^{99m} Tc\)}\)sestamibi and other tracers have been shown to detect P-gp transport activity in normal human tissues (40), in human tumors (41–43), and in patients treated with MDR modulators that inhibit P-gp transport activity (44–46). These types of tracers can be used as tools to predict failure of chemotherapy in breast cancer, lung cancer, lymphoma, and osteosarcoma (47–51). It is not yet clear which of the \(\text{\(^{99m} Tc\)}\) complexes would be the most clinically useful for evaluation of P-gp status in tumors by SPECT imaging.

5.3.4.2. PET Agents for Assessment of P-gp/MDR1-Mediated Transport Activity. PET allows enhanced spatial resolution and quantification capabilities compared to SPECT agents. To probe P-gp transport activity, PET-based radiopharmaceuticals have therefore been actively investigated at three
levels by using SPECT organic ligands capable of being labeled with PET radionuclides, bioinorganic radiolabeled complexes, and conventional PET organic medicinal compounds. Among the organic scaffolds that coordinate both SPECT and PET radionuclides, two validated examples use the PET radionuclides $^{94m}$Tc ($^{94m}$Technetium) and $^{68}$Ga ($^{68}$Gallium). The radiosynthesis and comparative biochemical validation of ($^{94m}$Tc)sestamibi and ($^{68}$Ga)ENBPI complexes have been reported (52, 53) with highly favorable P-gp-targeting properties of ($^{68}$Ga)ENBPI.

Organic scaffolds capable of accommodating PET radionuclides that generate novel metallopharmaceuticals through short synthetic pathways, such as radiolabeling with ($^{64}$Cu) ($^{64}$Copper), another positron emitter radionuclide, have been reported for the targeting of P-gp (54). Finally, a third level consists of incorporation of conventional PET radionuclides $^{11}$C ($^{11}$Carbon) or $^{18}$F ($^{18}$Fluorine) into small organic molecules characterized as substrates or inhibitors known to interact with P-gp (34). Various labeled PET agents have been reported with this approach with promising clinical applications to predict in vivo response to chemotherapy in cancer patients (55). They are: ($^{11}$C)colchicine, ($^{11}$C)verapamil, ($^{11}$C)daunomycin, and ($^{11}$C/$^{18}$F)paclitaxel (34).

In summary, a panel of MDR substrate transporters is amenable for incorporation of PET or SPECT radionuclides to allow functional diagnostic imaging of MDR phenotype in cancer patients. Clinically approved ($^{99m}$Tc)complexes have already been shown to be beneficial for the evaluation of functional P-gp transport activity in human tumors in vivo. Radiochemistry is therefore making a major contribution to the range of targeted agents suitable for noninvasive functional analysis of MDR phenotype in vivo.

5.4. THE CELLULAR AND TISSUE ABC TRANSPORTER EXPRESSION IN SOLID TUMORS

5.4.1. ABC Transporter Expression in Tumor Tissues as a Predictor of Drug Response

Although ABC transporter expression can be evaluated in tumor samples, allowing a snapshot of their status at a given point in time, most of their functions and regulations in tissues remain terra incognita. Table 5.6 summarizes the data concerning the expression of ABC transporters involved in drug resistance. However, this table is probably very incomplete and other ABC transporters located in human tissues known for functions other than detoxification will probably also be discovered to be involved in drug resistance, as suggested in the recent review by Gillet et al. (9).

Studies on P-gp or MDR1 expression detection have been extensively conducted in breast cancer, using various methods that were not always optimized and generating conflicting results. The association between P-gp or MDR1 expression and clinical outcome of chemotherapy (MDR phenotype) has been
TABLE 5.6. Examples of ABC Transporters Expression in Human Normal Tissues and Solid Tumors

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression in Normal Tissues</th>
<th>Expression in Human Solid Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-glycoprotein (ABCB1)</td>
<td>Adrenal gland, Kidney, liver, colon</td>
<td>Neuroblastoma, Breast, pancreas, colon, adrenal, liver, Renal, ovarian, Nonsmall cell lung carcinoma</td>
</tr>
<tr>
<td></td>
<td>Brain (endothelial cells)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Testis (endothelial cells)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Respiratory mucosa</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Placenta</td>
<td></td>
</tr>
<tr>
<td>MRP1 (ABCC1)</td>
<td>Placenta, testis, lung</td>
<td>Neuroblastoma, Nonsmall cell lung carcinoma</td>
</tr>
<tr>
<td></td>
<td>Skeletal muscle</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Heart, liver, monocytes</td>
<td>Breast, esophageal, gastric, Thyroid (anaplastic)</td>
</tr>
<tr>
<td>MRP2 (ABCC2)</td>
<td>Liver</td>
<td>Colon cancer</td>
</tr>
<tr>
<td></td>
<td>Duodenum, kidney</td>
<td></td>
</tr>
<tr>
<td>MRP3 (ABCC3)</td>
<td>Liver, colon, pancreas</td>
<td>Colon cancer</td>
</tr>
<tr>
<td></td>
<td>Duodenum, kidney</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adrenal gland</td>
<td></td>
</tr>
<tr>
<td>MRP4 (ABCC4)</td>
<td>Prostate, kidney, liver</td>
<td>Neuroblastoma</td>
</tr>
<tr>
<td></td>
<td>Erythrocytes, adrenal gland, platelets, brain</td>
<td></td>
</tr>
<tr>
<td>BCRP (ABCG2)</td>
<td>Placenta, breast, liver, intestine</td>
<td>Breast cancer, Nonsmall cell lung carcinoma</td>
</tr>
</tbody>
</table>

clarified in a meta-analysis including a large number of studies utilizing RNA hybridization methods as well as IHC methods (25). The authors reported that more than 40% of tumors displayed high levels of P-gp, but they evidenced large discrepancies due to the broad spectrum of methodologies. However, *MDR1* expression was clearly demonstrated to be associated with an increased risk of inefficacy of chemotherapy. Treatment failure was more than threefold higher in patients expressing high levels of transcripts or protein as compared with patients with nonexpressing tumors, and a high expression of P-gp or *MDR1* in samples collected after chemotherapy is strongly associated with treatment failure. In a more recent study not included in the meta-analysis of Trock et al. (25), Burger et al. (56) conducted a multiple gene analysis using RT-PCR on primary tumors from patients who received primary chemotherapy and demonstrated a large variation in expression levels: *MDR1* expression was inversely related to the efficacy of first-line chemotherapy, and a high expression level was predictive of poor prognosis for patients with advanced disease. In addition to *MDR1*, the expression levels of BCRP, MRP1, and LRP
a non-ABC protein related to drug resistance) could also have predictive values for clinical outcomes. In locally advanced breast cancer, RT-PCR studies of MDR1 and LRP showed them to be good candidates to predict axillary node invasion at the time of mastectomy after induction chemotherapy (40). In contrast, BCRP expression was not found to be predictive of resistance to anthracyclines (57), in contrast to MRP1, 2, or 3 (58). Finally, in breast cancer, the expression of ABC transporter genes may be related to other non-ABC genes involved in chemoresistance such as GSTP1 (59), whose expression may constitute an independent prognostic factor for disease-free survival in patients (60).

In ovarian carcinoma, high expression of MRP1 and MRP3 was found to be an unfavorable prognostic indicator (61), whereas higher levels of P-gp are significantly associated with an increased risk for disease progression (62). Several studies have been also conducted in lung cancer; testing MRP1–3 levels in tumor samples, Young et al. (63) evidenced higher levels of gene expression in samples from NSCLCs compared to cell lines from small-cell lung carcinomas. BCRP expression studies could possibly provide valuable information concerning drug resistance to topotecan in NSCLC, but this remains to be confirmed in clinical situations (64). ABC transporter expression has also been evaluated in nervous system tumors to test whether ABC transporter expression can affect progression, prognosis, or drug sensitivity. Although high expression of genes such as MDR1, MRP1, MRP5, and LRP was not predictive of clinical response or prognosis in patients with advanced neuroblastoma (65), MRP3 may modulate drug sensitivity to certain anticancer agents in human gliomas (66). In hepatocellular carcinoma, most tumors showed decreased expression of one or more canalicular transporters (MDR1, MDR3, BSEP), whereas MRP2 tended to be inconstantly overexpressed and appeared to be the major candidate of hepatic transporters involved in chemoresistance (67).

Apart from RT-PCR, pharmacogenomic analysis based on low-density microarrays will undoubtedly be used in the near future to detect MDR on biopsy specimens, as microarrays allow determination of the expression profile of a wide set of ABC transporters in a single hybridization experiment and may be useful as diagnostic tools to detect drug resistance in clinical situations (68, 69). Moreover, in their recent and exhaustive general review, Gillet et al. (9) indicate that at least 26 ABC transporters could be involved in chemoresistance in breast cancer.

5.4.2. ABC Transporters and Tumor Blood Supply

Before reaching and killing a large proportion of cancer cells, anticancer drugs must be distributed throughout the tumor blood supply and must then cross the vessel wall and enter the tumor. Tumor blood supply is known to be abnormal with marked variability in terms of vascular permeability. Expression of P-gp and BCRP has been shown in capillary endothelial cells of the central
nervous system (70) to be responsible for the blood–brain barrier, preventing passage of certain blood toxins. Functional P-gp activity demonstrated in cultured cerebral capillary endothelial cells (71) has been shown to decrease after irradiation (72). Similar barriers involving P-gp and BCRP have also been shown in the testis and placenta (73). IHC on conventional paraffin sections has also shown P-gp expression in endothelial cells of the microvasculature of most gliomas (86% in the work of Tóth [70]) and of tumors giving metastases in the brain, while this study did not detect P-gp in the tumor capillaries of a different set of “fresh” nonbrain malignant tumors (breast carcinoma, NSCLC, prostate carcinoma, and miscellaneous carcinomas and sarcomas). In contrast, P-gp has been localized in endothelial cells of bladder tumor capillaries with upregulation after chemotherapy, suggesting potential induction (74). P-gp has been localized by electron microscopy on the luminal surface of capillary endothelial cells of gliomas (75). Although the function of these ABC transporters in endothelial cells of tumor microvasculature needs to be determined, drug resistance in tumors bearing such microvessels may result not only from the characteristics of tumor cells but also from the characteristics of the endothelial cells which exert a barrier role in relation to drugs. However, the potential role of ABC transporters in endothelial cells of the tumor microvasculature is still speculative and needs to be thoroughly investigated.

5.4.3. Hypoxia and ABC Transporters

Solid tumors are usually heterogeneous tumors with hypoxic areas and an acidic extracellular microenvironment (76). Many types of human tumors have been demonstrated to contain significantly oxygen-deprived areas (77), and for many years data have suggested that hypoxia in solid tumors is associated with resistance to anticancer drugs (78). Transient hypoxia has been shown to cause amplification and increased expression of the genes encoding P-gp (76, 78). The \textit{MDR1} gene has been demonstrated to be responsive to hypoxia with a binding site for hypoxia inducible factor-1 (HIF-1) (79). Moreover, inhibition of HIF-1 expression by antisense oligonucleotides resulted in significant inhibition of hypoxia-inducible \textit{MDR1} expression and almost complete loss of basal \textit{MDR1} expression (79). In contrast, when cells were maintained under hypoxic conditions for a longer time in other experimental models, a decrease of P-gp and MRP1 functions was observed, probably related to the decrease of intracellular adenosine triphosphate due to inhibition of aerobic metabolism (80). However, although these authors concluded that pronounced hypoxia has only a minor effect on drug transporters, no \textit{in vivo} data are available concerning the effects of intermittent hypoxia, which is considered to be a major feature in tumors. In contrast, studies in prostate cancer cell lines have recently shown that incubation at a pH of 6.6 for a few hours induces an increase of P-gp with a persistent and constant level of protein expression, suggesting functional modulation (81). The rapid change of intracellular Ca$^{2+}$ in response to an acidic extracellular pH could be a modulator of P-gp func-
It is therefore possible that the factor inducing drug resistance via ABC transporters in hypoxic tumor areas is not hypoxia *per se*, but the acidic extracellular environment associated with hypoxia. A similar role has also been demonstrated for BCRP at low pH (82).

A hypoxic microenvironment of cancer cells may also have an action on cancer stem cells, as it has been demonstrated that BCRP confers a strong survival advantage to noncancer murine stem cells under hypoxic conditions (83, 84). In this model, it has been shown that BCRP expression was upregulated by hypoxia involving HIF-1. The authors of this study suggested that, in response to hypoxia, cells use BCRP to reduce heme or porphyrin accumulation, which is detrimental to cells. However, this type of study must be performed on cancer stem cells.

### 5.4.4. Cancer Stem Cells and ABC Transporters

A subset of stem cells termed the side population (SP) has been identified in various tissues, with the property of expelling rhodamine 123 and Hoechst 33342 through membrane ABC transporters, mainly BCRP (85, 86). Tumor-initiating cells capable of self-renewal and differentiation have also been identified in a variety of solid tumors and are predicted to be only a small fraction of the tumor, as in neuroblastoma (87) or lung cancer (88). Such pluripotent cancer stem cells may arise from either oncogenic transformation of normal tissue stem cells or from differentiated cells that have regressed to tumor-initiating stem cells during the oncogenic process. These cancer stem cells are assumed to share many properties of normal tissue stem cells. These properties include resistance to drugs and toxins via expression of ABC transporters, and it has therefore been suggested that this population has an intrinsic resistance to chemotherapy (89, 90). The two ABC transporters that have been extensively studied in normal stem cells as they constitute criteria of identification were P-gp and BCRP. P-gp is constitutively expressed at high levels in stem cells and in progenitor cell populations (44). As underlined by Dean et al. (89), “Although the expression of ABC transporters could render stem cells resistant to drugs, it is not the sole determinant of resistance as the DNA repair capacity of the cell and the reluctance to enter apoptosis could be equally or more important.”

The problem of chemoresistance of cancer stem cells applies to tumors which initially present an almost complete response but which recur after a short period of time, such as small-cell lung cancer, and these cancer stem cells may explain why most solid tumors cannot be eradicated if they are not surgically removed.

Tumor SP stem cells have been identified in a large variety of solid tumors from brain tumors to squamous cell carcinomas (91), and their clinical relevance is now a wide subject of research. There is a growing body of data concerning these cells and ABC transporters, and although BCRP and P-gp were the first two ABC proteins demonstrated to be associated with these cells,
other transporters have now been identified. For example, tumor SP stem cells in neuroblastoma have been shown to express high levels of BCRP and ABCA3 (87), and SP stem cells in head-and-neck squamous cell cancer express high levels of MRP1 and BCRP (91).

It has been recently shown that tumor stem cells in melanomas, giving rise to rapidly proliferating cells and then more differentiated cells forming the bulk of the tumor (92), constitutively express P-gp associated with \textit{NANOG} expression (an embryonic stem cell marker) and ABCB5 (93). In physiologic progenitor cells, ABCB5 functions to maintain membrane hyperpolarization, thereby serving as a regulator of cell fusion (94), particularly melanocyte progenitor cell fusion. ABCB5-expressing cells have been shown to be essential for melanoma induction. These ABCB5-expressing melanoma cells also possess the features of stem cells, such as expression of BCRP, CD133, CD144, and nestin. ABCB5-expressing cells are able to give rise to ABCB5-negative cells, strongly suggesting a tumor hierarchy in which ABCB5-positive cells are able to both self-renew and generate ABCB5-negative progeny (92). The fraction of ABCB5-positive cells in clinical melanomas is parallel to the fraction of CD133-positive cells, a marker of subpopulations enriched in tumor-initiating cells. Moreover, as previously described, ABCB5 is a highly active transporter of drugs like doxorubicin and is at least partly responsible for the usual chemoresistance of melanoma tumor-initiating cells/melanocytes (95).

Breast cancers are also considered to comprise subpopulations of cancer stem cells that play a major role in these tumors (96). Recent evidence supports the hypothesis that the \textit{BCRA1} gene plays a role in breast cancer stem cells, and studies in a mouse knockout model of \textit{BRCA1} have shown the cellular heterogeneity of these breast cancer stem cells (97). However, in this murine model, although transporters such as BCRP or P-gp have been shown to be overexpressed in some stem cells, no correlation with drug resistance has been demonstrated. Therefore, other potential mechanisms of chemoresistance of cancer stem cells remain to be elucidated, including overexpression of drug-metabolizing enzymes, changes in cell cycle kinetics, and overexpression of anti-apoptotic proteins (98).

### 5.4.5. ABC Proteins and Cancer Immunology

Ten years ago, Borst et al. (99) already reported that drug-transporting P-gp may also be involved in cancer immunity via other physiologic immune functions, such as its role in natural killer cells or mobilization of dendritic cells (99). However, few data are still available concerning the potential clinical relevance of these observations. Another aspect of ABC transporters, apart from drug transport, is their Transporter associated with Antigen Processing (TAP) transporter role. TAP is essential for peptide delivery from the cytosol through the reticulum, followed by their expression on the cell surface associated with the class I major histocompatibility complex (MHC I) allowing their presentation to cytotoxic T cells (100). TAP is a heterodimer composed of
TAP1 and TAP2, two subunits essential for antigen processing. Many tumors escape cytotoxic T cell recognition at least in part by the absence of surface presentation of cancer cell-associated peptides. Downregulation of TAP1 and/or TAP2 observed in some cancer cell lines and tumor samples (101) seems to be related to various mechanisms: low levels of mRNA restored by incubation with γ interferon (101) or low levels of mRNA due to a mutation in the TAP gene with marked instability of mRNA (100). Moreover, normal expression of TAP with a defective function has also been found to be associated with a mutation observed between the C loop and the Walker B sequence (100, 102). Frequent TAP mutations have been reported in cervical carcinoma tumor samples, more frequently on TAP1 than on TAP2 alleles (103). Reduced TAP expression or function has therefore been shown to be a factor of poor prognosis in head and neck cancer (101) or melanoma (104).

5.5. THE HOST: PHARMACOGENETICS OF ABC TRANSPORTERS AND SOLID TUMORS

Patients treated for cancer exhibit major interindividual variations in terms of drug response and toxicity, which might be due to inherited polymorphisms in genes encoding proteins involved in drug metabolism. These proteins include ABC transporters, especially ABCB1 which plays a role in so-called phase 0 (efflux of unmodified drug) of the metabolism of xenobiotics, ABCC1 involved in so-called phase III (efflux of drug metabolites, especially conjugates), and ABCG2/BCRP involved in both phases.

Since the first report of a functional polymorphism in ABCB1 gene (105), many studies including several on solid tumors have focused on polymorphisms in this ABC transporter and their potential clinical impact. More than 45 polymorphisms have been described in coding, promoting, and noncoding regions of ABCB1, including the synonymous C1236T (exon 12) and C3435T (exon 26), which was the first functional polymorphism to be reported, and the nonsynonymous A61G (exon 2) and G2677T/A (exon 21) polymorphisms, C3435T and G2677T/A being in strong linkage disequilibrium. Interethnic differences in the frequency of these polymorphisms have been observed. For example, the 3435C allele is more frequent in African populations than in Caucasian and Asian populations. The CC genotype in C3435T has been most often associated with higher levels of mRNA and protein, and increased drug efflux in normal tissues and tumors (106–109), although some discrepancies exist especially in Japanese population (110). How this silent C3435T polymorphism affects ABCB1 function was not understood, but it has been recently explained by altered folding of the protein due to slowing down of translation rate (111, 112).

Although many data are available concerning ABCB1 polymorphisms and pharmacokinetics of drugs, especially immunosuppressive and cardiac drugs, few studies dealing with anticancer drugs are available. No correlation was
found between C3435T polymorphism and the pharmacokinetics of the main drugs used in the treatment of solid tumors and effluxed by ABCB1, such as paclitaxel (113), docetaxel (114, 115), and irinotecan (116). An association was reported between the other C1236T polymorphism and docetaxel (117) or irinotecan (116) pharmacokinetics and patients with TT genotype presenting an increased exposure to these drugs. However, no correlation was found between C1236T and the pharmacokinetics of irinotecan in Korean patients (118).

Some ABCB1 polymorphisms have been associated with drug toxicity such as C3435T with paclitaxel-induced leukocytopenia or neuropathy (119, 120) and G2677T/A with irinotecan-induced neutropenia (118), although the association was not always related to the pharmacokinetics of the drug.

Few studies have been conducted on the relationship between ABCB1 polymorphisms and drug response in patients treated for cancer. Correlations between G2677T/A and response to paclitaxel in ovarian cancer (121) and between C3435T and clinical response to preoperative anthracycline-based chemotherapy in breast cancer (122) have been reported.

More than 50 polymorphisms of the ABCG2 gene have been found to date, including the nonsynonymous G34A (V12M) and C421A (Q141K) polymorphisms that are common in various populations, although with different frequencies. The C421A polymorphism, the most extensively studied and for which the A variant is the most frequent in Asian populations, appears to be a functional polymorphism. As reported in in vitro models (123) or human tissues such as the placenta (124), BCRP protein levels are lower in CA or AA cells than in CC cells.

It has been demonstrated in human cell lines that CA cells exhibit higher intracellular accumulation of topotecan, a well-known substrate of BCRP, than do CC cells (123). In patients treated with anticancer agents known to be effluxed by BCRP, a higher drug bioavailability was found for patients heterozygous for the C421A polymorphism than for those with the wild-type CC genotype, whether they received topotecan (125) or the EGFR inhibitor gefitinib (126). Two other ABCG2 polymorphisms located in the promoter have been recently reported, that is, C–15622T and C1143T, with variants associated with lower BCRP levels and higher pharmacokinetic parameters of erlotinib, another EGFR inhibitor (127).

Patients heterozygous for the ABCG2-C421A polymorphism have been shown to more frequently develop toxicity (diarrhea) with gefitinib than CC patients (128) although this relationship was not found with erlotinib (127). No data concerning the influence of ABCG2 polymorphisms on anticancer drug response are available at present time.

Results on polymorphisms of other ABC transporters, especially ABCC1 and ABCC2, and relationships to drug toxicity or response in patients treated for solid tumors are rare, although many polymorphisms have been described. No association was observed between the pharmacokinetics of irinotecan and ABCC1 (116) or ABCC2 (116, 118) polymorphisms. Acute doxorubicin-
induced cardiotoxicity has been associated with the Gly671Val variant of ABCC1 and the Val1188Glu-Cys1515Tyr haplotype of ABCC2 (129).

In conclusion, conflicting results have been reported concerning the role of ABC transporter polymorphisms on the clinical characteristics of solid tumors, including the most widely studied C3435T polymorphism in the ABCB1 gene. Although the effect of a given polymorphism might be specific to a given drug, contradictory results have been reported even with the same anticancer drug. The relatively small number of patients analyzed and the various populations tested could at least partly explain the discrepancies observed between studies. More studies on the pharmacogenetics of ABC transporters clearly need to be conducted in order to assess their precise role in anticancer drug response and toxicity.

5.6. CLINICAL RELEVANCE OF ABC TRANSPORTER DETECTION IN SOLID TUMORS

As discussed above, the clinical relevance of ABC transporter analysis in solid tumors is still a controversial issue. Several examples, some of which have already been quoted, address the following question: Are ABC transporters responsible for chemoresistance or are they markers of cancer cell behavior or both?

Breast cancer, often considered to be one of the more chemosensitive solid tumors in adults, has a high overall response rate to chemotherapy, but most of these tumors have a short duration of response and acquire an MDR phenotype. A recent review by Fojo and Coley (130) pointed out that time to progression after first-line treatment in metastatic breast cancer is about 6–12 months, and response to second-line therapy is obtained in only 20%–30% of patients, with a median response time lasting less than 6 months. The high percentage of nonresponders and failures following initial responses highlight the critical role played by drug resistance mechanisms in breast cancer management, and “breast cancer represents perhaps one of the best fields for the study of clinical drug resistance and its reversal” (131). As most of the chemotherapy regimens used in metastatic breast cancer include anthracyclines, taxanes and, more recently, capecitabine, resistance to such regimens has been suspected to involve, at least partly, MDR mechanisms, particularly P-gp expression. The 1997 meta-analysis by Trock et al. (25) was highly suggestive of P-gp involvement in breast cancer resistance. In this analysis, the overall proportion of breast cancer tumors expressing P-gp was 41.2% (36%–46.5%), and treatment with chemotherapeutic drugs or hormonal agents was associated with an increase in the proportion of tumors expressing P-gp (RR: 1.77). It was therefore concluded that patients with tumors expressing P-gp were three times more likely to fail to respond to chemotherapy than patients with P-gp-negative tumors (relative risk [RR]: 3.21); this relative risk increased to 4.19 when considering only those patients with tumor P-gp expression after
chemotherapy. P-gp, therefore, represents a potential biomarker of drug resistance, while a direct role of P-gp as a cause of clinical drug resistance can only be inferred from studies on cell lines. MRP1 has been detected in untreated breast cancer (49% by IHC; 98% by RT-PCR), and at lower levels in the normal breast. As a consequence, the potential role of MRP1 in breast cancer resistance is still unknown (131). In their recent and exhaustive general review, Gillet et al. (9) reported that at least 26 ABC transporters could be involved in chemoresistance in breast cancer. IHC of a large set of MDR-related ABC transporters (P-gp, MRP1, MRP2, MRP3, and BCRP) in nonsmall-cell lung cancer suggests that BCRP could be potentially involved in chemoresistance in patients treated by platinum-based chemotherapy (132). A case of acquired resistance to gefitinib in a “never-smoker” patient with advanced nonsmall-cell lung cancer associated with acquired expression of BCRP was recently reported (133). The hypothesis of a prominent role of BCRP in the chemoresistance of lung cancer therefore appears to be particularly promising.

Another clinically relevant aspect of ABC transporter expression is that ABC transporters must not only be considered to be markers of cell chemoresistance but also markers of cancer cell behavior and, therefore, prognostic markers. In 1995, Baldini et al. (134) reported a strong correlation between the presence of increased levels of P-gp assessed by IHC and the prognosis of patients with osteosarcoma. This finding suggested that P-gp may not only simply be a marker of tumor sensitivity to drugs but also a sign of tumor aggressiveness (135). Similarly, it has been observed that carcinoma cells at the invading edge of colon carcinoma express P-gp and that, even in P-gp-negative tumors, lymph node metastases express P-gp (136), suggesting a role for P-gp in cell invasion and metastasis. MRP2 expression has also been recently reported to be an independent prognostic factor in patients with completely resected nonsmall-cell lung cancer (136).

ABC transporter expression could simultaneously constitute a marker of chemoresistance and a marker of poor prognosis, regardless of tumor response to chemotherapy. Neuroblastoma is the most common extracranial solid tumor in children. MYCN amplification is observed in about 25% of neuroblastomas and is associated with rapid tumor progression and a rapid outcome. There is increasing evidence that MRP1 is an MYCN target gene involved in the development of MDR in neuroblastoma and that high levels of MRP1 correlate with MYCN amplification. It has been shown that MYCN interacts with the MRP1 promoter region. The level of MRP1 expression is also an independent prognostic factor per se (138). It must be emphasized that other MRP family members, such as MRP4 that has been found to be overexpressed in aggressive neuroblastoma, might also contribute to drug resistance in these tumors (138). Activation of P-gp in melanoma cell lines induced increased release of metalloproteases associated with increased invasive behavior (139). Likewise, ABCB5, a major efflux transporter of doxorubicin in melanoma, is also involved in the regulation of melanocyte progenitor cell fusion (94, 140).
5.7. CONCLUSION

Although a large body of evidence is in favor of a significant role of ABC transporters in solid tumors, the clinical relevance of the detection of these transporters in tumor samples is still under evaluation. One of the main problems is the heterogeneity of the methods used and therefore the results obtained in the same type of tumor; test methodology must therefore be standardized in order to obtain conclusive data (141). The use of modulators to circumvent MDR, particularly P-gp-mediated transport, has been tested in solid tumors in order to increase the chemosensitivity of cancer cells but with largely disappointing results. Moreover, toxicity of such modulators or increased toxicity of anticancer drugs were observed in these trials. The use of therapeutic modulation of ABC transporters therefore appears to be particularly difficult to apply in patients with solid tumors. In contrast, in the near future it is conceivable that pharmacogenomic as well as pharmacogenetic evaluation in patients, at least partly focused on ABC transporters, could predict individual response to a set of anticancer drugs, leading to improved treatment and tumor response by tailored selection of drugs used in solid tumor chemotherapy.

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PART III

ABC PROTEINS AND PATHOGENIC MICROORGANISMS
6

ABC TRANSPORTERS AND RESISTANCE TO ANTIBIOTICS

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6.1. INTRODUCTION

Different intelligent strategies have been developed by bacteria to avoid antimicrobial therapy: target modification, inactivation of drug, enzymatic degradation, or reduced intracellular drug concentrations (either by decreased permeability or by active efflux of the drug). Among them, efflux is one of the
most common and basic mechanisms, but it also seems to be currently one of
the most significant because of its capacity to confer multidrug resistance
(MDR) phenotype to bacteria, leading some strains to become resistant to
every known treatment (1).

Efflux pumps are involved in many physiological functions in bacteria:
They are characterized as cell cleaners mainly, but they also play important
role in the cell pathogenicity (2). Under antibiotic pressure, their over-
expression leads to the increase of the antibiotic minimum inhibitory
concentration (MIC), which in turn allows the development of further
resistance mechanisms leading eventually to treatment failure. Because some
of these transporters are encoded by mobile genetic elements, intra- and
interspecies transfers allow a rapid dissemination of resistance mechanisms,
which increases the difficulty of finding appropriate therapeutic solutions and
contributes to exerting selective pressure for resistance determinants in the
environment (3).

Bacterial efflux transporters are mainly composed of secondary-type trans-
porters (depending on the proton motive force or the sodium motive force)
belonging to the multidrug and toxic compound extrusion (MATE), the major
facilitator superfamily (MFS), the resistant nodule division (RND), and the
small MDR (SMR) families. These transporters have been the most described
in the antibiotic efflux processing, especially for MDR. However, ATP-binding
cassette (ABC)-type MDR transporters have been reported in Gram-positive
and Gram-negative species (4), and the genomic analysis of numerous pro-
karyotic species allowed the identification of many putative ABC-type efflux
pumps (5). Recently, the crystal structure of a bacterial ABC associated with
MDR (Sav1866 from Staphylococcus aureus) has been determined, providing
an insight into the molecular mechanism of energy coupling and transport
(6, 7). Several bacterial ABC transporters have been associated with antimic-
brbial resistance; among them, some confer MDR phenotype to bacteria,
rendering them resistant to multiple and diverse antimicrobials. Recently, an
ABC transporter of plant origin has been used as a resistant determinant in
the antimicrobial protection of transgenic plants, and significant levels of resis-
tance to kanamycin were achieved when the transporter was overexpressed,
showing that these efflux systems may efficiently protect living cells from
antibiotics’ effects (8).

In this chapter we will discuss the structural aspects of ABC-type transport-
ers described in prokaryotes and their implication in antibiotic resistance as
well as in antimicrobial therapeutic strategies.

6.2. ABC-TYPE TRANSPORTERS IN BACTERIA

ABC systems constitute some of the most abundant superfamilies of proteins
in eukaryotes as well as in prokaryotes. The common characteristic of all these
ABC-TYPE TRANSPORTERS IN BACTERIA

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systems is that they share a highly conserved ATPase domain of approximately 215 amino-acid consensus sequence, called nucleotide-binding domain (NBD).
In prokaryotes, these proteins are involved in many energy-dependant cellular processes since they are capable of binding adenosine triphosphate (ATP) and coupling the free energy relative to its hydrolysis to a determinate physiological function. Examples include transport of a wide variety of substances (import or export), which is crucial for the cell physiology (uptake of nutrients, elimination of waste products, energy generation, cell signaling, or cell pathogenicity), but they are also implicated in other cell functions like ribosomal biogenesis, translational control, DNA repair, telomere maintenance, or chromatin organization (9, 10).

In the case of ABC transporters, NBDs are associated with membrane-embedded transmembrane domain (TMD) usually composed of six transmembrane (TM) α-helices, which are believed to selectively distinguish the type of substrate transported. The minimal structural organization of an ABC transporter is composed of two NBDs and two TMDs. Several organizations of these four domains are encountered: It consists either of the arrangement of four different peptides (reported in prokaryotes only), or two (fusion of two NBDs, two TMDs, or one NBD with one TMD), or, as most of eukaryotic ABC, of one single polypeptide containing the four domains.

In prokaryotes, ABC transporters have been classified into two main functional groups relative to their structures and activities.

6.2.1. Importers (Uptake Systems)

These transport systems are composed of four different polypeptides with an additional extracytoplasmic solute-binding protein (SBP), which in Gram-negative bacteria is a periplasmic receptor and in Gram-positive bacteria is present either as a lipoprotein tethered to the external surface of the cytoplasmic membrane or as a cell surface-associated protein bound to the external membrane surface by electrostatic interactions. Uptake systems are involved in the supplying of essential nutrients and have been identified only in prokaryotes. This function has also been associated with bacterial virulence since knockout mutants (of oligopeptides or metal ions importers for example) showed attenuated virulence (11). Three of these transporters have been crystallized, and their full three-dimensional structures have been determined: the vitamin B_{12} transporter BtuCD from *Escherichia coli* (12), the metal-chelate type transporter HI1470/1 from *Haemophilus influenzae* (13), and the molybdate/tungsten transporter ModB_{2}C_{2}-A from *Archaeoglobus fulgidus* (14). Unlike NBDs that show conserved sequence motifs and common architecture for all ABC proteins, variations in TMDs can be observed between importers, like the number of TM helices (ranging from 10 to 20 depending on the mass and the structure of the substrates they translocate) or their spatial arrangement in the subunit. Unidirectional transport is allowed.
because only the extracytoplasmic receptor, not the TMDs, bind substrates effectively.

### 6.2.2. Exporters (Extrusion Systems)

By contrast to importers, extrusion systems are composed of TMDs fused to NBDs, creating “half-transporters” that form homodimers or heterodimers to generate the functional units. In Gram-negative bacteria, which present an outer membrane, the transporter is generally coupled to an adaptor protein and an outer membrane pore in order to achieve direct extrusion from the cytosol to the cell medium. The higher substrate affinity of the cytosol side of the protein conditions the unidirectional transport. Extrusion systems are divided into two main groups related to the type of substrate they translocate:

*Single drug resistance (SDR) transporters* are designated to export a specific class of compounds: for example, the transporter MsrA confers resistance to 14- and 15-membered macrolides and streptogramin B antibiotics in streptococci and staphylococci, but susceptibility to other antibiotics is not affected by this system.

*MDR transporters* are responsible for the efflux of structurally unrelated compounds such as antibiotics, fluorescent dyes, or anticancer agents. Since specific classes of compounds may be extruded by different transporters in the same cell, this nonsense energy wasting, which is uncommon in nature, leads us to believe that this ability to export various substances might be an opportunistic function of the transporter, where the substrate recognition mechanism is nonspecific enough to accommodate diverse structures.

Although differences in the three-dimensional structure of importers and exporters have been shown (for example, the contact surface between TMDs and NBDs), a common mechanism of action for both transporters has been hypothesized (15) (Fig. 6.1). From the three-dimensional structure of the importer ModB×C2-A and that of the exporter Sav1866, two distinct conformations were observed. The nucleotide-free state where the transporter adopts an inward facing (ModB×C2-A), and the ATP-bound state where the protein shows an outward facing (Sav1866). When ATP binds to NBDs (in open conformation), the latter adopts a closed conformation. The conformational changes occurring at the NBDs are transmitted to the TMDs via non-covalent interactions at the shared interface. Despite their difference in topological arrangement, all TMDs feature similar alpha helices that provide the greater part of the contacts to the NBDs. These “coupling helices” share little or no sequence similarity but superpose with NBDs in the same way for each transporter, and are believed to be the transmission between the engine (NBDs) and the effector (TMDs) (16). This conformational change makes the transporter adopt an outward facing where the substrate is released in the cell medium (exporter case) or where the substrate may be recruited
After hydrolysis of ATP, the protein adopts the nucleotide-free state (inward facing), which results in the entrance of the substrate (importer case), or the recruitment of another substrate (exporter case) (Fig. 6.1). This simple switch mechanism implies that, in a first step, ATP has to be bound to NBDs before transport occurs in importer case, whereas for exporters, it is only when the substrate has bound to TMDs that ATP may bind the nucleotide site. Other intermediate states between these two steps are likely to occur as exemplified by the crystallized BtuCD importer, which presents an outward facing of the TMDs with nucleotide-free NBDs in open dimer conformation.

6.3. BACTERIAL ABC TRANSPORTERS RESPONSIBLE FOR ANTIBIOTIC RESISTANCE

The first bacterial ABC-type transporter responsible for antibiotic resistance reported was MsrA, a plasmid-encoded transporter isolated in *Staphylococcus epidermidis* and responsible for 14/15-membered macrolides and type B
streptogramins resistance (17). LmrA, the first bacterial ABC-type transporter associated with MDR, was described in 1996 in *Lactococcus lactis* (18), although bacterial MDR mechanisms were reported in secondary antiport systems in the early 1990s (19). It is rather these secondary transporters that have been associated with antibiotic resistance and treatment failure (particularly from the MFS and the RND family for Gram-positive and Gram-negative bacteria, respectively). Many of these MDR transporters have been studied like, for example, NorA of *S. aureus* or the MexAB-OprM system of *Pseudomonas aeruginosa*, and different strategies to circumvent their involvement in antibiotherapy failure are being developed.

The contribution of bacterial ABC-type efflux systems in antibiotic resistance has remained less documented over the past years, and most of these transporters have been characterized in antibiotic-producing organisms as playing important roles in drug secretion and self-resistance to the produced antibiotics (20). At present, with the entire genome sequencing of many species and advances in molecular biochemistry (gene knockouts and/or overexpression studies), a large number of putative ABC-type transporters have been characterized, and among them a growing number are associated with antibiotic resistance in many bacterial strains (Table 6.1).

All these transporters are associated with significant levels of antibiotic resistance (at least fourfold increases in MICs between the control and the strain expressing the transporter). Almost every class of antibiotics is affected, and active efflux of antimicrobials, catalyzed by ABC-type transporters, occurs in both Gram-negative and Gram-positive bacteria. On the overall characterized transporters, few have been classified as MDR (4, 46), whereas the majority is rather described as SDR. Considering drug therapy strategy, MDR pumps are of higher importance since they contribute to the adaptation of bacteria to multiple drugs, allowing even more class-specific resistance mechanisms, which eventually lead to very hypersensitive strains that may threaten both clinical and community settings (1).

The most studied bacterial ABC-type MDR transport is LmrA of *L. lactis*. This gene-encoded protein shares structural homology with the human P-glycoprotein (P-gP) but, unlike P-gP, is composed of two half-transporters and functions as a homodimer. This transporter has been expressed in a drug-hypersensitive strain of *E. coli* and has been shown to confer resistance to multiple antibiotics and also other drugs, all functionally and structurally unrelated (18, 24). Other MDR ABC-type pumps with broad spectrum substrate affinity have been described in bacteria; here we only focus on those associated with resistance to clinically used antibiotics. For this purpose, drug-hypersensitive strains lacking several major MDR transporters are useful tools to characterize the potent substrate spectrum of MDR efflux pumps (40). Other main MDR ABC-type efflux pumps involved in antibiotic resistance include EfrAB, VcaM, and MacAB, the latter being more likely an SDR transporter given its name (macrolide-specific ABC-type efflux carrier) and its substrate
### TABLE 6.1. Bacterial ABC-Transporters Associated with Antibiotic Resistance

<table>
<thead>
<tr>
<th>Pump</th>
<th>Organisms</th>
<th>Antimicrobials</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abc7</td>
<td>Enterococcus faecalis</td>
<td>Chloramphenicol, ofloxacín</td>
<td>(21)</td>
</tr>
<tr>
<td>Abc11</td>
<td>Enterococcus faecalis</td>
<td>Chlorhexidine</td>
<td></td>
</tr>
<tr>
<td>Abc16</td>
<td>Enterococcus faecalis</td>
<td>Azithromycin</td>
<td></td>
</tr>
<tr>
<td>BbmAB (AbcAB)</td>
<td>Bifidobacterium breve</td>
<td>Bacteriocin, nisin, polymixin B</td>
<td>(22)</td>
</tr>
<tr>
<td>EfrAB</td>
<td>Enterococcus faecalis</td>
<td>Ciproflaxacin, doxycycline, norflaxacin</td>
<td>(23)</td>
</tr>
<tr>
<td>LmrA</td>
<td>Lactococcus lactis</td>
<td>Aminoglycosides, anthracyclines, β-lactams, chloramphenicol, clindamycin, macrolides, ciproflaxacin, ofloxacín, streptogramins, tetracyclins</td>
<td>(18, 24)</td>
</tr>
<tr>
<td>Lsa (ex Abc23)</td>
<td>Enterococcus faecalis</td>
<td>Clindamycin, lincomycin, virginiamycin, quinupristin-dalfopristin</td>
<td>(21, 25)</td>
</tr>
<tr>
<td>Lsa (B)</td>
<td>Staphylococcus sciuri</td>
<td>Clindamycin</td>
<td>(26)</td>
</tr>
<tr>
<td>MacAB (ex YbjYZ)-TolC</td>
<td>Escherichia coli, Neisseria gonorrhoeae</td>
<td>14/15-membered macrolides</td>
<td>(27, 28)</td>
</tr>
<tr>
<td>MD1/MD2</td>
<td>Mycoplasma hominis</td>
<td>Ciproflaxcin</td>
<td>(29, 30)</td>
</tr>
<tr>
<td>MsrB</td>
<td>Staphylococcus xylosus</td>
<td>14/15-membered macrolides, streptogramins</td>
<td>(32)</td>
</tr>
<tr>
<td>MsrC</td>
<td>Enterococcus faecium</td>
<td>14/15-membered macrolides, tylosin, quinupristin</td>
<td>(33, 34)</td>
</tr>
<tr>
<td>MsrD</td>
<td>Streptococcus pneumoniae</td>
<td>Ketolides, 14/15-membered macrolides, streptogramins</td>
<td>(35, 31)</td>
</tr>
<tr>
<td>Orf10-Orf11-Orf12</td>
<td>Pseudomonas spp.</td>
<td>Nalidixic acid, norflaxacin</td>
<td>(36)</td>
</tr>
<tr>
<td>(plasmid)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PatAB</td>
<td>Streptococcus pneumoniae</td>
<td>Ciproflaxcin, norflaxcin</td>
<td>(37)</td>
</tr>
<tr>
<td>RV2686c-RV2687c-RV2688c</td>
<td>Mycobacterium tuberculosis</td>
<td>Ciproflaxcin</td>
<td>(38)</td>
</tr>
<tr>
<td>SmdAB</td>
<td>Serriata marcescens</td>
<td>Norflaxcin, tetracycline</td>
<td>(39)</td>
</tr>
<tr>
<td>SP2073/SP2075</td>
<td>Streptococcus pneumoniae</td>
<td>Berberine, ciproflaxcin, norflaxcin, erythromycin</td>
<td>(40)</td>
</tr>
<tr>
<td>VcaM</td>
<td>Vibrio cholera</td>
<td>Ciproflaxcin, norflaxcin, ofloxacín, tetracycline</td>
<td>(41)</td>
</tr>
<tr>
<td>Vga (A/B)</td>
<td>Staphylococcus spp.</td>
<td>Streptogramins</td>
<td>(42, 43)</td>
</tr>
<tr>
<td>Vga (A)c</td>
<td>Staphylococcus haemolyticus</td>
<td>Lincosamides, streptogramin A</td>
<td>(44)</td>
</tr>
<tr>
<td>VmlR (ex expZ)</td>
<td>Bacillus subtilis</td>
<td>Lyncomycin, virginiamycin</td>
<td>(45)</td>
</tr>
</tbody>
</table>

* Encodes an ABC-MFP component of a probable ABC-MFP-OMF export system. MFP = membrane fusion protein; OMF = outer membrane factor.
spectrum. Regarding this classification, SP2073/SP2075 could be added to this nonexhaustive and ever-growing list.

Multidrug efflux mechanisms are broadly conserved in bacteria and are almost invariably chromosome-encoded, while drug-specific efflux mechanisms are more likely encoded by plasmids and/or other mobile genetic elements like transposons or integrons (47). The latter promote horizontal transfers between species and allow a rapid dissemination of resistant determinants like efflux pumps, which intensifies the development of multiresistant strains in pathogenic and nonpathogenic bacteria and may pose, in addition to health concerns, an environmental problem (48).

6.4. IMPLICATIONS OF ABC TRANSPORTERS IN ANTIMICROBIAL THERAPEUTIC STRATEGIES

In terms of human therapeutics, ABC transporters are essentially studied for anticancer drugs’ purposes. Regarding antibacterial therapeutics, interest in MDR and efflux has risen during the past 10 years, and this phenomenon is now recognized as an intrinsic and, also with overexpression, as an acquired mechanism of resistance, which highly contributes to recent antibiotherapy weaknesses. It is especially on efflux driven by bacterial secondary transporters that most studies were conducted and are still in progress (49, 50). Bacterial ABC transporters are less considered because their involvement in antibiotic efflux is described as minor (51). However, recent findings and advances in molecular biology and bacterial genetic engineering allow a better knowledge of the bacterial efflux process, and ABC transporters seem to play a nonnegligible role in multidrug (including antibiotics) resistance as more and more transporters are characterized; also, their relative importance in environmental adaptation and survival of bacteria, together with their recent three-dimensional structure resolved, make them potential targets for new antibacterial strategies.

6.4.1. Bacterial ABC Transporters as Targets for Immunotherapy

It is known that ABC transporters may be immunogenic, especially surface-located proteins like outer membrane proteins (OMPs) and/or SBPs, suggesting that they could be exploited as candidate subunits for vaccination against pathogenic strains (11). Recombinant antibodies raised against these proteins have shown a reduction in the bacterial population in the mouse model of infection. Thus, it is possible to make bacteria overexpress these immunogenic components through the use of DNA vaccines. Examples of such vaccines have been evaluated for Mycobacterium tuberculosis infections, and efficient protection against intravenous infection was demonstrated with the use of PstS-3, one of M. tuberculosis SBP involved in phosphate transport (52).
ABC transporter components, located in the inner membrane of Gram-negative bacteria, are also able to stimulate specific immune responses since antibodies directed toward cytoplasmic proteins were already used in convalescent sera (11). Further studies are required to confirm the process by which proteins with inner membrane location are able to stimulate antibody response. This will allow a more rational approach of ABC transporter proteins as DNA vaccines.

Furthermore, immunotherapy may be enhanced when it is used in combination with antibiotics. A combination of a recombinant antibody raised against an epidemic strain of methicillin-resistant \textit{S. aureus} (EMRSA-15) ABC transporter peptide with vancomycin has terminated phase 3 clinical trials under the name Aurograb\textsuperscript{®} (NeuTec Pharma), and the overall response (bacterial and clinical) to this drug combination was greater than the one observed for the placebo plus vancomycin combination.

6.4.2. Inhibition of Efflux-Mediated Resistance

Faced with the problem of MDR and efflux as a limitation of antibacterial therapy, several theoretical approaches have been postulated, and apart from developing agents that are less sensible to efflux, all other strategies concern the coadministration of an “efflux modulator” with an antibiotic for which efflux-mediated resistance mechanisms limit the clinical use. This approach is rationalized and promising because (i) the MDR phenomenon is extending to every class of antibiotics (even the more recently approved agents like quinupristin-dalfopristin, telithromycin, tigecycline, and linezolid have been reported as being substrates of efflux pumps); (ii) antibiotics used in this combination have already proven their efficacy on wild-type strains, and many data on their toxicity, biodisponibility, etc., are available; and (iii) the association of a resistance inhibitor with an antibiotic has been proved to be clinically efficient with, for example, the clavulanic acid/amoxicillin combination that has been widely used in hospital settings. In order to promote higher intracellular drug concentrations, the efflux modulator may act either by activating import of the drug or by inhibiting its export. These two approaches have been undertaken during the last 20 years by both academics and industrials, and some of them provide good hopes for the future of antibacterial therapy.

6.4.2.1. Antibiotics Import Activation. One of the ways to activate the drug uptake is to associate the antibiotic to a natural substrate of a bacterial ABC importer. Artificially created drugs have been reported several decades ago using peptide importer systems as targets (11). The main interest of this approach is its specificity toward prokaryotes with one limitation: the complex size, which has to respect the transport capacity of the protein as exemplified in the study conducted by Marshall et al. (53) However, this approach presents an attractive potential that is probably underestimated. Because ABC
importers are specific to prokaryotes, and also because their three-dimensional structure has recently been resolved, it is likely that antimicrobial drugs based on ABC importer systems as targets might rise in the next decades.

6.4.2.2. **Bypassing Efflux.** Two approaches are currently undertaken to lower and/or abolish enhanced efflux of antimicrobials (54).

6.4.2.2.1. **Biological Inhibition of Efflux.** Biological inhibition of efflux includes interference with efflux gene expression. In this case, researches on the regulatory molecules acting as activators or repressors of efflux pumps gene transcription may provide useful tools for a better understanding of the physiological role of efflux pumps, as well as potential routes toward inhibition of efflux. However, the limitation of this approach in therapy is that regulatory molecules often regulate more than one gene expression, and thus, inhibition of the transcription might have other unexpected consequences.

6.4.2.3. **Pharmacological Inhibition of Efflux.** Conceptually, pharmacological inhibition of efflux pumps may be achieved by using different nonspecific and specific approaches: decoupling energy and transport by dissipating the energy gradient (only possible for secondary transporters since ABC uses ATP as energy motive force); interfering with pump assembly (only possible in Gram-negative bacteria; and for multicomponents systems such as RND pumps or also ABC pumps with one example: MacAB-TolC); or using a chemical inhibitor acting competitively or noncompetitively in the pump itself. The latter approach is the most adopted in medicinal chemistry, and many patents on chemical structures of efflux pump inhibitors (EPIs) have already been deposited (54).

In eukaryotic MDR, EPIs are well documented, and they were the first to be reported, even though few of them are used for their clinical efficacy (cyclosporine). Considering the structural homology between P-gP and some bacterial ABC transporters like LmrA, for example, it is admitted that molecules could inhibit both types of transporters. For example, verapamil or reserpine are considered as reference or control substances in eukaryotic and prokaryotic cell-based assays, respectively, but both of these molecules are able to inhibit both efflux systems. Therefore, developing ABC pump inhibitors from natural or synthetic sources goes through an important challenge: finding the prokaryote specificity. Now that some three-dimensional structures of these transporters are available, this challenge might be easier to take up, even though toxicity toward eukaryotic cells is to be feared. This is the reason why most studies target secondary transporters such as those belonging to MFS (NorA), SMR (EmrE), MATE (NorM), or RND (MexAB-OprM) superfamilies which are mostly bacteria specific.

Finally, in order to have a targeted modulation of bacterial efflux-mediated resistance, it is worth collecting more data about three-dimensional structures, physiological functions, encoding genes, and regulatory proteins that drive
efflux systems, together with improved cell-based assays screening of chemical compounds from both natural and synthetic sources using genetically engineered bacterial strains. In this effort, and in order to access specific and efficient antimicrobial drugs, bacterial ABC-type transporters could be regarded as potential targets, especially importer systems, because of their prokaryotic specificity.

6.5. CONCLUSIONS

Prokaryotic ABC-type transporters are involved in diverse vital functions for bacteria, such as import of nutrients or export of various toxins and/or toxics, thus contributing greatly to bacterial fitness and survival in the environment. Recent studies reported the crystallization of four transporters, three importers, and one exporter classified as MDR, which allowed for hypothesizing a common mechanism of coupling energy and transport for both types of transporters.

Exporter systems have been associated with drug resistance, including antibiotics, and some confer MDR phenotype to bacteria although this capacity was first attributed mainly to secondary type transporters. With the recent advances of molecular biochemistry and the complete bacterial genome sequencing of a large number of species, many putative ABC-type transporters have been identified, and a growing list is associated with antibiotic resistance either as SDR (for the most part) or MDR.

Altogether, these findings lead us to believe that bacterial ABC-type transporters play a significant role in antimicrobial resistance and are also well implicated in bacterial pathogenicity, making them potential targets for the development of new antimicrobial therapeutic strategies. The combinatory therapy, which consists of the combination of an extruded antibiotic with an efflux modulator, is currently one of the most promising strategies. In the case of bacterial ABC-type transporters, the lack of specificity between eukaryotes and prokaryotes make them secondary choices for EPIs’ cell-based assays’ screenings. However, with the import function being exclusively reported in prokaryotic cells, antimicrobial strategies targeting importer systems (either as drug import activators or as DNA vaccines for immunotherapy) might bring new therapeutic tools to control or at least prevent the extension of resistant phenotypes in bacteria.

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ment plant is related to plasmids residing in phytopathogenic bacteria and carries eight different resistance determinants including a multidrug transport system. *Microbiology* 150: 3613–3630.


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ABC PROTEINS INVOLVED IN PROTOZOAN PARASITE RESISTANCE

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ABC Transporters and Multidrug Resistance, Edited by Ahcène Boumendjel, Jean Boutonnat and Jacques Robert
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7.1. INTRODUCTION

Parasitic protozoa are responsible for a wide spectrum of diseases in human and domestic animals. These diseases are the major causes of mortality and morbidity throughout the world. Among them, malaria is by far the most prominent disease, affecting 300–500 million people and killing 3 million people every year, including 200–300 children per hour. It is endemic in most tropical regions with 2 billion persons at risk in 100 countries. Other life-threatening protozoal infections include African sleeping sickness, Chagas disease, toxoplasmosis, amoebic dysentery, and cryptosporidiosis. The estimated annual incidence of African trypanosomiasis is now 300,000–500,000 cases, with 66,000 deaths annually and 2 million disability adjusted life years. American trypanosomiasis, also known as Chagas disease, infects 16–18 million people with an estimated annual loss of 2.7 million disability adjusted life years. Until now, chemotherapy has been the main line of defense against these infectious organisms. However, drug use has resulted in the selection and development of resistance mechanisms, which strongly limit the number of antiprotozoal agents that are effective in disease treatment. The emergence and spread of resistance to currently used antiprotozoal drugs indicate that new compounds need to be discovered and developed through identification of novel chemotherapeutic targets. The urgency of this situation is better appreciated in statistical terms: "of the 1233 new drugs identified as reaching the market between 1975 and 1997, only 13 were approved for tropical diseases." A large amount of scientific effort is spent on elucidating the mechanisms underlying this resistance with the hope of restoring/improving the efficacy of existing drugs and of developing new drugs that can bypass resistance mechanisms. This chapter focuses on the involvement of protozoal ATP-binding cassette (ABC) transporters and other integral membrane proteins in drug resistance, emphasizing recent insight into interactions between molecular compounds and transmembrane proteins involved in drug efflux or uptake.

Among the various mechanisms identified, those based on drug transport appear to play an important role by pumping drugs out of target sites. The transport proteins of the ABC family are known to provide the basis of multidrug resistance (MDR) in mammalian cancer cells and in pathogenic yeasts, fungi, parasites, and bacteria (1–5). These proteins are found in both prokaryotic and eukaryotic cells.

The ABC transporters are generally composed of four identified domains: two membrane domains (MDs), each usually with six putative α-helical transmembrane segments (TMSs), and two nucleotide-binding domains (NBDs). The NBDs contain the “Walker A” and “Walker B” motifs and a short but highly conserved sequence, which are typical for members of the ABC family. At least 20 ABC transporters have now been identified in parasitic organisms. We focus below on the role of ABC transporters in drug resistance of the protozoal parasites Trypanosoma, Leishmania, and Plasmodium.
7.2. ABC TRANSPORTERS AND MDR IN TRYPANOSOMA SPP.

Sleeping sickness (human African trypanosomiasis), caused by *Trypanosoma brucei*, remains a major scourge in the endemic areas of Sub-Saharan Africa. Chemotherapy, which is the mainstay in the control of this disease, relies on very few drugs. Drugs in use are still the melaminophenyl arsical derivative melarsoprol, and diamidines like pentamidine and suramin, which were introduced more than 50 years ago. There are increasing reports on relapses in some sleeping sickness endemic areas, especially in Mozambique (6), Uganda (7, 8), Angola (9), and Sudan (10). Some compounds are in preclinical and clinical development, such as eflornithine (fluoromethylornithine derivative) and nifurtimox (nitrofuran derivative) (11).

South American trypanosomiasis (Chagas disease), caused by *T. cruzi*, is endemic from northern Mexico to Argentina. Currently, there are 18–20 million people infected with *T. cruzi* and another 40 million people are at risk. Recent surveys indicate that there are 200,000 new cases and 21,000 deaths associated with this disease every year (12, 13). The drugs most frequently used for the treatment of Chagas disease are nitroheterocyclic compounds such as nifurtimox, a nitrofuran, and benznidazole, a nitroimidazole derivative that was discovered to have anti-*T. cruzi* activity three decades ago (14, 15). Nevertheless, their respective efficacy varies according to the geographic area, probably due to discrepancies in *T. cruzi* strain drug susceptibility (16, 17). In addition, *in vitro* and *in vivo* resistances to benznidazole and nifurtimox have been reported (18–21).

The recurrent association of reduced drug uptake with drug resistance stresses the importance of drug transport in *Trypanosoma*. Reduction of intracellular drug accumulation can be caused by either a decreased drug import or an increased drug expulsion. Either mechanism implies mutations that could induce overexpression of a drug exporter, loss of a drug importer, or change in substrate specificity of drug transporters. Table 7.1 provides an overview of all known ABC proteins identified in *Trypanosoma* spp.

**TABLE 7.1. ATP-Binding Cassette (ABC) Transporters in Trypanosoma spp.**

<table>
<thead>
<tr>
<th>Trypanosoma spp.</th>
<th>Gene</th>
<th>Protein</th>
<th>Family</th>
<th>Involvement in Drug Resistance</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trypanosoma cruzi</em></td>
<td>Tcpgp1</td>
<td>TcPGP1</td>
<td>ABC</td>
<td>No?</td>
<td>(22–24)</td>
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<tr>
<td></td>
<td>Tcpgp2</td>
<td>TcPGP2</td>
<td>ABC</td>
<td>No?</td>
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<td>ABC</td>
<td>No?</td>
<td>(26)</td>
</tr>
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<td><em>T. brucei</em></td>
<td>Tbabc1</td>
<td>TbABC1</td>
<td>ABC</td>
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<td>TbMRPE</td>
<td>ABC</td>
<td>Yes</td>
<td>(28)</td>
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</table>
ABC transporter genes were described in *T. brucei* and *T. cruzi* (22–27). Three partially cloned ABC genes, named *Tbabc1*, *Tbabc2*, and *Tbabc3*, were identified in *T. brucei* (27). All three genes are expressed in both bloodstream form and in procyclic life-stage one. *TbABC3* shows homology to various known ABC transporters. *TbABC2* is highly homologous to the MDR protein (MDR) of *Leishmania donovani* (*LdMDR1*). The sequence of *Tbabc1* has now been identified to be identical to the NBD2 sequence of the MRP homologue described by Shahi et al. in *T. brucei* and designated *TbmrpA* (28). The most recently discovered ABC genes in *T. brucei* are *TbmrpA* and *TbmrpE* (28). Sequence analysis suggests that *TbMRPA* is closely related to *LtPGPA* and *TbMRPE* to *LtPGPE*. Both proteins exhibit the typical MRP topological configuration (MRP cluster) (Fig. 7.1).

Overexpression of *TbmrpA* in trypanosomes causes a 10-fold increase in resistance to melarsoprol while susceptibility to suramin and pentamidin is preserved. *TbMRPA* is located in the plasma membrane of the *T. brucei* bloodstream form, indicating that the protein confers resistance by pumping the drug out of the parasite. Overexpression of *TbmrpE* in trypanosomes causes a threefold increase in resistance to suramin, marginal resistance to melarsoprol, but no change in the pentamidin susceptibility, suggesting that suramin and melarsoprol are substrates of this protein. *TbMRPE* is located in an intracellular organelle between the nucleus and kinetoplast, and hence, it may...
transport drugs into this organelle. Drug resistance in trypanosomes is based on a trypanothione and glutathione-dependent conjugation of drugs, followed by transport of the metal–thiol complex, as it is in Leishmania spp. However, in contrast to Leishmania in which high levels of oxyanion resistance arises due to a combination of elevated concentrations of trypanothione and increased metal–thiol transport activity, the levels of trypanothione or glutathione in melarsen-resistant cells are unaltered (29). Cytosolic trypanothione levels in T. brucei are about 10-fold higher than in Leishmania spp., and endogenous levels of glutathione and trypanothione are high enough for the conjugation of melarsen and other drugs, which is in contrast to Leishmania. MRP overproduction is sufficient to induce arsenical resistance. Resistance to suramin and pentamidine must be due to other transporters or alternative mechanisms of resistance. The uptake of diamines in trypanosomes is thought to be mediated by the P2 aminopurine transporter, which is encoded by the TbaTl gene in T. brucei, a low-capacity high-affinity pentamidine transporter (HAPT1), and a high-capacity low-affinity pentamidine transporter (LAPT1) (30–32). The loss of P2 function in resistant trypanosomes to diamines is involved in resistance to these drugs (30, 33–35).

The first isolation and molecular characterization of the P-glycoprotein (Pgp) gene came from T. cruzi. It was designated as Tcpgp2 and showed considerable homology with the H-region type P-gp (36), MRP, and other ABC members not associated with the MDR phenotype (25). The transfection of multiple copies of Tcpgp2 in L. tropica results in the overexpression of Tcpgp2 transcripts. Under these experimental conditions, Tcpgp2 is not associated with resistance toward nifurtimox, benznidazole, puromycin, and daunomycin (25). It seems that TcPGP2 is a trypanothione S-conjugate pump, but that the synthesis level of trypanothione is not high enough to confer detectable resistance. TcPGP2 might be involved in the transport of molecules necessary for T. cruzi growth or other physiological functions such as the regulation of intracellular pH. The molecular characterization of TcPGP1 shows an absence of typical sequences corresponding to the second NBD of ABC members; this is due to the insertion with deletion of a non-long terminal report (LTR) retrotransposon (23), with high homology to T1Tc previously described in T. cruzi (Fig. 7.2) (37). TcPGP1 is a truncated ABC transporter and perhaps acts as a homodimer unit or as a multimeric complex. The phenotype resistant to benznidazole or nifurtimox in T. cruzi is not related to P-gp overexpression. Tcpgp1 and Tcpgp2 are not amplified and there are no changes in karyotype (24). Alternatively, drug resistance may be based on qualitative rather than quantitative differences. Further studies are needed to investigate possible point mutations in Tcpgp1 and Tcpgp2 and their roles in the drug resistance phenotype in T. cruzi. Finally, an ABCA transporter, TcABC1, located in the plasma membrane, in the flagellar pocket and intracellular vesicles, has been identified in T. cruzi (26). Functional studies of TcABC1 in transfected parasites suggest that this protein is involved in intracellular trafficking, such as endocytic and exocytic pathways of T. cruzi (26). Using different specific
inhibitors of ABC transporters, a study suggested that the P-gp of *T. cruzi* is probably involved in heme transport through the plasma membrane (38).

7.2.1. Transporter Inhibitors and Modulators of MDR

Verapamil was first reported as an *in vitro* modulator of resistance to nifurtimox in *T. cruzi* (39). However, this compound, which has no significant anti-trypanosomal activity at the concentrations used, did not reverse the resistance of *T. brucei* strains to diminazene aceturate and isometamidium chloride (40). Similar results were recorded in *T. evansi* when verapamil, cyproheptadine, desipramine, or chlorpromazine were combined with melarsen, diminazene aceturate, or suramin (41). Modulators at concentrations at which no direct activity on trypanosomes is detected did not reverse the resistance to most of the trypanocidal drugs. As phenothiazines inhibit trypanotheine reductase through a peroxidase/H$_2$O$_2$ system (42), it may be concluded that mechanisms of resistance to trypanocides in *T. brucei* and *T. evansi* differ from MDR in cancer cells, in malaria parasites, and in *T. cruzi* and *Leishmania* spp. as well. It seems that the reduction of net drug uptake in *T. brucei* can be caused by decreased drug import and not by increased drug export mediated by ABC transporters.

7.3. ABC TRANSPORTERS AND MDR IN *LEISHMANIA* SPP.

Several species of *Leishmania* are human pathogens. They are endemic in several parts of the world and are involved in one of the three clinical
forms of the disease, which are cutaneous, mucosal, and visceral infection. Leishmaniasis is now an emerging zoonosis in the United States (43). In Iraq, peacekeeping corps and U.S. soldiers are experiencing large outbreaks of leishmaniasis with more than 600 cutaneous lesions and one visceral infection (44). An outbreak of cutaneous leishmaniasis occurred among 71 French soldiers who participated in various missions during a four-month period in French Guiana (45). Today, arsenic- and antimony-containing drugs are still the first line of treatment. Pentavalent antimonial compounds remain the choices of treatment for all forms of leishmaniasis, ranging from cutaneous lesions to fatal visceral infections. Resistance to antimonial drugs is encountered more and more frequently (46–48).

7.3.1. ABC C Subfamily Transporters and MDR in Leishmania spp.

In Leishmania, several genes have been reported to show homology with those of the ABC family (Table 7.2). The first mdr homologue described was identified in L. tarentolae (LtpgpA) (36). This gene was discovered on an extrachromosomal element amplified in a methotrexate-resistant L. tarentolae promastigote cell line. LtpgpA has also been amplified in cell lines resistant to arsenicals, primaquine and terbinafine (49). The pgpA gene was amplified in strains of L. mexicana (LmepgpA) (50) and L. major (LmpgpA) (51). Analysis of the sequence of LtpgpA points to a structure similar to that of other ABC transporters. Nevertheless, LtpgpA is more closely related to the mdr-related (MRP) gene than to the mdr itself (52, 53). In some studies, the PGPA protein has been renamed MRPA (54). Two other genes, LtpgpB and LtpgpE, were amplified in strains of L. tarentolae (52). A homologous gene,
ABC PROTEINS INVOLVED IN PROTOZOA N PARASITE RESISTANCE

LtrpgpE, was also described in methotrexate-resistant strains of L. tropica (55). All these P-gp or MRP proteins (LtPGPA, LtPGPB, LtPGPE, LtrPGPE, LmePGPA, LmPGPA) belong to the MRP cluster. Members of this cluster contain a [MD-NBD]₂ topologic configuration, similar to that of MDR proteins (P-gp cluster) (Fig. 7.2). They are composed of four domains: two MDs (MD₁ and MD₂), each of which comprise six putative TMSs, and two NBDs (NBD₁ and NBD₂). Moreover, they show a long hydrophilic N-terminal chain, which is representative of MRP members. PGPA and PGPE are involved in the resistance of Leishmania toward arsenic and antimony compounds (56–59).

The role of PGPA in resistance was proven by gene transfection (51, 60, 61). Transfection of LtpgpA results in a low level of resistance (twofold increase) to arsenicals and sodium stibogluconate. Disruption of the LtpgpA gene results in hypersensitivity to arsenic-containing compounds. Similar experiments with LmpgpA induce a 10-fold increase in the resistance level to arsenical and trivalent antimonial drugs, while the activity of pentavalent antimonials or other cytotoxic drugs such as vinblastine are not significantly modified (51). However, the variation of the resistance level depends on the species into which the gene is transfected and on which pgpA allele is used. These observations suggest a role for additional factors and/or genes in the high levels of resistance to arsenical and antimonial drugs (62). Thus, GSH1, which is an enzyme involved in the rate-limiting step of the glutathione biosynthesis (63) and a transporter that actively expels arsenic-glutathione conjugate (64–66) could be involved as an associate factor. PGPA recognizes metal (arsenic) conjugated to trypanothione, which is made of two glutathione molecules linked by a spermidine moiety and through the thiol groups in Leishmania cells (63). The possible contribution of species-specific factors, such as the level of trypanothione, may explain the large differences in the susceptibilities to arsenicals between wild type of L. tarentolae and pathogenic species, as well as the differences in the resistance levels found in pgpA transfecants (67). The LtPGPA tagged with green fluorescent protein was shown to be located in the intracellular vesicular membrane close to the flagellar pocket rather than in the plasma membrane of the L. tarentolae. This suggests that LtPGPA confers resistance by sequestering the thiol–oxyanion conjugate into an intracellular vesicle (63). In fact, antimony (SbIII) probably not only interacts with cellular targets but also forms conjugates with various thiol functions brought by cysteine, glutathione, or trypanothione molecules. This process may be potentially accelerated by a conjugase (68). The metal–thiol conjugate can be either sequestered into an intracellular organelle via PGPA or extruded outside the cell via a plasma membrane adenosine triphosphate (ATP)-dependent efflux system (Fig. 7.3).

7.3.2. ABC B Subfamily Transporters and MDR in Leishmania spp.

The second class of ABC transporters identified in Leishmania cells are proteins showing higher similarity with mammalian P-gps that confer a MDR
phenotype similar to that observed in cancer cells. The first Leishmania mdr gene was described in a vinblastine-resistant L. donovani strain (Ldmdr1) (69, 70). The mdr1 gene was also amplified in a vinblastine-resistant L. enrietti strain (Lemdr1) (71), in a vinblastine-resistant L. amazonensis strain (Lamdr1) (72, 73), and in a daunomycin-resistant L. tropica strain (Ltrmdr1) (55, 74). All these strains, stepwise-selected by vinblastine or daunomycin, exhibited an MDR phenotype associated with cross-resistance to structurally nonrelated hydrophobic compounds such as puromycin, adriamycin, doxorubicin, daunomycin, and vinblastine; a decrease in intracellular accumulation of daunomycin was also observed. All these MDR1 proteins (LdMDR1, LeMDR1, LaMDR1, LtrMDR1) are members of the P-gp cluster and contain an [MD-NBD]2 topologic organization similar to that of the P-gp transporters (MRP cluster) (Fig. 7.2). Nevertheless, in contrast with MRP proteins, they have a small hydrophilic N-terminal chain representative of the P-gp family. It was suggested that amplification of the mdr1 gene is the cause of the increase in resistance to vinblastine and daunomycin (71, 74). However, the level of resistance observed in transfectants with Lamdr1 do not correlate with gene copy number in the

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**FIGURE 7.3.** Mode of action and resistance to pentavalent antimonials in Leishmania spp. The trivalent antimony (SbIII) penetrated via the aquaglyceroporin transporter (AQP1) into amastigotes and the pentavalent antimony (SbV) via an unknown protein. In the parasite, SbV can be converted to SbIII by either thiols or by ACR2 (arsenic compound resistance) and TDR1 (thiol-dependent reductase). SbIII probably not only interacts with some cellular targets but can also form conjugates with various thiol functions brought by the cysteine, glutathione, or trypanothione molecules. The metal–thiol conjugate can be either sequestered into an intracellular organelle via PGPA or expelled outside the cell via a plasma membrane ATP-dependent efflux system.
parasites (72). This may be due to a difference in RNA processing signals in the transfected gene compared to the genomic copy, and to the role of additional factors that confer a higher level of drug resistance or an appropriate cellular location of the gene products. It was originally suggested that Leishmania MDR1 is located in intracellular vesicles close to mitochondria (75, 76). It seems that LeMDR1 and LaMDR2 are located in the tubular structure; this cell compartment may correspond to a multivesicular tubule lysosome. This vesicular structure, recently described, is involved in exocytosis and endocytosis in Leishmania spp. (72, 77–79). The subcellular location of this transporter suggests that mechanisms different from those acting in the conventional mammalian efflux pump P-gp MDR1, may participate in the drug resistance of Leishmania spp. Intracellular expression of Leishmania MDR1 indicates that drug resistance possibly involves the recovery of the molecule from its target by sequestration (58). LeMDR1’s function in mediating drug resistance is iron-dependent (80). A new member of the P-gp cluster has been isolated in L. amazonensis (Lamdr2) (81). The overexpression of LaMDR2 confers resistance to 5-fluorouracil, although this compound is not clinically used for the treatment of leishmaniasis. The subcellular location of LaMDR2 proteins is unknown, but some preliminary indirect immunofluorescence studies have shown that anti-LaMDR2 antibodies predominantly stain the multivesicular tubule lysosome as it is in LeMDR1. Nevertheless, LaMDR2 seems to be involved in the extrusion of xenobiotics, but it presents functional divergence with LaMDR1 (81).

7.3.3. ABC A Subfamily Transporters and MDR in Leishmania spp.

This third class of ABC transporters shows high similarity with members of the mammalian ABCA subfamily (82). LtrABC1.1 was isolated in L. Tropica (82). Drug resistance studies with Leishmania, which overexpresses LtrABC1.1, indicate that this transporter is not associated with resistance to a wide range of unrelated drugs. LtrABC1.1 appears to be involved in lipid movement across the plasma membrane of the cell. Thus, it has been proposed that ABCA1 works as a phosphatidylserine flippase (83).

7.3.4. Transporter Inhibitors and Modulators of MDR

A number of compounds, for example, calcium channel blockers, calmodulin antagonists, hydrophobic peptides, protein kinase inhibitors, antibiotics, hormone derivatives, and flavonoids, have been previously described to reverse in vitro MDR in mammalian cells (84). They are called modulators or chemo-sensitizers; those that reverse the multidrug resistant phenotype in Leishmania spp. are listed in Table 7.3.

Some of these compounds, like the calcium channel blocker verapamil, are known to efficiently overcome MDR phenotype in vitro, not only in mammalian cells (85–87), but also in some bacteria such as Mycobacterium spp.
TABLE 7.3. Major Multidrug Resistance Reversal Drugs Investigated in Leishmania spp.

<table>
<thead>
<tr>
<th>Class of Compound and Specific Modulators</th>
<th>Resistance to</th>
<th>Strains</th>
<th>References</th>
</tr>
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<tr>
<td>Calcium channel blockers</td>
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<td></td>
</tr>
<tr>
<td>Verapamil</td>
<td>Pentavalent antimonials</td>
<td>Leishmania donovani</td>
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<td></td>
<td>Pirarubicin</td>
<td>L. braziliensis</td>
<td>(99)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L. guyanensis</td>
<td>(99)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L. mexicana</td>
<td>(99)</td>
</tr>
<tr>
<td></td>
<td>Vinblastine</td>
<td>L. amazonensis</td>
<td>(73)</td>
</tr>
<tr>
<td></td>
<td>Adriamycin</td>
<td>L. amazonensis</td>
<td>(73)</td>
</tr>
<tr>
<td>Phenothiazine derivatives</td>
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<td></td>
<td></td>
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<tr>
<td>Thioridazine, chlorpromazine, trifluoperazine, procholorperazine, trifluoropromazine</td>
<td>Pirarubicin</td>
<td>L. braziliensis</td>
<td>(99)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L. guyanensis</td>
<td>(99)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L. mexicana</td>
<td>(99)</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>N-meglumine antimonate</td>
<td>L. donovani</td>
<td>(130)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L. major</td>
<td>(130)</td>
</tr>
<tr>
<td>Flavonoids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silymarin and silybin derivatives</td>
<td>Daunomycin</td>
<td>L. tropica</td>
<td>(103, 104)</td>
</tr>
<tr>
<td>Sesquiterpenes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sesquiterpene esters (agarofuran derivatives)</td>
<td>Daunomycin</td>
<td>L. tropica</td>
<td>(105, 115, 116)</td>
</tr>
<tr>
<td>Sesquiterpene C-3 (agarofuran derivative)</td>
<td>Daunomycin</td>
<td>L. tropica</td>
<td>(113)</td>
</tr>
<tr>
<td></td>
<td>Miltefosine</td>
<td>L. tropica</td>
<td>(113)</td>
</tr>
<tr>
<td></td>
<td>Edelfosine</td>
<td>L. tropica</td>
<td>(113)</td>
</tr>
<tr>
<td>Pyridine analogues</td>
<td></td>
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<td></td>
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<tr>
<td>PAK104P</td>
<td>Pirarubicin</td>
<td>L. braziliensis</td>
<td>(99)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L. guyanensis</td>
<td>(99)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L. mexicana</td>
<td>(99)</td>
</tr>
<tr>
<td>Acridonecarboxamide derivatives</td>
<td>Elacridar, zosuquidar</td>
<td>Miltefosine</td>
<td>L. tropica</td>
</tr>
</tbody>
</table>

(88, 89) or Enterococcus spp. (90), in parasites such as nematodes like Haemonchus contortus (91–93), and in protozoa like Entamoeba histolytica (94–97) or Plasmodium falciparum (97, 98). Previous studies have shown that verapamil increases the in vitro antimony activity on L. donovani (39). However, verapamil at nontoxic concentrations in vivo cannot efficiently overcome the multidrug resistant phenotype in Leishmania spp. (39). This drug partially reverses the resistance in vinblastine-resistant L. amazonensis, which
shows cross-resistance to adriamycin (73). The energy-dependent efflux of
pirarubicin, an anthracycline derivative, is inhibited by verapamil in *L. braziliensis*, *L. guyanensis*, *L. mexicana*, *L. peruviana*, and *L. panamensis* (99). The
apparent wide substrate specificity of the *Leishmania* transport system sug-
gests that it could be responsible for the intrinsic resistance of parasite pro-
mastigotes to drugs. Its physiological relevance is supported by the fact that it
was described in at least five different *Leishmania* species. It seems that vera-
pamil regulates drug susceptibility by downregulating P-gp expression in
arsenical-resistant *Leishmania* spp. (100). In tumor cells, verapamil stimulates
 glutathione transport through MDR protein 1 (MRP1), which is a primary
active transporter of many conjugated organic anions, such as cysteinyl leuk-
otriene and reduced glutathione (101, 102).

Natural compounds such as flavonoids have shown promise to reverse
multidrug resistant phenotypes in *L. tropica* (103–106). Flavonoids constitute
a well-known class of natural inhibitors of different proteins (107, 108), with
contradictory results concerning their modulation effects on different multi-
drug resistant cells (109–111). They bind to the two cytosolic NBDs of the
ABC transporters. Their interactions with the ATP-binding site and a vicinal
hydrophobic region (103, 105, 112) cause the inhibition of drug efflux and
reverse the resistance to daunomycin in *L. tropica*. In addition, flavonoids may
modulate the multidrug transporter by decreasing P-gp synthesis and inhibit-
ing the transcriptional activation of the *mdr* gene involved in the susceptibility
to daunomycin (113, 114). Synthetic flavonoid dimmers exhibit a significant
reversing activity on pentamidine and sodium stibogluconate resistance in
*L. enriettii* and *L. donovani* (106).

Agarofuran sesquiterpenes, for example, natural compounds isolated from
*Maytenus cuzcoina*, *M. chubutensis*, *M. macroparta*, and *Crossopetalum tondu-
zi*, are new promising reversal agents that overcome the MDR phenotype in
*Leishmania*, including the resistance to anthracyclins (daunomycin) and
alkyl-lysophospholipids (miltefosine and edelfosine) (115–119). These com-
pounds bind to the NBD$_2$ C-terminal of *L. tropica* P-gp-like transporter,
*LtrMDR1* (115). Sesquiterpene C-3 remarkably sensitizes multidrug resistant
parasites to miltefosine and edelfosine by increasing alkyl-lysophospholipid
accumulation (113). Moreover, *mdr1* gene transfections can alter membrane
fluidity in mammalian cells and change alkyl-lysophospholipid effects (120,
121).

Phenothiazines and reserpine can also reverse drug resistance in mamma-
lian cells and bacteria (122–126). Phenothiazine drugs, of which chlorproma-
zine is the leading molecule, are widely used for their antipsychotic, antianxiety,
and antiemetic effects. In addition, they also possess protozoacidal activity
against amastigotes and promastigotes of *L. donovani* and *L. chagasi in vitro
as well as *in vivo* (127–129). Chlorpromazine, trifluoropromazine, thioridazine,
trifluoperazine, and prochlorperazine are reported to inhibit the energy-
dependent efflux of pirarubicin, an anthracycline derivative in *L. braziliensis*,
*L. guyanensis*, and *L. mexicana* (99). A synergistic effect between chlorproma-
zine and N-meglumine antimonate is observed in multidrug resistant *L. donovani* and *L. major* cells *in vitro* (130). The effect of phenothiazine derivatives on *Leishmania* active expel systems may be explained by their ability to inhibit the activity of trypanothione reductase (131, 132). Indeed, if we consider that the reduced form of trypanothione is an important cofactor for the function of the *Leishmania* drug transporter, in the same way as reduced glutathione is required for the MRPI1 function (102, 133), phenothiazines may inhibit transport activity by decreasing the intracellular level of reduced trypanothione (99). However, no significant effect is observed *in vivo* against amastigotes of *L. major* and *L. mexicana*, in cutaneous lesions in mice (130). The toxic effects reported with the most frequently studied phenothiazine, chlorpromazine, have impaired the investigation of other phenothiazines as potential clinical agents.

A pyridine analog, PAK-104P, was demonstrated *in vitro* as well as *in vivo* to inhibit P-gp-mediated MDR to vincristine, adriamycin, doxorubicin, paclitaxel, antimonial, and arsenical drugs (134–139). PAK-104P also blocks the energy-dependent efflux of pirarubicin in *L. braziliensis*, *L. guyanensis*, and *L. mexicana* (99). This compound probably alters the activity of trypanothione reductase and the transport activity by decreasing the intracellular level of reduced trypanothione. This compound may be considered as a very promising compound for reversing MDR in *Leishmania*.

Acridonecarboxamide derivatives (elacridar and zosuquidar), modulators of human P-gp, can overcome P-gp (LtrMDR1)-mediated *Leishmania* miltefosine resistance by increasing intracellular miltefosine accumulation (117).

### 7.4. ABC TRANSPORTERS AND MDR IN *PLASMODIUM* SPP.

Of the four *Plasmodium* species that infect humans, *P. falciparum* causes the most serious pathology and the overwhelming parasitemia results in high mortality rates if untreated. An estimated 300–500 million cases with at least 2–3 million deaths occur annually. From virtual eradication in the 1960s, malaria has reemerged to such an extent that more than 41% of the world’s population is now at risk. The current expansion of the disease is due to the emergence and the rapid spread of multidrug resistant forms of the parasites (140). Actually, *P. falciparum* resistance to chloroquine causes a severe and increasing public health threat. This inexpensive and widely used drug has been the main defense against malaria, and its increasing failure accompanies a reemergence of malaria-related morbidity and mortality (141). Initially reported from independent regions in South America in 1961 (142) and Southeast Asia in 1962 (143), chloroquine resistance is now found in all areas where malaria is endemic (144). Furthermore, failures of antimalarial prophylaxis based on the combination of chloroquine and proguanil (145), or mefloquine (146, 147), and clinical failures with halofantrine (148) or quinine (149) have been observed in Africa. One strategy that can be pursued
to reduce the prevalence of malaria is to chemically reverse antimalarial drug resistance.

### 7.4.1. ABC Transporters and MDR in *Plasmodium* spp.

Resistance is associated with chloroquine confinement in the vacuole, which results from a reduced uptake of the drug, an increased efflux, or a combination of both processes (150). A number of *P. falciparum* candidate genes that play a role in membrane transport have been proposed to be involved in chloroquine resistance. Restoration of the drug’s effect by agents like verapamil is a characteristic feature of chloroquine resistant strains, which is similar to the MDR phenomenon in cancer cells (151). Indeed, the decrease of chloroquine accumulation in resistant parasites (152), and the *in vitro* modulation of resistance by verapamil suggest that *P. falciparum* MDR could be similar to the mechanism described in cancer cells and, consequently, that homologous proteins are involved in both cases (153). Four ABC transporter genes have been identified in *P. falciparum*: a member of the P-gp cluster, *Pfmdr1* (154–156); a member of the TAP (Transporter associated with Antigen Processing) cluster, *Pfmdr2* (157, 158); and two members of the MRP cluster, *Pfgcn20* (159, 160), which is a homologue of the yeast *Gcn20* gene, and *Pfa0590w* (161) (Table 7.4 and Fig. 7.4).

*Pfmdr1* is overexpressed in chloroquine-resistant strains of *P. falciparum* (155). This gene, which encodes a 162 kDa protein named *P. falciparum* homologue of the P-gp (Pgh1), is located on chromosome 5. Pgh1 comprises the four usual domains, two MDs and two NBDs (Fig. 7.5) and is located in the membrane of the parasite digestive vacuole (156). Competition experiments

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**TABLE 7.4. ATP-Binding Cassette (ABC) Transporters in *Plasmodium* spp.**

<table>
<thead>
<tr>
<th><em>Plasmodium</em> spp.</th>
<th>Gene</th>
<th>Protein</th>
<th>Family</th>
<th>Involvement in Drug Resistance</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. falciparum</em></td>
<td><em>Pfmdr1</em> (chr 5)</td>
<td><em>PfMDR1</em> (Pgh1)</td>
<td>ABC</td>
<td>Yes</td>
<td>(154–156)</td>
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<tr>
<td></td>
<td><em>Pfmdr2</em> (chr 14)</td>
<td><em>PfMDR2</em> (Pgh2)</td>
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<td>No</td>
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<tr>
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<td><em>Pfgcn20</em> (chr 11)</td>
<td><em>PfGCN20</em></td>
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<td></td>
<td><em>Pfa0590w</em></td>
<td><em>PfMRP</em></td>
<td>ABC</td>
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<td>(161)</td>
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<tr>
<td><em>P. yoelii</em></td>
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<td><em>PyMDR1</em></td>
<td>ABC</td>
<td>Yes</td>
<td>(214)</td>
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<tr>
<td><em>P. berghei</em></td>
<td><em>Pbmdr1</em> (chr 12)</td>
<td><em>PbMDR1</em></td>
<td>ABC</td>
<td>Yes</td>
<td>(215)</td>
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<tr>
<td><em>P. chabaudi</em></td>
<td><em>Pcmdr1</em></td>
<td><em>PcMDR1</em></td>
<td>ABC</td>
<td>Yes</td>
<td>(153, 212, 213)</td>
</tr>
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chr = chromosome.
ABC TRANSPORTERS AND MDR IN *PLASMODIUM* SPP.

**FIGURE 7.4.** Predicted topological configuration of ATP-binding cassette (ABC) transporters in *Plasmodium* spp. ABC proteins in *Plasmodium* spp. belong to P-gp, MRP, and TAP clusters. Members of the TAP cluster contain two membrane domains (MD₁ and MD₂) and only one nucleotide-binding domain (NBD). Typical members of the MRP cluster and the atypical ones, which contain two NBDs but no MDs, are found in *Plasmodium* spp.

**FIGURE 7.5.** Predicted structure of the membrane ABC-transporter Pgh1. Numbers indicate the amino acids that flank the intramembrane. Their positions are predicted due to Gene DB. White stars show mutated amino acids related to antimalarial drug resistance described in the literature.
carried out with photoaffinity labeling of purified parasite digestive vacuole preparations associated with ATP analogues have indicated that ATP binds a nucleotide site located on the NH₂ terminal moiety of the molecule exposed on the cytoplasmic side of the vacuolar membrane (162).

Three possible functions are proposed for Pgh1 (Fig. 7.6a). It was originally reported that Pgh1 pumps chloroquine out of the food vacuole and is expressed in chloroquine-resistant parasites (Fig. 7.6a) (152). Earlier studies demonstrated the relationship between Pfmdr1 amplification and overexpression in resistant parasites (155). Nevertheless, subsequent studies failed to correlate gene overexpression and the chloroquine resistance (154, 163, 164) and showed that selection of chloroquine resistance is associated with a decrease in copy number of Pfmdr1 (165). It was then speculated that Pgh1 plays an important role in chloroquine import into the food vacuole of the parasites (Fig. 7.6b) (163). Further studies are needed to investigate the possible role of Pfmdr1 point mutations in chloroquine resistance. Namely, there is considerable controversy as to whether specific mutations in Pfmdr1 actually confer clinical drug resistance. Fieldwork has shown that the predictive value for chloroquine resistance and point mutations in the Pfmdr1 sequence resulting in amino acid changes varies depending on the geographic area (166–169). Point mutations, most notably at codon 86, have been associated with a decrease in the chloroquine susceptibility (170–174). However, in some of these epidemiological studies the number of chloroquine-susceptible samples is too limited to provide statistically meaningful analysis (167, 175). When precautions are taken into account, no relationships or only weak relationships are established in P. falciparum between resistance to chloroquine and mutations in Pfmdr1 (166, 169, 176–179). Other polymorphisms in Pfmdr1, such as point mutations at codon 184, 1034, 1042 and 1246, were identified in both chloroquine-susceptible and chloroquine-resistant parasites (180, 181). It is tempting to conclude that Pgh1 is not involved in chloroquine pumping. In fact, Pgh1 could act as a chloride channel or as a modulator of such a channel (Fig. 7.6c) (182).

As a chloride channel, Pgh1 may be constitutively expressed to maintain an internal acidic pH via the proton motive force of the vacuolar H⁺-pump. The pH of the vacuole plays an important role in chloroquine action and resistance. It seems that chloroquine reaches the parasite food vacuole, where it accumulates due to its weak base properties and local acidic pH, which is higher in resistant parasites than in the susceptible ones (183, 184). However, it was recently shown that the pH value in the chloroquine-resistant parasites is lower than it is in chloroquine-susceptible parasites (185). These findings are divergent from the earliest ideas according to which reduced vacuolar pH leads to an increase in the chloroquine accumulation (184). It has been shown that vacuoles isolated from chloroquine-susceptible strains actively accumulate significantly more chloroquine (threefold increase) than those isolated from the chloroquine-resistant parasites (186). In addition, it was initially suggested that the most convincing explanation for chloroquine activity lies in its
capacity to inhibit crystallization of the free heme by the formation of a toxic heme-chloroquine complex in the food vacuole (187) and/or to increase vacuolar pH (188, 189). Nevertheless, it was recently found that a short-term exposure to chloroquine has no significant effect on susceptible or resistant parasites while longer exposure decreases vacuolar pH in susceptible parasites (190). It has been suggested that the cellular uptake of chloroquine is dependent on its binding to ferriprotochlorophyrin IX (FPIX) and is independent of Na⁺/H⁺ antiport activity (191). Increased acidity of the digestive vacuolar pH contributes to the drug resistance because of the strong pH effects on the solubility of unpolymerized heme in the vacuole. Changes in pH are reported to modify the conversion of soluble heme to insoluble aggregates (185, 192). The aggregate form of FPIX dimers promoted by lower pH, is still capable of crystalliza-

**FIGURE 7.6.** Proposed functions of P-gp1 in *P. falciparum*. (a) P-gp1 functions as an efflux transporter. (b) Pgh1 operates as a drug importer. (c) Pgh1 mediates chloride permeability either as a channel or as a channel modulator.
tion as hemozoin (193, 194) but does not bind chloroquine as avidly as freely soluble FPIX does (185). The pH turning point for this conversion is close to that of vacuoles in chloroquine-resistant parasites (195). The lower the vacuolar pH in chloroquine-resistant parasites, the better the formation of insoluble heme. The acidification of the digestive vacuole would decrease significantly the free heme concentration available for the formation of toxic complexes with chloroquine. In addition, antimalarial drugs can influence the pH-dependent solubility of heme via apparent nucleation phenomena. The presence of chloroquine reduces the solubility of FPIX (195). It has been found that verapamil normalizes vacuolar pH in chloroquine-resistant parasites to a value close to that measured in chloroquine-susceptible parasites. In contrast, no modification is apparent in vacuolar pH with chloroquine-susceptible parasites (190). Verapamil has been shown to lower the Kd (constant of dissociation) of chloroquine binding in intact infected cells (189).

Expression of the wild-type Pfmdr1 gene causes cellular resistance to quinine, mefloquine, and halofantrine in yeast cells (196). Amplification and overexpression of Pfmdr1 has been associated with mefloquine resistance and halofantrine decreased susceptibility in Palaparam (164, 197–200). In contrast, two studies report that the copy number and the level of expression of Pfmdr1 are not altered in mefloquine resistance (201) or in halofantrine-decreased susceptibility (202). Recently, Price et al. showed that amplification of Pfmdr1 is the main cause of resistance to mefloquine in P. falciparum (203); the Pfmdr1 copy number could be used as a molecular marker to monitor mefloquine drug resistance in areas of emerging resistance (181, 204).

It has been shown through heterologous expression that Pfmdr1 mutations at codons 1034 and 1042 abolish or reduce the level of resistance to mefloquine (196). Moreover, transfections with a wild-type Pfmdr1 allele at codons 1034, 1042, and 1246 confer mefloquine resistance to susceptible parasites (205). However, mutations at codons 1034, 1042, and 1246 in P. falciparum Pfmdr1 isolates are not sufficient to explain variations in mefloquine susceptibility (206–208). Analyses of P. falciparum isolates showed an association between mutation at the codon 86 and an increase in susceptibility to mefloquine, halofantrine, or artemisinin derivatives (204, 209–211). Pfmdr1 homologues have been identified in P. chabaudi, Pcmdr1 (153, 212, 213), P. yoelii, Pymdr1 (214), and P. berghei, Pbmdr1 (215). As yet described for Pfmdr1, no association is found between CQ susceptibility and Pcmdr1 (153, 212, 213), while overexpression of Pcmdr1, Pbmdr1, or Pymdr1 gene products is associated with mefloquine resistance or artemisinin resistance (212, 214, 215).

Concerning other ABC transporters, Pfmdr2, located on chromosome 14, encodes a 110 kDa protein, termed P/MDR2 (157, 158). P/MDR2 contains 10 transmembrane domains (TMDs) (two MDs) and a single NBD. This protein is a member of the TAP cluster (Fig. 7.4). The amplification and the overexpression of Pfmdr2 is still debated. Some works report increased transcription of Pfmdr2 in CQ-resistant parasites (216), while other studies have demonstrated neither amplification, nor overexpression of the gene (157, 158). To date, there is no evidence to support that P/MDR2 is involved in drug resistance.
PfGCN20, located on chromosome 11, encodes a 95.5 kDa protein termed PfGCN20 (159, 160). This protein contains two NBDs but no MD and is a member of the MRP cluster (Fig. 7.4). PfGCN20 is located in multiple regions of the infected erythrocyte, including the membrane and cytosolic compartments inside and outside the parasite (160, 217). PfGCN20 could be involved in plasmodial translational regulation or could be an ATP-binding subunit of a multimeric ABC transporter (160, 217).

Pfa0590w encodes a 210–215 kDa protein, termed PfMRP or CAB63558 (161). This protein contains two MDs and two NBDs and is a member of the MRP cluster (Fig. 7.7). When PfMRP is compared to Pgh1, they share only 7% overall sequence identity. PfMRP shows structural similarity with human MRP2 and the parasite Cryptosporidium parvum CpABC2. PfMRP contains the same structural domains described for Pgh1: TMD1, NBD1, TMD2, and NBD2. The NBDs are constituted with the conserved motifs Walker A, Walker B, and the ABC signature. The predicted protein lacks the additional NH2-terminal TMD (TMD0) that exists in human MRP2. This transporter is localized to the digestive vacuole membrane similarly to the transporters involved in drug resistance described above and is expressed both in chloroquine-sensitive and chloroquine-resistant strains. The structural similarities with human MRP2 and CpABC2 suggest a function as an oxidized glutathione (GSSG) transporter in P. falciparum.

Previous studies described two mutation points correlated with chloroquine resistance and quinine resistance (218, 219). Pfmrp single-nucleotide polymorphisms at position 191 and 437 were found to vary together so that associated haplotypes were either 191Y and 437A or 191H and 437S. The haplotype 191H and 437S is associated with a decrease in the chloroquine and quinine response.

![FIGURE 7.7. Predicted structure of the membrane ABC-transporter PfMRP. Numbers indicate the amino acids that flank the intramembrane domains. Their positions are predicted due to Gene DB. White stars show the two mutated amino acids described in the literature and related to chloroquine or quinine resistance. Both codons are either wild or mutated.](image)
7.4.2. Transporter Inhibitors and Modulators of MDR in *Plasmodium* spp.

7.4.2.1. Inhibition of Chloroquine Resistance. Many molecules, related or not to MDR mechanisms, have already shown a capacity to modulate *in vitro* the chloroquine response on chloroquine-resistant strains without any change on chloroquine-susceptible strains. There are calcium channel blockers, calmodulin inhibitors, antidepressant drugs, histamine H1-receptor antagonists and synthetic surfactants (Table 7.5).

Classical chemosensitizers, such as calcium channel blockers like verapamil, diltiazem, tiapamil, nifedipine, nicardipine, or β-blockers like propanolol, are known to effectively overcome chloroquine resistance *in vitro* (220–223). Verapamil reverses the chloroquine resistance of laboratory strains and of clinical isolates as well (222, 224, 225).

Differentials in drug uptake versus efflux in chloroquine-susceptible and chloroquine-resistant parasites have both been proposed as explanations for lower chloroquine accumulation by resistant parasites, but this debate remains (226–229). Some authors have concluded that a decrease in chloroquine uptake could account for the drug resistance (227, 229, 230), while others propose that chloroquine-resistant parasites rapidly expel the drug via a pump-mediated mechanism (152). Increased chloroquine accumulation and low extracellular concentration of chloroquine have been demonstrated in resistant parasites treated with verapamil (191, 231, 232).

Different doses of verapamil were tested. Verapamil, when used at concentrations between 0.5–1µM, has been found to reverse chloroquine resistance from 40% to 85% (97, 233–235). Moreover, verapamil at concentrations ranging from 2 to 10µM is able to increase the level of chloroquine accumulation in chloroquine-resistant parasites two- to sixfold (189, 191, 232, 236–238).

Chloroquine has little morphological effect on resistant parasites, while the combination of chloroquine and verapamil results in typical chloroquine-related food vacuolar swelling with increased amounts of granular matrix (239). These effects are similar to those observed in drug-susceptible parasites treated with chloroquine alone or with a chloroquine-verapamil combination. It has been found that verapamil buffers vacuolar pH in chloroquine-resistant parasites to a value close to that measured in susceptible parasites (190). However, as pointed out above, the resistance reversal capability of verapamil cannot be explained only by its effects on global drug accumulation.

Concerning models of malaria in mice, daily injections of verapamil, nicardipine, or diltiazem, in combination with chloroquine, reverse the resistance in chloroquine-resistant *P. chabaudi* parasites (240), and this reversal is dose-dependent. Amlodipine increases chloroquine accumulation in infected mouse erythrocytes and potentiates chloroquine’s action against CQ-resistant *P. yoelii* strains (241). Verapamil and nicardipine display dose-independent reversal activity *in vivo* against chloroquine-resistant *P. berghei* parasites, but
### TABLE 7.5. Modulators Used in Plasmodium spp. as Chloroquine Resistance Reversal Drugs

<table>
<thead>
<tr>
<th>Class of Compounds</th>
<th>Specific Modulators</th>
<th>Strains</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium channel blockers</td>
<td>Verapamil</td>
<td><em>Plasmodium falciparum</em></td>
<td>(97, 222, 224, 225, 223–235, 237)</td>
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<tr>
<td></td>
<td></td>
<td><em>P. falciparum</em></td>
<td>(240)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. chabaudi</em></td>
<td>(241)</td>
</tr>
<tr>
<td></td>
<td>Diltiazem</td>
<td></td>
<td>(221, 222)</td>
</tr>
<tr>
<td></td>
<td>Tiapamil</td>
<td><em>P. falciparum</em></td>
<td>(221, 222)</td>
</tr>
<tr>
<td></td>
<td>Nifedipine</td>
<td><em>P. falciparum</em></td>
<td>(222)</td>
</tr>
<tr>
<td></td>
<td>Propranolol</td>
<td><em>P. falciparum</em></td>
<td>(222)</td>
</tr>
<tr>
<td></td>
<td>Nicardipine</td>
<td><em>P. chabaudi</em></td>
<td>(240)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. berghei</em></td>
<td>(242)</td>
</tr>
<tr>
<td>Calmodulin inhibitors</td>
<td>Chlorpromazine</td>
<td><em>P. falciparum</em></td>
<td>(221, 223, 245–247)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. chabaudi</em></td>
<td>(254)</td>
</tr>
<tr>
<td></td>
<td>Trifluoperazine</td>
<td><em>P. falciparum</em></td>
<td>(222, 246)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. chabaudi</em></td>
<td>(254)</td>
</tr>
<tr>
<td></td>
<td>Prochlorperazine</td>
<td><em>P. falciparum</em></td>
<td>(223)</td>
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<tr>
<td></td>
<td>Methotrimeprazin</td>
<td><em>P. falciparum</em></td>
<td>(286)</td>
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<td></td>
<td>Fluphenazine</td>
<td><em>P. falciparum</em></td>
<td>(286)</td>
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<td>Tricyclic antidepressant drugs</td>
<td>Desipramine</td>
<td><em>P. falciparum</em></td>
<td>(222, 223, 255–259, 261)</td>
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<td></td>
<td></td>
<td><em>P. chabaudi</em></td>
<td>(254)</td>
</tr>
<tr>
<td></td>
<td>Imipramine</td>
<td><em>P. falciparum</em></td>
<td>(222, 261, 264, 265)</td>
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<tr>
<td></td>
<td></td>
<td><em>P. chabaudi</em></td>
<td>(254)</td>
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<td>Fluoxetine</td>
<td><em>P. falciparum</em></td>
<td>(235)</td>
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<td><em>P. falciparum</em></td>
<td>(231, 263)</td>
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<td></td>
<td></td>
<td><em>P. chabaudi</em></td>
<td>(263)</td>
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<td></td>
<td>Amitriptyline</td>
<td><em>P. falciparum</em></td>
<td>(231)</td>
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<tr>
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<td>Oxaprotiline</td>
<td><em>P. falciparum</em></td>
<td>(231)</td>
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<tr>
<td>Histamine (H-1) receptor antagonists</td>
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<td><em>P. falciparum</em></td>
<td>(255, 266, 267)</td>
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<td></td>
<td></td>
<td><em>P. yoelii</em></td>
<td>(269, 268)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. berghei</em></td>
<td>(268)</td>
</tr>
<tr>
<td></td>
<td>Ketotifen</td>
<td><em>P. falciparum</em></td>
<td>(223)</td>
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<tr>
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<td></td>
<td><em>P. berghei</em></td>
<td>(268)</td>
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<td></td>
<td><em>P. yoelii</em></td>
<td>(268)</td>
</tr>
<tr>
<td></td>
<td>Promethazine</td>
<td><em>P. falciparum</em></td>
<td>(224, 233, 234, 236)</td>
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<td></td>
<td><em>P. berghei</em></td>
<td>(270)</td>
</tr>
<tr>
<td></td>
<td>Azatadine</td>
<td><em>P. falciparum</em></td>
<td>(267)</td>
</tr>
<tr>
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<td><em>P. berghei</em></td>
<td>(268)</td>
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<tr>
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<td></td>
<td><em>P. yoelii</em></td>
<td>(268)</td>
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<tr>
<td></td>
<td>Pizotyline</td>
<td><em>P. berghei</em></td>
<td>(268)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. yoelii</em></td>
<td>(268)</td>
</tr>
<tr>
<td>Synthetic surfactants</td>
<td>Chlorpheniramine</td>
<td><em>P. falciparum</em></td>
<td>(272–275)</td>
</tr>
<tr>
<td></td>
<td>Nonylphenolethoxylates</td>
<td><em>P. falciparum</em></td>
<td>(277)</td>
</tr>
</tbody>
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**ABC TRANSPORTERS AND MDR IN PLASMODIUM SPP.**
unlike *P. falciparum*, the chloroquine-susceptible strains are the most susceptible to the modulators (242). This result supports the hypothesis that *P. berghei* has an innate low-level efflux mechanism which is verapamil-susceptible. In addition, the efficacy of verapamil and that of nicardipine decreases while chloroquine resistance increases. Verapamil also chemosensitizes resistant parasites to quinine and amodiaquine *in vitro* (220, 243, 244).

Antipsychotic agents such as phenothiazine drugs (chlorpromazine, trifluoperazine, prochlorperazine) can enhance *in vitro* the potency of chloroquine against *P. falciparum* chloroquine-resistant strains (221–223, 245–247). Phenothiazines have also been shown to reduce or reverse antibiotic resistance in bacteria (248–250) and in yeast (251). It now seems increasingly likely that phenothiazines may exert their chloroquine resistance reversal activity by interacting with Pgh1 (205). Phenothiazine drugs can reverse chloroquine-resistant South American parasites with a lesser degree of susceptibility compared to African and Southeast Asian isolates as verapamil does (244, 246, 247, 252). Chlorpromazine has been shown to bind to human P-gp and modulate MDR in cancer cells (253). Combinations of chlorpromazine or prochlorperazine with chloroquine confirm the reversal effect of these drugs on chloroquine resistance as shown by the results in *Aotus* monkeys infected with chloroquine-resistant *P. falciparum* strains (223). Moreover, daily injections of chlorpromazine or trifluoperazine reverse resistance to chloroquine in *P. chabaudi* resistant parasites (254).

Desipramine and other tricyclic antidepressant drugs, such as imipramine, fluoxetine, oxaprotiline, amitriptyline, or citalopram, reverse *in vitro* chloroquine resistance in *P. falciparum* at concentrations observed in the plasma of human patients treated for depression (255–260). However, although desipramine reverses chloroquine resistance *in vitro* (261), this drug does not enhance the efficacy of chloroquine in clinical trials (262). Daily injections of desipramine, imipramine, or citalopram in combination with chloroquine inhibit the growth of chloroquine-resistant *P. chabaudi* parasites in a dose-dependent way (254, 263). Combinations of desipramine with chloroquine reverse resistance in *Aotus* monkeys infected with chloroquine-resistant *P. falciparum* strains (255). It has been shown that an aminoalkyl substitution at the N5-position of the heterocycle of the tricyclic antidepressants and a secondary or tertiary aliphatic aminoalkyl nitrogen with a two or three carbon long side-chain branched to the heteroatomic nitrogen (N5) are required for the resistance reversal activity (264, 265). Tricyclic antidepressant drugs, with different reuptake-blocking properties on various neurotransmitter systems were examined as potential reversal agents for chloroquine resistance. Unfortunately, chloroquine resistance reversal properties on *P. falciparum* with these compounds are not associated with their previous activities as transporter blockers (231).

Tricyclic histamine (H-1) receptor antagonists, such as cyproheptadine, promethazine, ketotifen, or azatadine, also reverse *in vitro* the chloroquine resistance in *P. falciparum* (255, 266, 267). These compounds produce *in vivo* a
marked reversal of chloroquine resistance of *P. berghei* and *P. yoelii* strains (268–270). Aotus monkeys that have been infected by chloroquine-resistant *P. falciparum* parasites are cured following treatment with chloroquine and promethazine (271). The combination of chloroquine with chlorpheniramine enhances the efficacy of the former in *P. falciparum* malaria and is effective on chloroquine- and pyrimethamine/sulfadoxine-resistant malaria in Nigerian children (272–275). In addition, significant synergy in halofantrine efficacy has been demonstrated in halofantrine-resistant *P. yoelii* strains when administered concurrently with cyproheptadine or ketotifen (276).

Nonylphenolethoxylates (NPEs) are synthetic surfactants used as intestinal permeability enhancers to improve oral drug delivery. They have synergistic effects in combination with chloroquine (277). Nevertheless, there is a subset of *P. falciparum* isolates that display polyethoxylated nonylphenol (NP30)-insensitive chloroquine resistance (278). The differences between NP30 activities against these isolates cannot be explained by point mutations in *Pfmdr1* since these mutations are present in all of the parasites used in the study. In addition, only poor interactions have been identified between mammalian P-gps and long-chain NPEs (279).

### 7.4.2.2. Inhibition of Quinine Resistance.

The mode of action of quinine is less documented than that of chloroquine. Like chloroquine, quinine is a quinoline drug and in some cases shows cross-resistance to chloroquine. Quinine shares some common characteristics with chloroquine. The parasite’s resistance to chloroquine and quinine is directly related to the relative content of acid phospholipids and is inversely related to that of cholesterol in red blood cell membranes (280).

The role of mutations on the 1034, 1042, and 1246 alleles of the *Pfmdr1* gene was investigated by using a transfection-based approach (205). The results suggested that these mutations affect quinine susceptibility. *Pfmdr1* mutations contribute to quinine resistance (281). Recent findings have suggested that other loci may be involved in modeling quinine responses, such as the *pfmrp* gene (161, 218).

Although selection and transfection experiments have implicated these genes in the quinine response, their relative contribution to the trait and the way in which they interact with each other and additional genes to generate the specific drug response have not been elucidated.

The quinine response in *P. falciparum* can be modulated by some molecules (Table 7.6) that are often found to modulate the chloroquine response in *P. falciparum*, namely, calcium channel blockers such as verapamil, diltiazem, or tiapamil (221, 222, 244), calmodulin inhibitors (chlorpromazine) (221), and synthetic surfactant (NP30) (278).

### 7.4.2.3. Inhibition of Mefloquine Resistance.

The emergence of mefloquine-resistant *P. falciparum* has been documented over two decades. In preliminary studies, resistance to mefloquine and halofantrine was linked to mutations in
TABLE 7.6 Modulators Used in *Plasmodium* spp. as Quinine Resistance Reversal Drugs

<table>
<thead>
<tr>
<th>Class of Compounds</th>
<th>Specific Modulators</th>
<th>Strains</th>
<th>References</th>
</tr>
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<tr>
<td>Calcium channel blockers</td>
<td>Verapamil</td>
<td><em>Plasmodium</em></td>
<td>(221, 243, 244)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>falciparum</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diltiazem</td>
<td><em>P. falciparum</em></td>
<td>(221)</td>
</tr>
<tr>
<td></td>
<td>Tiapamil</td>
<td><em>P. falciparum</em></td>
<td>(221)</td>
</tr>
<tr>
<td>Calmodulin inhibitors</td>
<td>Chlorpromazine</td>
<td><em>P. falciparum</em></td>
<td>(221)</td>
</tr>
<tr>
<td>Synthetic surfactants</td>
<td>NP30</td>
<td><em>P. falciparum</em></td>
<td>(278)</td>
</tr>
</tbody>
</table>

*pfmdr1* (204, 282) and to increases in this gene’s copy number (203, 283). The tyrosine-86 allele of the *pfmdr1* gene is associated with increased susceptibility to mefloquine and increased resistance to chloroquine (209, 284). Mefloquine interacts with ABC proteins, MRP1 (ABCC1), and MRP4 (ABCC4) that are present in human erythrocyte membranes (285). Mefloquine stimulates ATPase activity and thus may itself be a substrate for transport.

Interestingly, mefloquine efflux from resistant parasites was blocked by penfluridol (286, 287), but not by verapamil. Few molecules (Table 7.7), generally others different from those known to modulate a chloroquine response, are able to modulate the mefloquine response in *P. falciparum*, such as penfluridol (calmodulin inhibitors) (286–288) and synthetic surfactants NP30 (278).

7.5. CONCLUSIONS

The most prevalent mechanisms of resistance in *Plasmodium* spp., *Leishmania* spp., and *Trypanosoma* spp. are mutations of proteins involved in the drug
transport (uptake or efflux) and amplification of transporter genes. The role of ABC transporters in drug resistance in Leishmania is well established. The impressive progress in understanding chloroquine resistance mechanisms in *P. falciparum* has shown that transporters belonging to the ABC families are involved in this pathway. Several modulators have been described to reverse MDR in vitro in mammalian cells, *Plasmodium* spp., *Leishmania* spp., and *Trypanosoma* spp. They are calcium channel blockers, calmodulin antagonists, tricyclic antidepressants, antihistaminic drugs, anthracenic derivatives, antibiotics, hormone derivatives, and flavonoids. Most of these drugs remain to be evaluated in vivo. Hence, clinical evaluation of therapeutic regimens is now required to validate the efficacy of these promising compounds or combinations for the treatment of these protozoal diseases.

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synthesis, and evaluation of new chemosensitizers in multi-drug-resistant 


PART IV

MULTIDRUG RESISTANCE (MDR) MODULATION THROUGH INHIBITION OF ABC TRANSPORTERS: DESIGN OF INHIBITORS AND MECHANISM OF ACTION
8

REVERSAL AGENTS FOR P-GLYCOPROTEIN-MEDIATED MULTIDRUG RESISTANCE

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8.1. INTRODUCTION

Drug resistance is the major reason for failure of cancer therapy. When one drug elicits a response in tumor cells resulting in resistance to a large variety of chemically unrelated drugs, this is called multidrug resistance (MDR). ATP-binding cassette (ABC) transporters contribute to drug resistance via adenosine triphosphate (ATP)-dependent drug efflux (1).

A diverse array of drugs that sensitize multidrug-resistant cells to chemotherapy have been identified. The drugs selected for the initial clinical studies were the ones already approved for clinical use, and modulators such as verapamil (VPL) (2), calmodulin inhibitors (3), immunosuppressors (cyclosporin A) (4–7), and antimalarials (quinine) (8) were the agents most frequently evaluated.

Plasma concentrations equivalent to the concentrations necessary to inhibit drug efflux in vitro were difficult or impossible to achieve with these drugs because of toxicity. It became clear early on, that reversing the resistance of malignancies like renal cell cancer and colorectal cancer would not be possible despite high levels of expression of ABC transporters in these tumors. It also became clear that modulators block normal excretory function and delay clearance of chemotherapy.

The limitation of the potency of the modulators has been addressed by the development of compounds that are less toxic and more effective as inhibitors. These second-generation modulators include cyclosporine A (9–12) and the cyclosporine D analog valspodar (PSC833) (13–16).

Results from clinical trials with PSC833 have clearly demonstrated a need to reduce the dose of anticancer agents used in combination with it (17, 18), probably because of decreased clearance of the anticancer agents and inhibition of cytochrome P450. Recent clinical studies on the third-generation inhibitors have shown no significant drug interactions with common chemotherapy agents (19).

In this chapter, we propose to review the interaction of the most important modulators with P-glycoprotein (P-gp)-mediated drug efflux.

8.2. DRUGS EXTRUDED BY P-gp AND COMMON SUBSTRATES WITH OTHER PUMPS

8.2.1. Drugs Extruded by P-gp

Drug resistance was first documented experimentally in mouse leukemia cells that acquired resistance to 4-amino-N[10]-methyl-pteroylglutamic acid (20). In 1973, Dano discovered active outward transport of daunorubicin by drug-resistant cells that were cross-resistant to other chemotherapeutic agents, such as vinca alkaloids (vincristine, vinblastine) and other anthracyclines (doxorubicin [DOX]) (21).
Other authors studying MDR phenotype noted a constant overexpression of a 170kDa membrane protein termed P-gp (22). The gene encoding P-gp was cloned and identified as MDR1 (23). Riordan and Ling purified also this protein from plasma membrane vesicles of Chinese hamster ovary cell mutants with reduced colchicine permeability (24).

Later, taxotere and taxol, other molecules from the cytoskeleton poisons group, have been identified as exclusive substrates of P-gp (25, 26).

8.2.2. Common Substrates with Other Pumps

P-gp is a highly complex transporter and has the ability to recognize and transport a large number of structurally diverse, mainly hydrophobic, compounds. In addition to its overlapping substrate specificity with other transporters such as MDR-associated protein (MRP) and breast cancer resistance protein (BCRP), P-gp can handle unique compounds. P-gp is a transporter for large hydrophobic, either uncharged or slightly positively charged compounds while the MRP family primarily transports hydrophobic anionic conjugates and extrudes hydrophobic uncharged drugs. The MRPI-related uncharged drug transport is linked to the transport or allosteric effect of cellular-free reduced glutathione (27). The exact spectrum of the BCRP (mitoxantrone resistant protein [MXR]) transported substrates has not yet been explored in detail, and these studies are complicated by the variable substrate-mutants of BCRP observed in the most recent studies (28). Some of the key molecules are presented in Table 8.1 and are unfortunately also MDR substrates for the patients.

8.3. MODULATION OF P-gp ACTIVITY

The clinical importance of P-gp might also be determined through trials designed to abrogate P-gp function. Toward this end, less than 10 years after

<table>
<thead>
<tr>
<th>Compound</th>
<th>Type of Compound</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>Anthracyclines</td>
<td>P-gp, MRP1, BCRP</td>
</tr>
<tr>
<td>Idarubicin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daunorubicin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>Anthraquinone</td>
<td>P-gp, BCRP</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>Antimetabolite</td>
<td>MRP1, MRP3, BCRP</td>
</tr>
<tr>
<td>Topotecan and SN38</td>
<td>Camptothecins</td>
<td>BCRP</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>Vinca alkaloids</td>
<td>P-gp</td>
</tr>
<tr>
<td>Navelbine</td>
<td></td>
<td>P-gp</td>
</tr>
<tr>
<td>Vincristine</td>
<td></td>
<td>P-gp, MRP1</td>
</tr>
<tr>
<td>Etoposide (VP16)</td>
<td>Epipodophyllotoxin</td>
<td>P-gp, MRP1</td>
</tr>
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</table>
the discovery of P-gp-mediated MDR, the first Phase I and II clinical trials began to test the clinical potential of P-gp inhibitors.

Initial trials used “first-generation” P-gp inhibitors, including VPL, quinine, and cyclosporine A. A randomized Phase III clinical trial showed the benefit of the addition of cyclosporine A to treatment with cytarabine and daunorubicin in patients with poor-risk acute myeloid leukemia (AML) (12). Similarly, quinine was shown to increase the complete remission rate as well as survival in P-gp-positive myelodysplastic syndrome cases treated with intensive chemotherapy (29), suggesting that successful P-gp modulation is feasible. However, several other trials failed to show improvement in the outcome (30).

The second generation of inhibitors was devoid of side effects related to the primary toxicity of the compounds (R-enantiomer of VPL, PSC833 “Valspodar,” and VX-710 “biricodar”). The two first molecules were able to inhibit P-gp without blocking calcium channels or immunosuppressive effects (31).

Third-generation inhibitors are designed specifically for high transporter affinity and low pharmacokinetic interaction. Table 8.2 summarizes the inhibitors of P-gp, and more details on the pharmacological and clinical trial aspects of some of these molecules have been reported (32, 33).

| TABLE 8.2. Summary of Molecules That Are Able to Inhibit P-gp |
|------------------|------------------|
| **Generation**   | **P-gp Inhibitor** | **References** |
| First            | Amiodarone       | (34)          |
|                  | Cyclosporine A   | (35)          |
|                  | Quinidine        | (32, 36)      |
|                  | Quinine          | (29, 37)      |
|                  | Verapamil        | (2)           |
|                  | Nifedipine       | (32)          |
|                  | Dexniguldipine   | (38)          |
| Second           | PSC833           | (35)          |
|                  | VX-710 (Biricodar)| (39)         |
|                  | GG918            | (40, 41)      |
| Third            | LY475776         | (42)          |
|                  | LY335979 (Zosuquidar) | (43, 44) |
|                  | XR-9576 (Tariquidar) | (45, 46)    |
|                  | V-104            | (47)          |
|                  | R101933 (Laniquidar) | (48, 49) |
|                  | S9788            | (50, 51)      |
| Other            | Disulfram        | (52)          |
|                  | Pluronic L61     | (53)          |
8.3.1. VPL and Derivatives

As early as 1987, results from the first clinical trial of MDR (mediated by P-gp) reversal in ovarian cancer with VPL (Fig. 8.1) in combination with DOX were published (54). Because of significant cardiac toxicity, the study was discontinued. In myeloma and non-Hodgkin’s lymphoma, VPL was clearly shown to be active on resistance to chemotherapy protocols with anthracyclines and/or vinka alcaloids (55, 56).

However, patient survival was not increased in a phase III study combining VPL and cytotoxic drugs in the treatment of multiple myeloma (57). In contrast to hematological malignancies, refractory solid tumors were never shown to respond to VPL when this reverter was added to classical chemotherapy, likely because the doses administered were too low as a consequence of the fear of cardiac toxicity (58–60). The encouraging results obtained in non-small-cell lung cancer and pediatric cancers were not confirmed (61, 62). An interesting randomized study performed in anthracycline-resistant metastatic breast cancer patients treated by vindesine with or without VPL revealed a significant increase of survival of patients receiving VPL (63).

Proof of activity of dextroverapamil was found in a phase I study (64) and in lymphoma (65) and breast cancer (66) patients in phase II studies but not in colorectal (67) or renal (68) cancer patients. Despite potential interest in this drug, it has not been developed because its cardiac toxicity was judged to be unacceptable (69).

![FIGURE 8.1. Structure of known inhibitors of P-gp.](image-url)
8.3.2. Quinolines

Quinidine entered very early in a randomized trial in breast cancer, aimed at reversing anthracycline resistance (70) (Fig. 8.1). Quinidine is much less toxic than quinidine and can be used at higher doses. Bennis et al. showed that quinine probably modulates resistance without any increase in intracellular accumulation of DOX and concluded that quinine was able to alter nucleocytoplasmic distribution since the target of the anthracycline is the nucleus (71). Later, Belhoussine et al. showed that modulation of resistance by quinine was not accompanied by an increase in nuclear accumulation of pirarubicin (72).

In a phase II study, Solary et al. showed that its association with mitoxantrone (MIT) and cytarabine could improve the response rate of acute leukemias with poor prognosis (73). This was not confirmed in a phase III randomized study (74). Quinine was also devoid of any effect on the resistance of non-Hodgkin’s lymphomas to paclitaxel (75), which may not be surprising because this drug has no major effect on lymphomas. Phase III studies remain to be performed, with an early introduction of the modulator in the therapeutic strategy. Cinchonine is a demethoxy derivative of quinidine that has shown interesting reversing properties in vitro and in vivo. A phase I trial has recently been completed, showing no interaction with the pharmacokinetics of the anticancer drug but presenting a dose-limiting cardiac toxicity (76).

8.3.3. Immunosuppressors

Cyclosporin A (CSA) and PSC833 (Fig. 8.1) belong to the group of MDR modulators that are able to inhibit the P-gp associated ATPase activity. The best characterized is PSC833, which at nanomolar concentrations inhibits the transport of CSA and PSC833. Several works favor the view that CSA and PSC833 are transport substrates for P-gp (77–79) while other data do not (80). The last report considers that PSC833 is indeed a substrate of P-gp, but a slow one. That is, when PSC833 competes with a substrate, it will win out due to its higher affinity (a larger interaction surface) (81). But since its transport rate is slower, it will slow down the turnover rate, which will then be reflected in the decrease in ATPase activity. So PSC833 seems to be a partial antagonist, since it does not completely block P-gp function, but just slows it down due to the bulkiness of this molecule acting as an “obstructive” substrate, slowing down the P-gp machinery.

Studies performed in vitro on samples from leukemia patients showed that CSA and PSC833 are appropriate inhibitors that can be used in MDR diagnosis. In fact, Merlin et al. have shown that in the P-gp-positive samples, a significant increase in cellular daunorubicin accumulation is observed in the presence of CSA (82). Later, the same group demonstrated the influence of PSC833 on daunorubicin intracellular accumulation in bone marrow specimens from patients with AML (83). Legrand et al. have also demonstrated
that there was a good correlation between P-gp expression and the in vitro modulatory effect of CSA on calcein-acetoxymethyl uptake in sample patients with AML (84). A functional study of calcein uptake and efflux in the presence of CSA and probenecid (a specific inhibitor of MRP1) respectively has shown that it was possible to discriminate the P-gp and MRPI transport activities (85).

CSA entered early into trials of reversal of MDR. The proof of reversing activity of CSA was found in phase II studies with myeloma (9) and acute leukemia (10). Phase III studies were conducted in hematological malignancies and there was no effect of CSA on the overall response rate and progression-free survival in myeloma patients (11), whereas among several studies, only one showed a positive effect of CSA in acute myeloblastic leukemia (12).

A widely tested second-generation compound is PSC833 (valspodar), a derivative of cyclosporin D that is 10 times more potent than CSA (86). PSC833 was the first molecule from cyclosporins group without immunosuppressive properties (6) and showed modulation of MDR in vivo with a lower renal toxicity when compared with CSA (7). During phase I studies, an important effect of this compound on the pharmacokinetics of the associated drugs was shown, the anticancer drugs being etoposide (VP16) (13), DOX (14), MIT (15), and Taxol (16). In most cases, either a doubling of the time-plasma concentration area under the curve or an important increase in elimination half-life was found. Therefore, it was not possible to separate the pharmacokinetic effects of PSC833 from its pharmacodynamic effects. Those results were in general disappointing, particularly in acute myeloblastic leukemia trials. Then it was necessary to reduce the dose of anticancer agent used in combination with PSC833. Reduction of the cancer agent dose ranged from 25% for etoposide to 66% for taxol (87, 88). Those dose reductions were required to prevent toxicities of the anticancer agent in combined therapy and compromised drug concentration in the tumor even with complete inhibition of P-gp.

One treatment that has not been fully explored is that of prevention of the emergence of resistance through the use of P-gp inhibitors. In the laboratory, PSC833 reduced the mutation rate for DOX-selected resistance in sarcoma cells by 10-fold, thus reducing the development of resistant clones via the MDR mechanism. In those sarcoma cells treated with PSC833, resistance was mediated by an alternative pathway with reduced expression of topoisomérase IIα, the target enzyme for anthracyclines (89). Another study examined six agents for their ability to prevent vincristine resistance in a rhabdomyosarcoma cell line (90). MDR modulators, particularly PSC833, prevented the development of resistance, suggesting the role of the use of P-gp inhibitors prior to cytotoxic therapy.

8.3.4. Third-Generation and Common Modulators with Other Pumps

S9788 (Fig. 8.2) was selected among thousands of compounds because of its important action against MDR cells in vitro and in vivo (50). Phase I studies
revealed cardiac toxicity at relatively high doses. Despite its potential interest and the absence of data on the frequency and lethal risk of this toxicity, this compound has not been further developed (51).

Biricodar (VX-710) (Fig. 8.2) was studied in two phase I trials in combination with doxorubicin112 or paclitaxel. This drug had been shown to reverse MDR \textit{in vitro} and \textit{in vivo} by acting on both P-gp and MRP1 (91, 92) (Table 8.3). The phase I studies of this compound have shown an acceptable toxicity together with an absence of effect on DOX pharmacokinetics but with a reduction in the clearance of paclitaxel (93). An increase in 99mTc-sestamibi hepatic uptake and retention was observed in all patients, bringing good arguments for further evaluation of biricodar.

Elacridar (GG918) (Fig. 8.2 and Table 8.3) was selected by theoretical considerations of characteristics of its structure. It is probably one of the most active compounds \textit{in vitro} (40, 41). It has also been shown to be active against another ABC pump, BCRP (or MXR) (Table 8.3) (94), which is especially expressed in leukemias. However, it is not active on MRP1. A phase I study has been completed showing no major pharmacokinetic interaction with DOX (95), but no phase II study has been undertaken with the aim of reversing
MODULATION OF P-gp ACTIVITY

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However, its use as an enhancer of intestinal uptake of oral paclitaxel or topotecan might be an interesting clinical application (96, 97).

Zosuquidar (LY335979) (Fig. 8.2), a difluorocyclopropyldibenzo[23]sudene derivative, is also active in the nanomolar range for inhibition of P-gp in vitro and in vivo (98). It was developed for its high affinity for P-gp and appears devoid of other pharmacological properties on MRP1- or BCRP-mediated drug resistance (43). This modulator has been recently shown to restore drug sensitivity in P-gp expressing AML (44). Phase I results were recently reported showing some risk of neurotoxicity at a high dosage and no pharmacokinetic interaction with DOX (99). Zosuquidar has been evaluated in phase III trials in AML as a first-line therapy in combination with chemotherapy with daunorubicin and cytarabine. Data reported by Gerrard et al. have shown that Zosuquidar is a specific inhibitor of P-gp efflux and can be given safely to patients with AML in combination with induction doses of conventional cytotoxic drugs (100).

Tariquidar (XR9576) (Fig. 8.2 and Table 8.3), an anthranilic acid derivative, is also active in the nanomolar range and is also devoid of pharmacokinetic interactions with paclitaxel (101). A phase I study in healthy volunteers has shown activity on rhodamine 123 uptake by P-gp expressing lymphocytes and without toxic symptoms (102). Tariquidar showed limited clinical activity to restore sensitivity to anthracycline or taxane chemotherapy in advanced breast carcinoma (103).

8.3.5. Immunization with Synthetic P-gp-Derived Peptides

In order to overcome MDR, several approaches using antibodies specific to P-gp have been reported. The monoclonal antibody MRK16 has been shown to enhance intracellular accumulation of anticancer drugs and to reverse MDR in a model of transgenic mice whose bone marrow cells express the mdr1 gene (104, 105). Pawlak-Roblin et al. have previously shown that

<table>
<thead>
<tr>
<th>Compound</th>
<th>P-gp</th>
<th>MRP1</th>
<th>BCRP</th>
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<tbody>
<tr>
<td>Verapamil</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Quinine, quinidine</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>PSC833 (Valspodar)</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Bircodar (VX-710)</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>S9788</td>
<td>+</td>
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<td>Elacridar (GG918)</td>
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<td>Zosuquidar (LY335979)</td>
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<td>−</td>
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<tr>
<td>Tariquidar (XR9576)</td>
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MDR. However, its use as an enhancer of intestinal uptake of oral paclitaxel or topotecan might be an interesting clinical application (96, 97).

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liposome-grafted palmitoylated peptides mimicking the external-loops 1, 2, and 4 of the murine P-gp reconstituted elicited specific antibodies that inhibited in vitro P-gp activity in multidrug resistant leukemia P388 cells (106).

Using synthetic palmitoylated peptides reconstituted in liposomes and aluminium hydroxide, Perrin et al. have shown that the highest immunoglobulin level was observed after the third immunization, and the immune response against lipopeptides was still detected more than 200d after immunizations (107). Immunocytochemical studies revealed that these antibodies were specific for P-gp. When incubated with P-gp-expressing MDR cell lines, serum from immunized mice restored sensitivity to either DOX or vinblastine or had no effect in a cell type-specific manner, suggesting that several mechanisms may occur in the establishment of the MDR phenotype.

In a recent work, synthetic peptides corresponding to fragments from extracellular loops 1, 2, and 4 of the murine P-gp were coupled to polyethylene glycol-distearoylphosphatidylethanolamine and inserted into empty or monophosphoryl lipid A-containing liposomes. This formulation elicited specific antibodies that blocked P-gp-mediated efflux of DOX, resulting in increased intracellular drug accumulation and subsequent potentiation of the cytotoxic effect of DOX in multidrug-resistant P388 cells (108). In vivo activity of the elicited antibodies, in combination with chemotherapy, showed that the immunization with extracellular loops 1 induced an increase of mean survival time of 20d compared to the control, which corresponds to an improvement of 83%. Mice that did not respond to the immunizations died early after injection of tumor cells. The other mice, which developed an immune response against the peptides, died between days 30 and 63 after tumor inoculation. In the group immunized with extracellular loops 2, mean survival was 27 days and mice died between days 22 and 31 (108).

Overall, these results suggest that this approach can modulate P-gp activity by blocking drug efflux and may have clinical relevance as an alternative strategy to toxic modulators in drug-resistant cancer therapy (109).

8.3.6. Other Alternative Approaches for Targeting P-gp

Using technologies that enable the targeted regulation of genes, antisense oligonucleotides, hammerhead ribozymes, and short-interfering RNA has produced mixed results. Sufficient downregulation of P-gp has proved difficult to attain, and the safe delivery of constructs to cancer cells in vivo remains a challenge (110, 111). However, transcriptional repression is a promising new strategy that is not only highly specific but also enables the prevention of P-gp expression during the progression of disease (33).

Several novel anticancer drugs are exported by ABC transporters. Moreover, a significant portion of the compounds in the drug development studies are substrates of ABC transporters and particularly P-gp (87, 112). Epothilones are novel microtubule-targeting agents with a paclitaxel-like mechanism of action and are not recognized by P-gp, providing proof of the concept that
new classes of anticancer agents that do not interact with the multidrug transporters can be developed to improve response to therapy (33). Another attractive solution would be to chemically modify the susceptibility of anticancer drugs to being transported while retaining antineoplastic activity. Although such modifications frequently decrease the bioavailability or efficacy of drugs, some new agents have been developed using this approach (113).

The apparent circumvention by increasing the rate of influx of anticancer drugs can be achieved by increasing the lipophilicity of compounds. For example, highly lipophilic anthracycline analogues (114), such as annamycin and idarubicin, were shown to elicit a high remission rate in P-gp-positive AML cases with primary resistance to chemotherapy (115). The efficacy of these drugs is currently being evaluated in other trials (116).

8.4. CONCLUSION

A number of lessons have been learned from the evolution of the field of MDR. A very recent study has demonstrated well that prediction of drug sensitivity and resistance can be accessed by profiling ABC transporter genes in cancer cells (104). However, the reversal of MDR mediated by P-gp by small molecules has not yet reached the level of routine clinical applications. The future of the potential therapeutic area remains uncertain. This is not for lack of molecules, since hundreds of compounds have been selected or designed with comprehensive studies on structure–activity relationships in several chemical families. Rather, the reason for this failure originates from the inadequate design of the clinical trials. The pharmacological and toxicological properties of MDR modulators should have been taken into consideration with those of anticancer drugs in terms of the benefit-risk ratio of combination.

REFERENCES


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REVERSAL AGENTS OF MULTIDRUG RESISTANCE MEDIATED BY MULTIDRUG RESISTANCE-ASSOCIATED PROTEINS (MRPs)

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9.1. INTRODUCTION

The ATP-binding cassette (ABC) superfamily of proteins contains a number of adenosine triphosphate (ATP)-driven transporters that pump various drugs or metabolites. These transporters are characterized by the presence of two ATP-binding motifs named Walker A and Walker B, as well as the signature motif ALSGGQ. The human ABCC subfamily (MRP-ABC) of transporters consists of 12 members, ABCC1 through ABCC12. The ABCC comprises the multidrug resistance-associated protein (MRP) class (ABCC1–6, 10–12), the cystic fibrosis transmembrane conductance regulator (CFTR) (ABCC7), and the ATP-dependent sulfonylurea receptors (ABCC8, ABCC9).

The MRPs contain two or three membrane-spanning domains (MSD0, MSD1, and MSD2) and two nucleotide-binding domains (NBDs). The MSDs are made of five (MSD0) or six (MSD1 and MSD2) transmembrane segments. One NBD is located near the C-terminus, and the other one is positioned between MSD1 and MSD2. Based on the number of spanning domains, two groups can be described. The first group consists of transporters which contain three spanning domains (MSD0, MSD1, and MSD2) and includes ABCC1 (MRP1), ABCC2 (MRP2), ABCC3 (MRP3), ABCC6 (MRP6), and ABCC10 (MRP7), and the second group consists of the transporters harboring two MSDs (MSD1 and MSD2), namely, ABCC4 (MRP4), ABCC5 (MRP5), ABCC11 (MRP8), and ABCC12 (MRP9).

The importance of the role of these MRPs in drug resistance is more or less potent and can be linked to each individual transporter’s relationship to the glutathione (GSH), as most of the drugs which are extruded by MRPs must previously be linked to GSH (see Part I, Chapter 2).

9.2. SUBSTRATES OR DRUGS EXTRUDED BY MULTIDRUG RESISTANCE-ASSOCIATED PROTEINS

9.2.1. Substrates of MRPs Containing Three MSDs

9.2.1.1. MRP1 (ABCC1). The first ABCC1 physiological substrate to be identified was the conjugated cysteinyl leukotriene (LTC4). ABCC1 was also shown to mediate ATP-dependent transport of anionic GSH, glucuronide, and sulfate conjugates. Using membrane vesicle preparations, it has been demonstrated that, in various conditions, transport activity of ABCC1 is closely related to GSH levels (Fig. 9.1) (1, 2). Following this requirement of GSH for MRP transport activities, the use of buthionine sulfoximine (an agent able to block GSH synthesis through γ-glutamyl cysteine synthetase inhibition) has been described to decrease MRP-mediated chemoresistance phenomena. MRP1 is involved in chemoresistance to:

(1) Nonconjugated antineoplastics such as anthracyclines, epipodophylo-toxins, vinca alkaloids, camptothecins and derivatives, antimitabolites,
and antiandrogens; however, concerning the taxanes, the literature is ambiguous.

(2) Conjugated antineoplastics such as alkylating agents and anthracyclines conjugated to GSH through GSH S-transferase enzymes, and etoposide which becomes conjugated to glucuronic acid during physiological detoxification reactions.

The broad diversity of MRP1 substrates is represented in Table 9.1 (3). MRP2 is a canalicular multispecific organic anion transporter, present in human liver cells in an apical localization. This protein is able to export a wide variety of both conjugated and unconjugated anionic compounds into the bile (4, 5). The substrates of MRP2 are quite similar to those of MRP1, including LTC4, bilirubin glucuronides, and conjugated drug metabolites, but sometimes with a lower affinity (6). Unlike MRP1, however, MRP2 confers chemoresistance to cisplatin. An overexpression of MRP2 was demonstrated in cisplatin-resistant cell lines as well as in patients treated for colorectal cancer by cisplatin (6). The diversity of MRP2 substrates is also represented in Table 9.1.

9.2.1.2. MRP3 (ABCC3). MRP3 is an organic anion transporter located in a basolateral position as MRP1. Even if this protein has 58% amino acid homology with MRP1, the substrate spectrum especially for antineoplastic drugs is more limited. In particular, MRP3 cannot transport GSH, and this could explain why overexpression of MRP3 does not confer resistance to anticancer agents which are cotransported with GSH (7).

Overexpression of MRP3 confers resistance to etoposide, teniposide, vincristine, and methotrexate but in a less potent way than that of MRP1 and MRP2 (8). Glucuronide and sulfate conjugates of bile salts are substrates of MRP3 as are monovalent bile salts, including glycocholate (9).

9.2.1.3. MRP6 (ABCC6). MRP6 was initially cloned from rat liver (10). Murine MRP6 shows more than 78% amino acid homology with human MRP6. Transfection of MRP6 in Chinese hamster ovary cells confers resistance to various anticancer agents such as doxorubicin, daunorubicin, etoposide,
<table>
<thead>
<tr>
<th>MRP1</th>
<th>MRP2</th>
<th>MRP3</th>
<th>MRP6</th>
<th>MRP7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukotrienes C4 (LTC4), D4 (LTD4) and E4 (LTE4)</td>
<td>Leukotrienes C4 (LTC4), D4 (LTD4) and E4 (LTE4)</td>
<td>Monoanionic and conjugated bile acids; etoposide; methotrexate, teniposide, vincristine</td>
<td>Small peptides (BQ123); glutathione conjugates</td>
<td>Estradiol-17β-glucuronide; leukotriene C4; docetaxel</td>
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<tr>
<td>GSH (-GS) conjugates</td>
<td>GSH (-GS) conjugates</td>
<td>Dinitrophenyl-GS</td>
<td>Prostaglandin A, GS</td>
<td>Smothe 3-sulfate</td>
</tr>
<tr>
<td>Ataxoloxin B1 epoxide-GS</td>
<td>Dinitrophenyl-GS</td>
<td>Ethacrylic acid-GS</td>
<td>Ethacrylic acid-GS</td>
<td>Metal (As, Bi, Cd, Cu, Zn,..) GS</td>
</tr>
<tr>
<td>Dinitrophenyl-GS</td>
<td>Prostaglandin A, GS</td>
<td>Bromosulphalein-GS</td>
<td>Melphanal-GS</td>
<td>Oxidized glutathione (GSSG)</td>
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<td>Prostaglandin A, GS</td>
<td>Ethacrylic acid-GS</td>
<td>Bromosulphalein-GS</td>
<td>GSSG (Oxidized glutathione)</td>
<td>Metal (As, Bi, Cd, Cu, Zn,..) GS</td>
</tr>
<tr>
<td>Ethacrylic acid-GS</td>
<td>Prostaglandin A, GS</td>
<td>GSSG (Oxidized glutathione)</td>
<td>Metal (As, Bi, Cd, Cu, Zn,..) GS</td>
<td>Metal (As, Bi, Cd, Cu, Zn,..) GS</td>
</tr>
<tr>
<td>Melphanal-GS</td>
<td>GSSG (Oxidized glutathione)</td>
<td>Metal (As, Bi, Cd, Cu, Zn,..) GS</td>
<td>Metal (As, Bi, Cd, Cu, Zn,..) GS</td>
<td>Metal (As, Bi, Cd, Cu, Zn,..) GS</td>
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<td>Glucuronide (-G) conjugates</td>
<td>Glucuronide (-G) conjugates</td>
<td>Glucuronide (-G) conjugates</td>
<td>Glucuronide (-G) conjugates</td>
<td>Glucuronide (-G) conjugates</td>
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<td>Bilirubin-G (mono and bis)</td>
<td>Bilirubin-G (mono and bis)</td>
<td>Bilirubin-G (mono and bis)</td>
<td>Bilirubin-G (mono and bis)</td>
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<td>Estradiol 17β-D-G</td>
<td>Estradiol 17β-D-G</td>
<td>Estradiol 17β-D-G</td>
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<td>Etoposide-G</td>
<td>Triiodothyronine-G</td>
<td>Triiodothyronine-G</td>
<td>Triiodothyronine-G</td>
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<td>Hydroxycholeolate-G</td>
<td>SN-38-G</td>
<td>SN-38-G</td>
<td>SN-38-G</td>
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<td>Sulfate conjugates</td>
<td>Sulfate conjugates</td>
<td>Sulfate conjugates</td>
<td>Sulfate conjugates</td>
<td>Sulfate conjugates</td>
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<tr>
<td>Estone 3-sulfate</td>
<td>Acetaminophen-G</td>
<td>Acetaminophen-G</td>
<td>Acetaminophen-G</td>
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<td>Taurocholate 3-sulfate</td>
<td>Dicoloten-G</td>
<td>Dicoloten-G</td>
<td>Dicoloten-G</td>
<td>Dicoloten-G</td>
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<tr>
<td>Dehydroepiandrosterone 3-sulfate</td>
<td>Indomethacin-G</td>
<td>Indomethacin-G</td>
<td>Indomethacin-G</td>
<td>Indomethacin-G</td>
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<tr>
<td>Anticancer drugs</td>
<td>Cholate-3-O-G</td>
<td>Cholate-3-O-G</td>
<td>Cholate-3-O-G</td>
<td>Cholate-3-O-G</td>
</tr>
<tr>
<td>Anthracycline antibiotics (daunorubicin, doxorubicin)</td>
<td>Lithochocholate-3-O-G</td>
<td>Lithochocholate-3-O-G</td>
<td>Lithochocholate-3-O-G</td>
<td>Lithochocholate-3-O-G</td>
</tr>
<tr>
<td>Camptothecins (topotecan, irinotecan, SN-38, methotrexate)</td>
<td>Chenodeoxychocholate-3-O-G</td>
<td>Chenodeoxychocholate-3-O-G</td>
<td>Chenodeoxychocholate-3-O-G</td>
<td>Chenodeoxychocholate-3-O-G</td>
</tr>
<tr>
<td>Epipodophyllotoxins</td>
<td>Sulfate conjugates</td>
<td>Sulfate conjugates</td>
<td>Sulfate conjugates</td>
<td>Sulfate conjugates</td>
</tr>
<tr>
<td>Imatinib, vinca alkaloids</td>
<td>Nordochocholate-3-sulfate</td>
<td>Nordochocholate-3-sulfate</td>
<td>Nordochocholate-3-sulfate</td>
<td>Nordochocholate-3-sulfate</td>
</tr>
<tr>
<td>Other</td>
<td>Anticancer drugs</td>
<td>Anticancer drugs</td>
<td>Anticancer drugs</td>
<td>Anticancer drugs</td>
</tr>
<tr>
<td>Folate, p-Aminohippurate</td>
<td>Cisplatin for MRP1</td>
<td>Cisplatin for MRP1</td>
<td>Cisplatin for MRP1</td>
<td>Cisplatin for MRP1</td>
</tr>
<tr>
<td>Fluorescent probes</td>
<td>Other</td>
<td>Other</td>
<td>Other</td>
<td>Other</td>
</tr>
<tr>
<td>Calcein, fluo-3, BCECF, SNARF</td>
<td>Pravastatin, amicillin</td>
<td>Pravastatin, amicillin</td>
<td>Pravastatin, amicillin</td>
<td>Pravastatin, amicillin</td>
</tr>
</tbody>
</table>

Note: Adapted from References 3, 17–21. SNARF = seminaphthorhodafloures.
cisplatin, but not to vinblastine or vincristine. This pump is also able to extrude GSH conjugates such as LTC4 and DNP-SG (2,4-dinitrophenyl-S-glutathione), but not glucuronate conjugates, BQ123 (cyclopentapeptide) or methotrexate (11).

Evaluation of MRP6 expression in various human tumor specimens did not show any overexpression, suggesting the minor role of this protein in tumor multidrug resistance (MDR) (12, 13).

9.2.1.4. MRP7 (ABCC10). MRP7 exhibits a membrane topology similar to that of MRP1, MRP2, MRP3, MRP6 and is expressed in various tissues such as colon, testis, and skin (14). Interestingly, this protein essentially confers resistance to taxanes, to docetaxel, and to a lesser extent, to paclitaxel (15). MRP7 can also extrude vinca alkaloids but not methotrexate or DNP-SG, monovalent bile salts, or cyclic nucleotides (16). At present, there is no evidence that MRP7 is able to transport GSH.

9.2.2. Substrates of MRPs Containing Two MSDs

9.2.2.1. MRP4 (ABCC4). MRP4 is expressed in various tissues, but a marked expression was shown in prostate and kidney (22, 23). The involvement of MRP4 in cancer resistance was evaluated essentially through transfected cell line models. Chen et al. (24) have demonstrated that this protein is able to confer resistance to purine analogs, with an increase of 6-mercaptopurine and 6-thioguanine efflux. In 2002, the same authors showed an MRP4-mediated transport of methotrexate. MRP4 can also play a role in the efflux of acid $N^5$-formyl tetrahydropholic folic acid (leucovorin) but not that of polyglutamated methotrexate metabolites. Another anticancer agent demonstrated to be effluxed by MRP4 is cisplatin. Following the establishment of a small cell lung carcinoma cisplatin-resistant cell line, Savaraj et al. (25) demonstrated a decrease of DNA-platinum complexes in the resistant cell line compared to the parental form. The authors also showed that in a small number of lung cancer patients, MRP4 expression increased following cisplatin treatment. Recently, Leggas et al. (26) demonstrated an increase of topotecan in brain tissue in MRP4-deficient mice, pointing to a potential role of this protein in the blood–brain barrier.

MRP4 is also involved in extrusion of GSH and of various compounds that are not anticancer drugs (Table 9.2).

9.2.2.2. MRP5 (ABCC5). MRP5 is ubiquitously expressed in the highest levels in skeletal and heart muscles and lung and brain tissues (22, 27, 28). The profile of drugs extruded by MRP5 is very similar to that of MRP4. Cyclic nucleosides are extruded by MRP5, but the implication of GSH in this extrusion is not clearly established (29, 30). MRP5 can also confer resistance to the antiviral agent 9-(2-phosphomethoxyethyl) adenine (PMEA) and to some purine analogs.
9.2.2.3. MRP8 (ABCC11). MRP8 is the third MRP with two MSDs. Structure homology and resistance profile are quite similar to those of MRP4 and MRP5. MRP8 protein was found in breast, testis, brain, liver, lung, and kidney (31–33). This protein can extrude pyrimidine analogs (5′-fluoro-5′-deoxyuridine, 5′-fluorouracil, 5′-fluoro-2′-deoxyuridine), a purine analog (PMEA), and cyclic analogs (34).

MRP8 is also able to mediate transport of E217bG, dehydroepiandrosterone sulfate, LTC4, taurocholate, and glycocholate, but not prostaglandin E1 or E2 (35, 36).

9.2.2.4. MRP9 (ABCC12). Concerning MRP9 expression, some discrepancies that were related to multiple transcript variants have been shown. Expression of MRP9 was shown in testis, brain, and breast tumors (33, 37, 38). Physiological roles as well as substrates for MRP9 remain to be described.

9.3. METHODS TO EVALUATE INHIBITORS OF MRP ACTIVITY

In contrast to P-glycoprotein (P-gp) or breast cancer resistance protein (BCRP), MRPs consist of a family of nine proteins. As shown above, the substrates are various, and GSH sometimes plays an important role in the transport mediated by these transporters. The methods used to evaluate MRP functions have to take into account the great diversity of this class of proteins, as well as the strategy used to identify potential inhibitors (identification of specific or of broad range inhibitors), knowing that the different MRPs are often expressed in a ubiquitous way.

Among the classical methods used to evaluate MRP activity and the spectrum of drugs exported by these proteins are the plasma membrane vesicles obtained from cells transfected by the genome of one subclass of MRPs or from cells expressing one or more MRPs (Table 9.3) (11, 45). This method also

|**TABLE 9.2. Known Substrates of MRP4, MRP5, MRP8, and MRP9** |
|-----------------|-----------------|-----------------|-----------------|
|**MRP4** | **MRP5** | **MRP8** | **MRP9** |
|Cyclic nucleotides (cAMP, cGMP) | Cyclic nucleotides (cAMP, cGMP) | Nucleotide analogs (PMEA) | N.A. |
|Nucleotide analogs (PMEA, azidothymidine-monophosphate) | Nucleotide analogs (PMEA, stavudine-monophosphate) | Fluoropyrimidines | |
|Prostaglandins methotrexate | | | |

*Note:* Adapted from references 15, 16, 30, 37, 39–44.
cAMP = cyclic adenosin monophosphate; cGMP = cyclic guanosin monophosphate; DHEAS = dehydroepiandrosterone; PMEA = phosphonylmethoxy adenine; N.A. = not applicable.
<table>
<thead>
<tr>
<th>Type of Model</th>
<th>Type of Assay</th>
<th>Description</th>
<th>Applications</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane-based</td>
<td>ATPase assays</td>
<td>Measurement of ATPase activity in cell membrane preparations based on the amount of inorganic phosphate (Pi) released from ATP in the presence or absence of vanadate</td>
<td>Evaluates the interaction of compounds with different transporters, allowing identification of MRP ligands (51)</td>
<td>Low sensitivity. Inhibition of hydrolysis can be produced by poor substrates or pure inhibitors, and lack of obvious effect does not imply lack of interaction.</td>
</tr>
<tr>
<td>Photolabeling</td>
<td></td>
<td>Nucleotide binding and hydrolysis properties were investigated using photoactive ATP analogs such as [α-32P]8-azidoATP for MRP1-MRP4 (45) or [(125)I]-IAARh123 for MRP1 and MRP6 (52)</td>
<td></td>
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<tr>
<td>Membrane vesicular transport</td>
<td></td>
<td>Determination of the role of GSH in transport. Evaluation of various compounds on the uptake of substrates more or less specific for each MRP. For example: MRP1 has a high affinity for dinitrophenyl-S-glutathione (DNP-SG) and a low affinity for cGMP transporters, whereas it is the opposite for MRP4.</td>
<td></td>
<td>Necessity to take care of the lipophilicity which can induce nonspecific binding</td>
</tr>
</tbody>
</table>
### TABLE 9.3. Continued

<table>
<thead>
<tr>
<th>Type of Model</th>
<th>Type of Assay</th>
<th>Description</th>
<th>Applications</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellular assays</td>
<td>Uptake</td>
<td>Direct or indirect evaluation of cellular accumulation of compounds, depending on MRP expression</td>
<td>Direct evaluation by flow cytometry fluorescence measurement of the dye efflux (calcein-AM for MRP1 and BCECF for MRP4) (45) carboxydichlorofluoresceine for MRP5 (53) Indirect evaluation by cell cytotoxicity assays Cell lysates and quantification of drugs or metabolites by HPLC.</td>
<td>Limitations: A major limitation is due to the low specificity of the substrates toward a specific MRP, requiring transfected models to evaluate this specificity (or lack thereof). Another limitation is brought by the sensitivity and wavelength of the methods used to quantify the fluorescence (spectrofluorimeter, flow cytometer). Cytotoxicity evaluation is limited by the proper antiproliferative compounds. This method does not allow or allows only partially to study the role of glutathione in the different MRPs.</td>
</tr>
<tr>
<td>In vivo studies</td>
<td>Knockout (MRP&lt;sup&gt;−/−&lt;/sup&gt;) mice</td>
<td>Evaluation of an oriented transport on epithelial cells A substrate may have a directional cellular transport with different flux from apical-to-basolateral and basolateral-to-apical directions An inhibitor can diminish the directional transport of the substrate Due to an important crossover in the substrates of MRPs, the need of knockout mouse models is of interest especially if they are associated to the measurement of other transporters</td>
<td>Caution needs to be taken when using these transgenic animal models in the interpretation of the results because deletion of one transporter can cause upregulation of another.</td>
<td></td>
</tr>
</tbody>
</table>

BCECF = Z,7′-bis(2-carboxyethyl)-5-(6)-carboxyfluorescein; BCECF-AM = BCECF-acetoxyethyl ester; BeFx = beryllium fluoride; calcein-AM = calcein-acetoxyethyl ester.
allows for determination of whether GSH or ATP is necessary for protein activity. In the same kind of experiments, ATPase activity and photolabeling have been widely used. As MRPs contain two ATP-binding domains and possess an ATPase activity, it is of interest to determine whether efflux is associated with inorganic phosphate (Pi) release and to determine whether phosphodiesterase modulators are able to act on their activity. Flow cytometry has also been largely used to evaluate MRP-mediated efflux, using especially the fluorescent probe calcein-acetoxyethyl (AM) to evaluate MRP1- and MRP2-mediated transport. This dye is a nonfluorescent and lipophilic AM ester of calcein that diffuses through the plasma membrane into the cells. In the cytosol, calcein-AM is metabolized to calcein by an esterase, which is fluorescent and hydrophilic and not extruded by P-gp (46, 47). For MRP1, other dyes have also been used, like daunorubicin, carboxyfluorescein diacetate, and 2′,7′-bis-(carboxypropyl)-5(6)-carboxyfluorescein coupled with AM ester. These last two dyes have the same metabolism profile as calcein-AM and can be measured by flow cytometry. This method is easily reliable and allows a rapid selection of potent inhibitors of MRPs. Cell viability assays have also been widely used for MRPs, which are able to extrude anticancer drugs to determine the action of modulators. The use of knockout models allows for determination of the physiological role of MRPs (48, 49), as well as the interaction between the drug and the transporter (50). However, in this kind of model, it is necessary to take care that other ABC proteins cannot supply for the invalidated MRP.

9.4. MRP MODULATORS

9.4.1. Reversion of MRP1 Protein

Contrary to what is observed for P-gp, few compounds have been shown to modulate drug efflux mediated by MRP1. The structural and functional homology shared by P-gp and MRP1 led to the screening of the most reported P-gp-mediated MDR modulators for their effect on MRP1. Unfortunately, most P-gp modulators were inactive on MRP1, even if this was somehow predictable because P-gp substrates are hydrophobic molecules whereas MRP1 recognizes hydrophilic substrates. There were exceptions where P-gp and MRP1 could be inhibited by the same molecule, such as the quinoline derivative MS-209, which went through clinical trials as a dual inhibitor of both P-gp and MRP1 (55, 56).

In order to give a clear picture of scaffolds governing MRP1 inhibition, we have classified them according to their structure. Attempts were made to exclusively shed light on potent and specific MRP1 inhibitors.

9.4.1.1. Agosterol A and Analogs. Agosterol A (Fig. 9.2) is a naturally occurring sterol isolated from a marine sponge and for which an MRP1-
mediated MDR reversing activity was reported (57). The total synthesis of agosterol A as well as of several analogs has been described, and some of them were found to be powerful modulators (29, 58–60). The structure–activity relationship studies conducted on KB-CV60 cells, which overexpress MRP1, concluded that 3,4,6-acetoxy groups as well as hydroxyl groups at positions 11 and 22 were important for the reversing activity (58). In addition, agosterol A was found to competitively inhibit the transport of LTC4 by MRP1. A photoaffinity analog of agosterol A was obtained by attaching an azido moiety (photoactivatable) to the 11-hydroxy group of agosterol A with a C-4 linker followed by an iodination with \([^{125}\text{I}]\)NaI and chloramine T. The resulting azidophenyl photoaffinity analog (Fig. 9.2) was shown to bind to the C-terminal (C<sub>932–1531</sub>) half of MRP1 in a GSH-dependent manner (61).

9.4.1.2. Verapamil Derivatives. The calcium channel blocker verapamil is generally considered as a reference for P-gp inhibition (Part IV, Chapter 8). In contrast, verapamil has been reported in most instances to be only weakly effective, if effective at all, in restoring drug sensitivity in MRP1-overexpressing cells (62, 63). The basis for this apparently variable effect of verapamil on MRP1-related resistance is unknown. However, whereas verapamil alone poorly inhibited LTC4 transport by MRP1, its inhibition in the presence of GSH was enhanced more than 20-fold on MRP1-enriched membrane vesicles (64–66). Structure–activity relationships on sulfur-containing verapamil derivatives showed that in the presence of GSH, the more lipophilic dithiane compounds were the best inhibitors of LTC4 transport (67).

The effects of verapamil and its iodinated derivative (Fig. 9.3) were investigated on MRP1-transfected BHK21 cells resistant to vincristine. Unexpectedly, both derivatives behaved as apoptogens in these MRP1-expressing cells while no death was observed in the parental cells under the same conditions. This apoptosis was triggered through stimulation of MRP1-mediated GSH extrusion, and cells were committed to die as they became deprived of intracellular GSH (68). The di-iodinated derivatives behaved as powerful agents with a 10-fold higher potency than verapamil (IC<sub>50</sub> ~ 0.1 μM) (69). This newly
described mechanism of apoptosis induction in MDR cells may represent a novel approach for the selective treatment of MRP1-positive tumors.

9.4.1.3. **Flavonoid Derivatives.** A growing interest concerning the use of dietary flavonoids as MRP1 inhibitors is observed. Structure–activity relationships have been drawn from inhibition of drug efflux activity with either LTC4 or DNP-SG, two GSH-conjugated substrates (Table 9.4). Flavones (luteolin and apigenin) and flavonols (quercetin, kaempferol, and myricetin) were more efficient than isoflavones (genistein) and flavanones/flavanonols (naringenin, taxifolin). The latter compounds were even less active upon glycosylation. This draws forward the role of the planar ring structure, requiring a 2,3-double bond, and the importance of hydroxyl groups at both positions 3′ and 4′ (70). Quantitative structure–activity relationship studies have determined that the total number of methoxylated moieties, the total number of hydroxyl groups, and the dihedral angle between the B- and C-rings are the major elements governing the inhibition of MRP1 (71).

Interestingly, the lower potency of prenylated dihydrosilybin (DHS) derivatives was improved in the presence of GSH, by contrast to DHS, suggesting that these derivatives might be cotransported with GSH whereas DHS might not. This was indeed confirmed by chemosensitization of cell growth to vincristine (72). Apigenin was more efficient than quercetin in stimulating the transport of GSH (73), and slightly different structure–activity relationships of inhibitory flavonoids were observed when studying the efflux of two non-conjugated drugs, daunomycin and vinblastine (74), in an MRP1-expressing human pancreatic adenocarcinoma cell line (75). This is consistent with variations observed in the transport mechanism of different drug substrates related to the involvement of a large polyspecific site.

9.4.1.4. **Raloxifene-Based and Isoxazole-Based Compounds.** Scientists at Eli Lilly have used the nonsteroidal estrogen receptor mixed agonist/antagonist raloxifene as a pharmacophore model to conceive selective modulators of MRP1-mediated MDR (Fig. 9.4). Among raloxifene analogs, compounds LY17018 and LY329146 were reported to reverse doxorubicin resistance in the MRP1-expressing HL60/ADR cell line (76, 77). Furthermore, LY329146 was found to inhibit LTC4 uptake into membrane vesicles, indicating that the
TABLE 9.4. Structure and Activity of Known Flavonoids as Inhibitors of MRP1

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>IC₅₀ (µM)</th>
<th>LTC4 Transport</th>
<th>DNP-SG Efflux</th>
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<tr>
<td></td>
<td></td>
<td>-GSH +GSH</td>
<td>-GSH +GSH</td>
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<tr>
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<td>7–12</td>
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<tr>
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<td>7–12</td>
<td>1.3</td>
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<tr>
<td>Kaempferol 5,7,4′-OH</td>
<td>7–12</td>
<td>7–12</td>
<td>4.8</td>
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<tr>
<td>Naringenin 5,7,4′-OH</td>
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<td>Apigenin 5,7,4′-OH</td>
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<tr>
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<td>Luteolin 5,7,3′,4′-OH</td>
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<tr>
<td>Taxifolin 3,5,7,3′,4′-OH</td>
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<tr>
<td>Galangin 3,5,7-OH</td>
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<tr>
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<td>1.1</td>
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<td>8-prenyl-DHS</td>
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<td>7.7</td>
<td>4.1</td>
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<td>6-geranyl-DHS</td>
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<tr>
<td>Silybin 3,5,7-OH, 3′,4′-0-monolignol flavononol</td>
<td>16</td>
<td>4.7</td>
<td></td>
</tr>
</tbody>
</table>

Note: Adapted from References 70–74.
LTC4 = leukotriene C₄; DNP-SG: = 2,4-dinitrophenyl glutathione; DHS = dihydrosilybin.

FIGURE 9.4. Structures of raloxifene-based and isoxazole-based inhibitors.
modulator indeed interacts with the MRP1 transporter. Since estradiol 17-β-D-glucuronide is a known MRP1 substrate, the three-dimensional structure of LY117018 was overlayed with estradiol and showed that the 6 and 4'-hydroxyl groups in LY117018 were respectively comparable to the 3 and 17-β-hydroxyl groups in estradiol. Therefore, it was hypothesized that the higher activity of LY329146 over LY117018 was due to the contribution of the bis-(methylsulphonyl) group in LY329146 that overlapped the glucuronide moiety of the substrate.

Based on Eli Lilly’s results, there have been several recent reports dealing with the synthesis and structure–activity relationships of indolopyrimidines and pyrrolopyrimidines as selective modulators of MRP1-mediated MDR. The best derivative exhibited high efficiency of inhibition of MRP1, with half-maximal values in the range of 0.02–0.2 µM toward daunomycin cellular efflux and cell growth sensitization to doxorubicin. Interestingly, some of them were rather specific for MRP1 over P-gp and displayed limited interactions with enzymes involved in drug metabolism such as cytochrome P450s. A small number of compounds exhibited acceptable pharmacokinetics, and appeared indeed active in vivo as shown by trials on xenografted mice models where they potentiated the cytotoxic effects of vincristine (78, 79).

Through screening and chemical modifications studies, a series of isoxazole derivatives were reported by Eli Lilly (80–85). Among them, LY402913 was identified as a potent MRP1-mediated MDR modulator (86), by potentiating doxorubicin cytotoxicity in vitro and enhancing the antiproliferative activity of vincristine in vivo with a high selectivity for MRP1 over P-gp (Fig. 9.6). In addition, it was evidenced that the modulator interacts with MRP1, as shown by its inhibition of MRP1-mediated LTC4 transport into membrane vesicles. The photoactivatable LY475776 derivative binds in a GSH-dependent manner to the C-terminal part of MRP1 containing TM17 (87).

9.4.1.5. Quinazolinones, Quinoxalinones Derivatives. Quinazolinones were first reported as dual inhibitors of P-gp and MRP1 (88). By using quinazolinone 1 as the core structure, structure–activity relationships were examined at the N-3 and C-2 positions and helped in the designs of selective MRP1 inhibitors (Fig. 9.5). A recent study reported by Smith and Lawrence has confirmed the successful use of quinazoline- and quinoxaline-derived molecules in the inhibition of MRP1-mediated drug-resistant cells. Some of the compounds disclosed are MRP1-specific. For example, quinoxalinone II (Fig. 9.5) inhibits MRP1 with an IC₅₀ of 0.9 µM versus 12.4 µM for P-gp and shows a very low cytotoxicity and a strong reversing activity (89).

9.4.1.6. Modulation by Peptides, Pseudopeptides, and Peptidomimetics. As pointed out previously, it is well established that MRP1 mediates MDR by effluxing drugs either conjugated to GSH or by cotransport of free GSH (without covalent bond between drug and GSH). Formation of GSH conjugates is catalyzed by GSH S-transferases (GST), and overexpression of GSTs has
often been associated with resistance to chemotherapeutic agents. Therefore, inhibitors of GST have been targeted, and two classes of MRP1 modulators have been investigated: (a) compounds which are able to mimic GSH and (b) GSH conjugates.

There are three types of compounds used to target GSTs. The first one concerns GSH-unrelated structures of drugs generally used in clinics. These include ethacrynic acid (90, 91), gossypol derivatives (92), or disulfiram (93). The main drawback of these drugs is their activity in other therapeutic areas, which makes them not useful for selective MDR treatment.

The second type of GST inhibitor consists of GSH analogs that can act as substrate competitors. Thus, in a 1999 study, O’Brien et al. (94) have described the MRP1-mediated MDR modulation by a GSH analog, named TER199 (Fig. 9.6) (95). The compound was obtained by substituting the glycine moiety of GSH with a phenyl glycine, benzylation of the thiol group, and ethyl esterification of both carboxylic acids. TER199 acts as a prodrug with facilitated membrane diffusion: After cellular uptake, the esters are hydrolyzed and carboxylic acid groups are thus generated. TER199 was found to bind to GST with an affinity 1,000-fold higher than GSH, potentiating the cytotoxicity of chlorambucil and improving melphalan activity (96).
The interaction of several GSH analogs with human MRP1 has been recently reported (Fig. 9.6) (97). These compounds were obtained by replacing either the γ-Glu of GSH by other amino acids (Gly, β-Asp, α-Glu) or the Gly unit by β-Ala or α-Glu with the aim of establishing the structural requirements of GSH to interact with MRP1. The ability of the series of GSH analogs to substitute for GSH in enhancing the transport of [3H]estrogen 3-sulfate conjugate indicated that γ-Glu substitution is harmful for the activity and that substitution of Gly is more tolerable since only a partial loss of activity was observed. Moreover, substitution of Cys with hydrophobic amino acids such as Leu or Phe resulted in more active peptides.

The third type of GST inhibitors is composed of GSH conjugates, including various compounds recently reviewed by Burg and Mulder (98). GST mimics were especially investigated (Fig. 9.7) (99). To synergize the activity of peptidomimetics, the cysteine sulfhydryl was conjugated to ethacrynic acid, based on the fact that GSH–ethacrynic acid conjugate is a good substrate for MRP1 and is known for its inhibitory potency of GST isoenzymes (Fig. 9.7A) (99, 100). In order to build up molecules that are resistant to peptidases, new compounds where the peptide bonds were replaced by N-methylamide (Fig. 9.7b), reduced
amide (Fig. 9.7c), and methylene linkage (Fig. 9.7d) were synthesized. Overall, it was found that the γ-Glu-Cys peptide bond is critical whereas the Cys-Gly amide is not essential. Replacement of the γ-Glu-Cys peptide bond (C-CO-N) by a urethane isostere (O-CO-N) led to an MRP1 competitive inhibitor (Fig. 9.7e). The resulting analog inhibited the efflux of calcein from ovarian carcinoma cells and partially restored the sensitivity of these cells to methotrexate (101).

Other series of MRP1-mediated MDR modulators based on GST inhibition have been reported by Burg et al. (102), including the GSH-conjugate analog containing a tetrazole carboxylate isostere at the glycine position (Fig. 9.7f) and a compound obtained by alkylating the thiol in order to increase membrane permeability. Yet, no higher activity was achieved.

The relevance of peptide-based compounds as inhibitors of MRP1 has been recently confirmed with a naturally occurring cyclic peptide named dendroamide A (Fig. 9.7), which was first isolated in 1996 from alga (*Stigonema dendroideum*) and synthesized in 2001 by Smith et al. (103, 104). Dendroamide A was shown to restore vincristine sensitivity in MRP1-overexpressing MCF-7/VP cells. The advantage of this hexapeptide is its cyclic nature and the absence of charges, making it penetrable through the cell membrane. In addition, the presence of oxazole and thiazole units in the structure is favorable for protection against protease action. Unspecific trimers of N-trialkylglycine were reported to act as potent inhibitors of MRP1 and P-gp with a potency that rivaled that of verapamil (105).

GSH and GSH-conjugate mimics are interesting compounds as supported by *in vitro* and *in vivo* data. Since P-gp does not require GSH for its function, these compounds are more likely to be MRP1 selective. However, the main drawback of these compounds might be their interference with the physiological role of GSH as a detoxifier of reactive oxygen by acting as a substrate of GSTs and GSH reductase (106).

By surveying the compounds reported so far as MRP1 inhibitors, we can distinguish two types of modulators: (a) compounds acting on GSH and GST pathways which are generally peptidomimetics and (b) compounds that do not hold any GSH framework and are thought to act independently from GSH and GST. Therefore, it is highly difficult to draw structure–activity relationship conclusions by comparing these two classes of compounds, which should in fact be treated separately. However, from a careful examination of compounds, which seem to be GSH/GST-independent acting agents, we can draw some structural requirements. The presence of at least two aromatic or/and heteroaromatic nuclei and two nitrogens (tertiary amines or imines) is frequently observed. The presence of polar groups such as hydroxyls, primary amines, and carboxylic acids is quite rare, whereas the presence of a carbonyl group is frequent, which leads to the assumption that these compounds are more hydrogen bond acceptors than donors.

At this stage, studies aimed at determining the potency of large series of compounds (belonging to the different classes described here) for MRP1
modulation with a single cell model are needed. Such studies would help to
define a topological description, to predict with acceptable accuracy the activity of untested compounds, and especially to design and select compounds to be tested as MRP1-mediated MDR modulators. Taking advantage of multidimensional quantitative structure–activity relationship studies would certainly be helpful for the design of potent and perhaps specific MRP1 inhibitors (107).

The binding site(s) and mechanism(s) of action of MRP1 inhibitors remains an unresolved issue. This is mainly due to the absence of X-ray structure for MRP1. The polyspecificity of MDR proteins may also contribute to the poor understanding of their mechanism of action.

The X-ray structures of two bacterial ABC transporters have been recently resolved. These are lipid A flipase MsbA (108) and vitamin B<sub>12</sub> importer BtuCD (109), for which significant differences in the modes of action have been reported, suggesting that ABC proteins involved in MDR may act differently. In recent studies, substantial differences between structural changes of P-gp and MRP1 during drug transport versus ATP hydrolysis cycle have been observed (110–112). Nevertheless, considerable efforts are being made to localize the precise binding sites of MRP1 modulators. Borst et al. (113) proposed a model for MRP1 with two drug-binding sites, one interacting with GSH (G-site) and the second one displaying high affinity for the drug (D-site). In a 2001 report, Ren et al. (61) localized the two sites in the L<sub>G</sub> loop and C-terminal half of MRP1, respectively. By using a series of MRP1 mutants, Daoud et al. have attempted to determine more precisely the location of binding sites and concluded that different ABC transporters may hold similar drug-binding domains (114). The use of various radioactive photoaffinity labels confirmed the existence of two drug-binding sites within MRP1 (115). All this molecular information may help in designing specific inhibitors that are able to function in vivo to reverse MRP1-mediated MDR of cancer cells.

**9.4.2. Reversion of MRPs Other Than MRP1**

**9.4.2.1. MRP2.** MRP2 substrates are closely related to those of MRP1. Concerning the inhibitors of MRP2, a good correlation with those of MRP1 is also found. Some α, β-unsaturated carbonyl compounds are able to inhibit MRP2. Wortelboer et al. (116) have explored a series of unsaturated carboxyls, including curcumin and two derivative forms (demethoxy and bisdemethoxy). The latter two drugs were described as able to inhibit calcine efflux from an MDCKII cell line transfected with MRP2. The discrepancy of activity between curcumin and the two derivative forms could be due to differences in metabolism and/or to better membrane diffusion. It was also demonstrated that two nonnucleoside reverse transcriptase inhibitors (NNRTI) (delavirdine, efavirenz) and one nucleoside reverse transcriptase inhibitor (NRTI) emtricitabine could achieve more than 50% inhibition of MRP2 (117). In 2005, van Zanden et al. (71) studied the activity of a series of flavonoids on MRP1
and MRP2. Robinetin and myricetin appeared to be good modulators of MRP2 with a percentage of inhibition around 70%. The author proposed that the replacement of the flavonol B-ring with a pyrogallol group induces a higher inhibition of MRP2 and that an A-ring pyrogallol group decreases the capacity to inhibit MRP2. As demonstrated by Asakura et al. (118), MRP2 activity can also be modified by azithromycin, which belongs to the macrolide class of antibiotics. By using a rat model, this study shows the role of azithromycin in the inhibition of the hepatobiliary excretion of drugs that are substrates for P-gp or MRP2.

Phenobarbital, a prototypical inducer of cytochrome P450, is well known to alter the biliary excretion of some organic anions. Patel et al. (119) have demonstrated in a rat model that phenobarbital or its hydroxylated derivative was able to alter MRP2 function or expression.

Glibenclamide, which is known to reverse MRP1 activity, has been shown to also reverse MRP2 (120). This sulfonylurea classically used in the treatment of noninsulin-dependent diabetes is a wide inhibitor of ABC proteins. The major disadvantage of this compound is that the concentration required to obtain a modulation of MRP is 10-fold higher than the concentration needed to regulate glucose concentration.

9.4.2.2. MRP3. In a recent work, Weiss et al. (117) evaluated the action of non-nucleoside reverse transcriptase inhibitors (NNRTIs) and nucleoside reverse transcriptase inhibitors (NRTIs). They showed that, concerning the NNRTIs, delavirdine and efavirenz were the more active. Among the NRTIs, emtricitabine was active, as well as efavirenz. MK571, which is a well-known inhibitor of MRP1, was also tested, and it was shown to inhibit MRP3 but twofold lower than MRP1. In 2003, Bodo et al. (121) described that E217BG transport by MRP3 is inhibited by the organic anions such as indomethacin, furosemide, probenecid, and by several conjugated bile acids.

9.4.2.3. MRP6 and MRP7. No data are available concerning the modulation of MRP6, probably due to its lack of impact in chemoresistance. Concerning MRP7, a competitive MRP7 inhibitor, 17-β-estradiol-(17-β-D-glucuronide) was demonstrated to be active but with a lack of specificity (122).

9.4.2.4. MRP4/MRP5. As mentioned above, MRP4 and MRP5 confer resistance to nucleoside and nucleobase analogs that are classically used as anticancer or antiviral agents, including compounds such as 6-mercaptopurine, 6-thioguanine (6TG), 9-(2-phosphonylmethoxyethyl)adenine (PMEA), and azidothymidine. Overexpression of MRP4 also results in resistance to camptothecins. In this context, modulation of these ABC transporters represents an important challenge.

Various compounds have been shown to inhibit the activities of MRP4, such as probenecid, sulfipyrazone, indomethacin, dipyridamole, and compounds containing purine (e.g., 6TG and PMEA) and pyrazolo[4,3-d]pyrimidin-7-one templates (e.g., sildenafil) (Table 9.5) (40). In a recent work, Tan et al. (123)
OTHER APPROACHES TO MRP MODULATION

9.4.2.5. MRP8/MRP9. Even if MRP8 was demonstrated to extrude pyrimidine and purine analogs, no inhibitor has been described to date. Concerning MRP9, inhibitors and substrates remain to be determined.

9.5. OTHER APPROACHES TO MRP MODULATION

Reversal of MRPs can also be achieved using molecular constructs. Two approaches were developed for MRP2.

(1) A hammerhead ribozyme directed against the transcripts of MRP2 (124). This molecular construct consisted in three transacting hammerhead ribozymes directed against MDR1/P-gp, BCRP, and MRP2. This interesting tool provides a new way for the modulation of ABC transporters, since most of the time, the overexpression of more than one ABC transporter is involved in chemoresistance.

(2) Antisense strategy against MRP2 (125). In this work, the authors have developed an antisense construct associated to an adenoviral vector, allowing a chemosensitization of a hepatocarcinoma cell line to various drugs, such as doxorubicin, vincristine, cisplatin, and etoposide.

The major advantage of this kind of strategy is the specificity of the ABC transporter inhibition compared to the classical reversing agents, which can

<table>
<thead>
<tr>
<th>Inhibitors of MRP4</th>
<th>Inhibitors of MRP5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probenecid</td>
<td>Probenecid</td>
</tr>
<tr>
<td>Sulfopyrazone</td>
<td>Sulfopyrazone</td>
</tr>
<tr>
<td>MK571</td>
<td>Dipyridamole</td>
</tr>
<tr>
<td>Zaprinast</td>
<td>Zaprinast</td>
</tr>
<tr>
<td>Trequensin</td>
<td>Trequensin</td>
</tr>
<tr>
<td>Sildenafil</td>
<td>Sildenafil</td>
</tr>
<tr>
<td>Dipyridamole</td>
<td></td>
</tr>
<tr>
<td>Indomethacin</td>
<td></td>
</tr>
<tr>
<td>Ibuprofen</td>
<td></td>
</tr>
</tbody>
</table>

Note: Adapted from Reid et al. (2003) and Jedlitschky et al. (2000).
induce side effects. The disadvantage is that a tumor can express more than one kind of ABC transporter, thus limiting the effects of this strategy.

9.6. CONCLUSION

Since the discovery of the first MRP transporter by Cole et al. in 1994 (62), an important work has been done to characterize the different actions and identify new proteins of the same family. This class of transporters contains at least nine proteins with various tissue expressions and physiological actions. The diversity also resides in the cellular topological expression and the necessity or not for various substrates to be linked to GSH or glucuronic acid. As a consequence to the great diversity of substrates, MRPs have been involved not only in the chemoresistance of anticancer drugs but also of anti-HIV drugs. As MRP1 and MRP2 are the first discovered and are related to extrusion of anticancer drugs, numerous chemical inhibitors have been developed against them, with more or less specificity and efficacy. An unexpected result is the lack of the use of these inhibitors associated with cancer treatment, probably secondary to the importance of their side effects. Thus, the development of new modulators of MRPs on the basis of structure-activity relationships, showing a higher specificity and an acceptable toxicity, remains an important issue.

REFERENCES

REFERENCES


REVERSAL AGENTS OF MULTIDRUG RESISTANCE MEDIATED BY MRPs


REVERSAL AGENTS FOR BREAST CANCER RESISTANCE PROTEIN (BCRP)-MEDIATED MULTIDRUG RESISTANCE

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2 Université de Grenoble, Grenoble, France

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Breast cancer resistance protein (BCRP) is a multidrug transporter also named ABCG2 in the international classification of ATP-binding cassette (ABC) proteins. This protein is the most recently discovered multidrug transporter (1). Various cytotoxic drugs (topoisomerase I inhibitors, anthracyclines, methotrexate [MTX]), endogenous substrates, and fluorescent dyes have been found to be extruded by BCRP (2–4).

BCRP is expressed in important tissues which have a role in (i) absorption (e.g., lung and gut), (ii) metabolism and elimination (kidney and liver), and (iii) maintenance of barrier functions for sanctuary sites, such as the blood–brain barrier (BBB), blood–testis barrier, placenta (5–7). Mutations in the BCRP gene have been found after drug selection. Two acquired mutations were described by Honjo et al. (8); they were associated with a transformation of the arginine in position 482 to a threonine or a glycine after drug exposition. These mutations were able to modify the size and/or the conformation of the third transmembrane domain, as well as the nature of the molecules extruded. According to its tissue distribution and its broad substrate specificity, BCRP plays an important role in the pharmacokinetics of many drugs.

The development of modulators of BCRP represents a strategy to circumvent chemoresistance induced by the transporter’s expression or overexpression. We will discuss here how to evaluate the activity of new inhibitors, the description of the different inhibitors with their structure–activity relationships, and future developments.

**10.2. DRUGS EXTRUDED BY BCRP**

BCRP is an adenosine triphosphate (ATP)-transporter that can extrude a broad spectrum of compounds. These compounds can be natural compounds, drugs used in therapy, or related compounds used in cell biology. In order to evaluate BCRP activity, as well as new modulators, it seems important to know the various compounds that are substrates for BCRP (Table 10.1).

An important point is to clearly state the specificity of each BCRP substrate toward wild-type BCRP and the various mutant types of transporters that
## TABLE 10.1. Drugs and Substances Extruded by ABCG2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Type of Compound</th>
<th>Specificity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antitumor drugs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>Anthraquinone, anthracycline</td>
<td>WT/MT MT</td>
<td>(1, 9)</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>Topoisomerase II inhibitor (fluorescent compounds)</td>
<td>MT</td>
<td></td>
</tr>
<tr>
<td>Idarubicin</td>
<td></td>
<td>MT</td>
<td></td>
</tr>
<tr>
<td>Methotrexate</td>
<td>Antimetabolite</td>
<td>WT/MT</td>
<td>(10)</td>
</tr>
<tr>
<td>Camptothecins (SN-38, topotecan)</td>
<td>Topoisomerase I inhibitor (fluorescent compounds)</td>
<td>WT/MT</td>
<td>(10)</td>
</tr>
<tr>
<td>CI 1033</td>
<td>EGFR tyrosine kinase inhibitor</td>
<td>WT</td>
<td>(10)</td>
</tr>
<tr>
<td>NB-506 and J-107088</td>
<td>Topoisomerase I inhibitor</td>
<td>WT</td>
<td>(11)</td>
</tr>
<tr>
<td>Flavopiridol</td>
<td>Cyclin-dependent kinase inhibitor</td>
<td>US</td>
<td>(12)</td>
</tr>
<tr>
<td>JNJ-770661</td>
<td>Inhibitor of cyclin-dependent kinases</td>
<td>WT</td>
<td>(13)</td>
</tr>
<tr>
<td>Other drugs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abacavir (ABC) and zidovudine (AZT)</td>
<td>Nucleoside reverse transcriptase inhibitors</td>
<td>WT</td>
<td>(14)</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>H2 receptor antagonist</td>
<td>WT</td>
<td>(15)</td>
</tr>
<tr>
<td>Toxins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pheophorbide a (PhA)</td>
<td>Fluorescent chlorophyll catabolite</td>
<td>WT/MT</td>
<td>(16)</td>
</tr>
<tr>
<td>2-Amino-1-methyl-6-phenylimidazo(4,5- b)pyridine (PhIP)</td>
<td>A carcinogen present in baked food and cigarette smoke</td>
<td>WT</td>
<td>(15)</td>
</tr>
<tr>
<td>Endogenous substrates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrone 3-sulfate</td>
<td>Natural steroid</td>
<td>WT</td>
<td>(17, 18)</td>
</tr>
<tr>
<td>17β-estradiol sulfate</td>
<td>Steroids metabolites</td>
<td>WT</td>
<td>(19)</td>
</tr>
<tr>
<td>17β-estradiol</td>
<td></td>
<td>WT</td>
<td>(19)</td>
</tr>
<tr>
<td>17-(β-D-glucuronide)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folic acid or folates</td>
<td>Natural compound acting in the purine biosynthesis</td>
<td>US</td>
<td>(3, 20, 21)</td>
</tr>
<tr>
<td>Protoporphyrin IX (PPIX)</td>
<td>Photosensitive toxin</td>
<td>WT</td>
<td>(22)</td>
</tr>
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</table>
have been described, as well as whether these compounds are also substrates of other ABC transporters.

The most widely used and reported BCRP substrate is mitoxantrone, a fluorescent compound with an emission wavelength accessible to the most commonly used flow cytometers. A strong resistance is often seen in drug-selected cell lines that overexpress BCRP, even if the compound that has induced selection is not mitoxantrone.

Anthracyclines are potent and widely used chemotherapy drugs. The fluorescence of these molecules allows easy flow cytometry measurement, and they have been widely used to evaluate P-glycoprotein (P-gp)-mediated efflux (28). In cell lines overexpressing BCRP obtained after longtime drug exposure, different doxorubicin efflux phenotypes can be observed. Using genomic DNA sequencing, Honjo et al. (8) demonstrated that doxorubicin efflux was closely related to the type of residue in position 482. For example, cells having a threonine or glycine at position 482 (Thr^{482} or Gly^{482}) were able to efflux doxorubicin, whereas cells harboring an arginine were not.

MTX is a folate analog that inhibits dihydrofolate reductase, a key enzyme in the formation of reduced folates. BCRP can extrude not only MTX but also MTX-polyglutamate, but only in the protein’s wild-type form. Transport of MTX can be decreased by coincubation with mitoxantrone, suggesting a competition between both drugs.

Topoisomerase I inhibitors are a class of compounds used to treat colorectal cancers (irinotecan) and ovarian cancers (topotecan). Irinotecan, its active metabolite 7-ethyl-10-hydroxycamptothecin (SN-38), and topotecan are fluorescent compounds, and their uptake can be followed using flow cytometry or high pressure liquid phase chromatography (HPLC) (10, 29).
10.2.1. BCRP Transport of Fluorescent Dyes Used in Cell Biology

The BCRP pump is able to transport various compounds, including fluorescent dyes (Hoechst 33342, rhodamine 123 [Rh123] specifically for the mutant forms) and different compounds such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) or pheophorbide A (PhA). These fluorescent dyes can be more or less easily used in flow cytometry. Transport efficiency is highly variable depending on whether BCRP protein is in its wild or mutant form. Rh123 is a well-known fluorescent dye used to evaluate P-gp activity but is also extruded by BCRP mutant type Gly<sup>482</sup>. Rh123 fluorescence can be followed by a classical flow cytometer and is commonly used at a final concentration of 1 µM. In order to determine the type of ABC transporter involved in the drug resistance, it is recommended to use specific inhibitors, such as elacridar for P-gp, or a fumitremorgin C (FTC) derivative such as Ko143 for BCRP.

PhA can be measured by flow cytometry using a 635-nm red diode laser and a 661-nm band-pass filter. It is transported by wild-type BCRP and Thr<sup>482</sup> and Gly<sup>482</sup> mutants (16, 22). This compound can be used at 10 nm in association with Ko at 2 µM, for example.

10.3. METHODS TO EVALUATE THE ACTIVITY OF BCRP INHIBITORS

10.3.1. Cellular Evaluation
10.3.1.1. Flow Cytometry. This method is widely used for the identification of modulators that are able to inhibit BCRP. Mitoxantrone is the BCRP-substrate of choice used in flow cytometry because it is extruded by BCRP wild or mutant types. BCRP wild-type (possessing an arginine at the position 482) can efflux mitoxantrone but not Rh123 or doxorubicin. According to these types of efflux, it is easy to distinguish between BCRP wild-type and BCRP Gly<sup>482</sup> and Thr<sup>482</sup> mutants (8). Ozvegy et al. (30) have established that mitoxantrone and Rh123 are transported by all BCRP variants excepted by Lys<sup>482</sup> and Tyr<sup>482</sup> for Rh123. The latter data, combined with the use of specific inhibitors of BCRP such as FTC and derivatives (26, 31) and using a cytometer equipped with an argon laser emitting at 488 nm, makes it easy to identify BCRP function and differentiate wild and mutant types (Table 10.2) (32).

Hoechst, a fluorescent dye classically used in cell cycle studies but needing a UV laser, is extruded by wild-type BCRP and all 482 variants except Lys<sup>482</sup> and Tyr<sup>482</sup>. This latter property has been shown on primitive stem cells, which are also called “side population” (SP) cells, referring to the aspect of the flow cytometry dot plot obtained with the fluorescent dye in these cells. Another way to test for transporter specificity is to combine P-gp and BCRP substrates such as calcine-acetoxymethyl and Hoechst 33342, respectively (24). PhA is an orange/red fluorescent chlorophyll catabolite which can be excited at
488 nm. This fluorescent dye is a specific substrate for BCRP and is transported by wild and mutant types of BCRP (33).

10.3.1.2. Cytotoxicity Assays. Cell cytotoxicity determination is indirectly correlated to the accumulation of BCRP substrates inside the cells. Most of BCRP substrates have antiproliferative activities and were identified by the IC<sub>50</sub> of cells overexpressing BCRP compared to unselected cells (34). A means for selecting BCRP-specific inhibitors is the increase of cell cytotoxicity when they are co-incubated with anticancer BCRP substrates (Table 10.2) (35, 36).

<table>
<thead>
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<th>TABLE 10.2. Methods Related to the Evaluation of BCRP Activity</th>
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<td><strong>Models</strong></td>
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<td>Membrane-based assays</td>
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<td>• Cell cytotoxicity</td>
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<td>• Cell lysis followed by HPLC quantification, for example (reference 13)</td>
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<td>Evaluation of an oriented transport on epithelial cells</td>
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<td>Polarized epithelial cells with tight junctions</td>
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10.3.1.3. Evaluation of an Oriented Transport on Epithelial Cells. Bidirectional transport can be studied on a confluent cell monolayer on transwell insert. Experiments are initiated by adding a solution containing the test compound to either the apical or basolateral compartment. The amount of the radiolabeled drug added to inhibitors is determined by liquid scintillation spectrophotometry. This method allows studying of the transport through the entire cell and eventually a polarization of this transport.

10.3.2. Membrane-Based Assays

10.3.2.1. ATPase Assays. ATPase activity of BCRP in crude membrane extracts can be measured by the end point inorganic phosphate (Pi) assay. This assay measures the amount of phosphate released in the ATPase assay buffer. The amount of Pi released can be measured by colorimetric or radioactive methods (37). The drawback of the method is that some compounds are able to increase ATPase activity of BCRP mutant type while they are potent and specific inhibitors of wild-type and mutant BCRP (Table 10.2) (38).

10.3.2.2. Photolabeling. This method allows the study of a specific interaction between a multidrug resistance protein (MRP) and compounds that are potential inhibitors. [3H]azidopine and [125I] are the most often used compounds to study P-gp-mediated transport, whereas iodoaryl-azido-Rh123 (IAARh123) is the compound mostly used for studying BCRP-mediated transport (39–41).

Briefly, membranes from cell lines are preincubated in the absence or presence of various concentrations of drugs prior to the addition of a photoreactive drug analogue. Membranes are submitted to UV irradiation. Following photoaffinity labeling, samples are mixed in a detergent solution. Immunoprecipitation is carried out using a monoclonal antibody. Immuno-precipitated proteins are resolved on gels, which are then dried and exposed to a film.

10.3.2.3. Membrane Vesicular Transport/Inside-Out Vesicles. Briefly, plasma membrane vesicles are obtained from frozen cell lines, and vesiculated membranes are enriched for inside-out vesicles by a slow passage through a column of wheat germ agglutinin. Vesicles are incubated with a radiolabeled substrate (such as [3H] methotrexate) and then separated from the free drug by filtration through a glass microfiber filter. The amount of the drug in the vesicles that remains on the filter is quantified by liquid scintillation counting. This method also allows the evaluation of the osmotic sensitivity of the transport as well as the need for ATP (3). This method is also an effective in vitro assay to assess kinetic constants such as Km for substrate and Ki for inhibitors (42).
10.3.3. In Vivo Evaluation

In vivo evaluations can be realized using genetically or chemically BCRP knocked-out animals and can provide information about the role of this protein in absorption and tissue distribution of its substrates.

10.3.3.1. Chemically Knocked-Out Animals. In order to evaluate the action of BCRP in tissue distribution and specifically in the maternal–fetal barrier, Jonker et al. (43) developed P-gp knockout mice. Pregnant mice were treated with elacridar, a known inhibitor of BCRP, and topotecan was measured in fetuses and in plasma. This study revealed that BCRP could play a role in protecting the fetus against drugs. Breedveld et al. (44) also used chemically knocked-out mice (with elacridar and pantoprazole) to determine the penetration of imatinib in brain tissue. They pointed out that BCRP and P-gp are both molecular determinants in drug diffusion to the brain.

10.3.3.2. Genetically Knocked-Out Animals. In the literature, these models have been widely used to determine the role of MRPs in pharmacokinetic studies of various drugs. ABC transporters are expressed and involved in absorption (intestine), excretion (kidney, liver), and blood–tissue barriers. For example, Breedveld et al. (44) used genetically knocked-out mice to evaluate the action of BCRP in the clearance of imatinib. Cisternino et al. (45) also evaluated the role of ABC transporters in the BBB. Using real-time polymerase chain reaction (PCR), they demonstrated that in P-gp knockout mice, BCRP was three times overexpressed compared to wild-type, and this overexpression was correlated to an increase of activity. This overexpression must be taken into account with transgenic animals when measuring transport mechanisms.

Zaher et al. (46), using BCRP or P-gp knocked-out mice, investigated whether Bcrp (abcg2) is involved in the disposition of sulfasalazine. It was concluded that Bcrp is an important determinant for the oral bioavailability and the elimination of sulfasalazine in the mouse, and that sulfasalazine has the potential to be used as a specific in vivo probe of Bcrp.

10.4. BCRP INHIBITORS: DESIGN AND ACTIVITY

10.4.1. Immunosuppressive Agents as Inhibitors of BCRP

Cyclosporin A (CsA) is an immunosuppressive agent discovered in 1970. This antibiotic is produced by the fungus Tolypocladium inflatum gans and is now used worldwide to prevent rejection following organ transplantation. CsA is a substrate and inhibitor of P-gp (48, 49). Until a few years ago, the role of CsA on BCRP was debated. Several studies concluded that CsA had marginal effects on BCRP and that these effects depended on protein expression levels
These findings were later confirmed by Ejendal et al. (51) who showed that CsA was neither an inhibitor of BCRP (using fluorescent dyes by flow cytometry) nor an inhibitor of the ATPase activity of BCRP. On the other hand, some studies have reported that CsA is able to enhance uptake and cytotoxicity of mitoxantrone in both wild-type and mutant BCRP-overexpressing cells (47, 52). Most studies agree that CsA is an unspecific inhibitor as it inhibits P-gp, BCRP, and MRP1.

A variety of other immunosuppressive agents were also assayed for BCRP inhibition and showed that CsA was the most potent agent (53, 54).

The discrepancies of the literature on the role of CsA as a modulator of BCRP could be due to many factors, such as the different sensitivities of the various fluorescent dyes used for efflux studies, the cellular model, or even the nature of the solvent used for the experiments.

In an original article, Gupta et al. (54) showed the reversing activity of three immunosuppressive agents, CsA, tacrolimus, and sirolimus (rapamycin), on BCRP (Fig. 10.1). These three compounds significantly inhibited the efflux of PhA (a specific substrate of BCRP), mitoxantrone, and BODIPY-prazosin. They were also able to reverse the resistance to topotecan and mitoxantrone. Direct efflux assays of these compounds using radiolabeling or liquid chromatography-mass spectrometry showed that none of the molecules were transported by wild-type BCRP. The mechanism of action of CsA may be through the inhibition of the ATPase activity of BCRP, as was shown on Arg482 and Gly482 BCRP (47, 55, 56). In a recent report, Xia et al. (47) demonstrated that CsA can also reduce the ATPase activity of the BCRP Thr482 mutant.

To date, it is not known if sirolimus and tacrolimus have an inhibitory effect on BCRP mutant types. Pawarode et al. (53) also demonstrated that CsA,

FIGURE 10.1. Structures of immunosuppressives, tacrolimus and sirolimus.
tacrolimus, and sirolimus were able to modulate BCRP transport. In this report, it was shown that these drugs could more or less compete with the photoaffinity analog $^{125}$I-iodoarylazidoprazosin and inhibit ATP hydrolysis by BCRP in a concentration-dependent manner.

Sirolimus is structurally closer to tacrolimus than to CsA and is also able to bind FKBP12. This latter complex binds and inhibits the phosphorylation of mTOR substrate P70S6K. However, the molecular bases of the inhibition of the kinase activity remain unclear.

In addition to being a competitive inhibitor of BCRP, sirolimus also decreases the surface expression of BCRP, probably through the inhibition of mTOR, which regulates cell growth, transcription, and motility.

The clinical tolerable ranges of tacrolimus and sirolimus are lower than that of CsA and are lower than the concentrations which have been found to be effective on BCRP modulation.

In 2005, Qadir et al. (52) reported that PSC833, a CsA derivative, is inactive as a modulator on Arg$^{482}$, Thr$^{482}$, and Gly$^{482}$ BCRPs; in the same article it was reported that VX-710 Biricodar is active on Arg$^{482}$ BCRP, but not on Thr$^{482}$ and Gly$^{482}$ BCRPs.

10.4.2. Anti-HIV Drugs as Modulators of BCRP

The emergence of resistant HIV-1 mutants makes treatment of HIV-1-infected patients not always successful. It is now well established that factors other than viral mutations may also contribute to treatment failure. For example, it has been reported that P-gp is a key determinant of the oral bioavailability of HIV-1 protease inhibitors. It was found more recently that the expression of BCRP in a CD4+ T-cell line confers cellular resistance to nucleoside reverse transcriptase inhibitors (NRTIs) (57). Thus, BCRP is considered to be an interesting target to improve the treatments using antiretroviral agents. In this context, considerable efforts have been made to check the influence of clinically used anti-HIV agents on BCRP. Ideally, an antiretroviral agent with an inhibitory activity on BCRP would be very useful because it would help decrease the doses and, therefore, the side effects.

The influence of important anti-HIV drugs on BCRP activity was assessed by an increase in PhA accumulation in MDCKII-BCRP cells compared with the corresponding parental cell line MDCKII lacking human BCRP. According to the IC$_{50}$ estimation, the rank order for BCRP inhibition was lopinavir > nelfinavir > delavirdine > efavirenz > saquinavir > atazanavir > amprenavir > abacavir (Fig. 10.2). The well-known anti-HIV drug zidovudine (AZT) exerted a weak inhibition. Ritonavir/tipranavir could not be evaluated in vitro due to lack of solubility (58).

Superior hydrophobicity of antiproteases compared to the NRTIs and the nonnucleoside reverse transcriptase inhibitors might be responsible for the higher activity.
10.4.3. Tyrosine Kinase and Protein Kinase Inhibitors Acting as BCRP Inhibitors

Protein kinases play a major role in cellular signaling and are considered among major targets for anticancer drugs. In this context, tyrosine kinase inhibitors (TKIs) are among the most studied molecules as BCRP inhibitors.

In the literature, four TKIs have been described for their interaction with BCRP (Fig. 10.3) (10, 35, 44, 59–62). In 2001, Erlichman et al. (10) showed the action of an HER family TKI CI1033 (PD183805) on BCRP (Fig. 10.3). This compound was studied in conjunction with the active metabolite of irinotecan 7-ethyl-10-hydroxycamptothecin (SN-38) or the related agent topotecan in cell lines expressing endogenous BCRP and in a BCRP transfected cell line. CI1033 increased uptake and cytotoxicity of SN-38 and topotecan. It was also demonstrated that accumulation of CI1033 alone was reduced in cells expressing BCRP, indicating that the compound is also a substrate.

Imatinib (Gleevec®) (Fig. 10.3) is a well-known TKI which is widely used (i) for treatment of chronic myelogenous leukemia (CML) by inhibiting BCR-ABL fusion protein, and (ii) for gastrointestinal stromal tumors by inhibiting c-KIT. Various clinical trials with other types of cancer are in progress, such as glioblastoma, prostate, and lung carcinoma. Imatinib mesylate, similar to many other TKIs such as members of the 4-anilinoquinazoline class, competes for ATP binding.

However, in patients, a limited penetration of imatinib into the brain has been reported. Imatinib is transported in vitro and in vivo by P-gp. Overexpression of BCRP has been shown to be involved in imatinib resistance. Imatinib’s effect on BCRP has been a subject of controversial and opposite debates. First, it was reported that imatinib potently reversed BCRP-mediated resistance, but the authors concluded that it was not a BCRP substrate for efflux (35). In the same year, an independent study came to challenge
the latter assumption because it concluded that imatinib was a substrate for BCRP (61). Shortly after, a third study showed that imatinib was a competitive substrate for BCRP (44).

Large hydrophobic molecules such as the TKIs have a great potential to interact with ABC MRPs, which are ATP-dependent active transporters. Ozvegy et al. (59) showed that low concentrations of the three studied TKIs (imatinib, ZD1839, and EKI-785) modulated BCRP activity (Fig. 10.3). At 0.1–1 µM, ZD1839 and EKI-785 stimulated ATPase activity, whereas imatinib strongly inhibited this activity. At higher concentrations, all three TKIs inhibited ATPase activity. This discrepancy at low concentrations could be explained by the fact that ZD1839 and EKI-785 could be substrates for BCRP. Due to the expression of BCRP on epithelium intestinal cells, TKIs’ oral absorption as well as their plasma concentrations could be modified.

Protein kinase C (PKC) inhibitors are promising anticancer agents. As staurosporine is among the most potent inhibitors of PKC, there has been an intensive search for staurosporine analogs (Fig. 10.3) (63). Among such analogs, compounds derived from bisindolylmaleimide and indolocarbazole have been identified, and some of them are currently under clinical
trials for cancer treatment. During clinical evaluation, indolocarbazoles and bisindolylmaleimide were shown to inhibit P-gp as well as MRP1 (Fig. 10.3). Later, several carbazoles and bisindolylmaleimides were evaluated as inhibitors of BCRP. At a concentration of 10µM, they were able to induce 1.3- to 6-fold increases in fluorescence of the BCRP-specific substrate PhA. It is noteworthy to highlight that these compounds were inhibitors but not substrates of BCRP.

10.4.4. Inhibition of BCRP with Flavonoids and Analogs

Flavonoids represent a large class of naturally occurring polyphenols, which have been extensively studied on P-gp and more recently on BCRP. Several naturally occurring flavonoids have been studied using mitoxantrone accumulation and cytotoxicity assays (Fig. 10.4) (64, 65). It appears that biochanin A and chrysin are the most active flavonoids on MCF7 and NCI-H460 resistant to mitoxantrone. Other molecules, such as genistein and kaempferol were active but not on both cell lines.

Among all the compounds evaluated by Zhang et al., only two (naringenin and phloretin) were tested in their aglycone and glycosylated forms. As could be expected, the aglycone analogs were the most active. The glycosylated forms were more hydrophilic and lowered the concentration of the compound in the membrane matrix.

The inhibition of BCRP was confirmed by cytotoxic assays. Except for silymarin, all active flavonoids were able to reduce the IC₅₀ value with mitoxantrone from 199 to 5.5µM. This work was reported at the origin of several articles dealing with flavonoids analogs synthesis.

FIGURE 10.4. Naturally occuring flavonoids and synthetic analogs studied as BCRP inhibitors.
Recently, Katayama et al. (66) reported a comparative study of a panel of 32 natural flavonoids with various glycosylated forms. Using topotecan accumulation and cytotoxicity assays, they identified 3',4',7-trimethoxyflavone and apigenin as the strongest BCRP inhibitors. In addition, these compounds seemed to have a good specificity toward BCRP. What is surprising is that glucosylated flavonoids, such as diosmin, are active on BCRP. This was the first time that highly hydrophilic molecules were reported to be inhibitors of BCRP. Knowing that the flavonoids studied by Katayama et al. (66) are widely present in fruits and vegetables, such molecules might have positive effects on pharmacokinetics of anticancer agents.

Techtochrysin (5-hydroxy-7-methoxyflavone) and 6-prenylchrysin were reported to be powerful inhibitors of wild-type BCRP (Fig. 10.4) (38). The substitution pattern among these molecules is critical. For example, the position of the methoxy group at position 7 of techtochrysin is necessary for higher activity. When this group is moved to other positions, the activity drops. In the same way, the position of the prenyl group at position six or eight is important.

Flavonoids behaving as weak estrogens called “phytoestrogens” have been studied as reverters of BCRP-mediated multidrug resistance (MDR). Since few sulfated estrogens have been identified as BCRP-substrates, Imai et al. conducted a study with the aim of checking the activity of phytoestrogens on BCRP (67). Genistein, naringenin, acacetin, and kaempferol potentiated the cytotoxicity of mitoxantrone and SN-38 (7-ethyl-10-hydroxycamptothecin) in BCRP-transduced K562 cells. In addition, these molecules were found to be selective inhibitors as no effect was observed on P-gp or on BCRP. Here again, some glucosylated flavones such as naringenin-7-glucoside were found to display BCRP-inhibitory activity.

Based on previously reported studies dealing with flavonoids as inhibitors of BCRP, hybrid molecules containing the chromanone moiety of flavonoids and a phenylethylamine similar to that found in elacridar were designed. Compounds A and B (Fig. 10.4) were reported as having a BCRP inhibitory potential equivalent to that of FTC, as shown by mitoxantrone uptake and cytotoxicity assays (68). The reported data emphasize the role of the length of the alkyl chain between the benzopyranone and the phenylalkylamine unit and the number of methoxy groups linked to the last unit.

Nonprenylated rotenoids, namely boeravinones obtained from Boerhaavia diffusa (Nyctaginaceae), have been assayed for the inhibition of the multidrug transporter BCRP (69). The inhibitory activity was measured by flow cytometry on HEK-293 human cells transfected by wild-type (Arg^{482}) BCRP and exposed to the antitumor drug mitoxantrone. The most active boeravinones (Fig. 10.4) were active at IC_{50}s ranging from 0.7 to 2.5µM. The close relationship among the structures of the tested compounds allowed the drawing of structure-activity relationships. Among others, the presence of a methoxy group at positions 6 and 9 on the one hand and a hydroxyl group at C-11 on the other hand are critical for higher activity.
10.4.5. Reversal of BCRP by Estrogens

Steroidal and nonsteroidal estrogen receptor mixed agonist/antagonist were used by Eli Lilly as a pharmacophore model to conceive selective modulators of MRP1-mediated MDR (70).

These findings led to the study of the effects of estrogens on BCRP. Imai et al. reported that estrone and 17β-estradiol were able to potentiate the cytotoxicity of mitoxantrone and topotecan in BCRP-transduced K562 cells (18). The same authors also reported that metabolites of estrogen, and especially sulfated estrogens, are transported by BCRP.

Following this study, estrogen antagonists and agonists have been evaluated as BCRP-reversal agents (Fig. 10.5). The pharmacophore of tamoxifen was used as a basis for the synthesis of analogs, and diversity was achieved by introducing small substituents on the A and/or B cycles (Fig. 10.5). Diethylstilbestrol showed the strongest BCRP-reversing activity by increasing the intracellular accumulation of topotecan and reversed drug resistance in K562/BCRP cells. Tamoxifen and toremifene were less active in enhancing topotecan uptake in the same cell line. The higher activity of diethylstilbestrol is expected to be correlated to its higher hydrophobicity.

10.4.6. Inhibition of BCRP by Derivatives of Acridones

Elacridar (GF120918) was the first of the series to be studied as a BCRP inhibitor. This compound was initially developed as a P-gp inhibitor (71) and was later demonstrated to be an efficient inhibitor of BCRP (Fig. 10.6). Studies related to elacridar have covered almost all types of cancers both in human and murine models. Using flow cytometry and confocal microscopy, scientists from the National Institutes of Health were among the first to show the effect of elacridar on BCRP-expressing human cell lines (72). The acridone was able to block both rhodamine and mitoxantrone effluxes. Later studies showed the effect of elacridar on resistance to topoisomerase I inhibitor, camptothecin. The compound was found to be a potent inhibitor with almost complete reversal at 100nM (5).

FIGURE 10.5. Structures of estrogens and analogs.
Maliepaard et al. (5) have studied the effect of elacridar on resistance to topoisomerase I inhibitor, camptothecin. The acridone derivative was found to be a potent inhibitor with almost complete reversal at 100nM. Following these results, the structure of elacridar was used as a work base to conceive new BCRP inhibitors (Fig. 10.6). In this regard, analogs have been made by shortening the aryllic side chain and were found to be slightly more active than elacridar, as shown by mitoxanthrone efflux from human wild-type (R482) transfected cells (73).

10.4.7. FTC and Related Analogs

FTC was discovered by Rabindran et al. in 1998 (74), the same year BCRP was described by Doyle et al. (1). This compound was isolated from a large library of microbial extracts and tested on a colon carcinoma cell line resistant to mitoxantrone (S1-M1-3.2). FTC and related molecules, FTA and FTB, belong to a class of diketopiperazines which are mycotoxins isolated from Aspergillus fumigatus (Fig. 10.7). FTA and FTB, which differ from FTC at two positions, are less effective on BCRP activity, with an increase of side effects in vivo, especially on the brain stem and spinal cord. When taken orally at 25 mg/kg, FTC is able to cause tremors in cockerels. FTC was demonstrated to be a specific inhibitor of a new class of transporters by its lack of modulation on P-gp (S1-B1-20) or MRP1-positive cells (HL60/AR). FTC was able to increase the amount of mitoxantrone and increase cell toxicity of mitoxantrone, doxorubicin, and topotecan in the cell lines expressing this new transporter.

Using cells transfected with the BCRP gene, Rabindran et al. (26) demonstrated that FTC could reverse resistance mediated by this transporter. With a cytotoxicity assay based on a sulforhodamine B probe, it was shown that FTC restores the sensitivity and the accumulation of mitoxantrone, topotecan, and doxorubicin in MCF7 cells transfected by BCRP. FTC is also able to increase the accumulation of the fluorescent probe, BBR3390 (26, 75).

FTC is a specific and potent inhibitor of BCRP but induces neurotoxic effects, thereby excluding any clinical use (74). In order to provide less toxic and specific BCRP inhibitors, FTC analogs have became attractive targets.
Shinkel’s group screened a variety of libraries derived from indolyl diketopiperazine (corresponding to A/B/C/D-rings of FTC) and found three compounds, namely Ko132, Ko134, and Ko143, as the most promising leads. The three compounds were potent, less toxic than FTC, and specific inhibitors of BCRP (31). Ko143 increased significantly the oral availability of topotecan in mice. Tryprostatin A (TPS-A) is another fungal secondary metabolite and is an analog of FTC. TPS-A was reported as an inhibitor of microtubule-associated protein (MAP)-dependent microtubule assembly (76). Using a classical approach with mitoxantrone uptake and cytotoxicity assays, Woehlecke et al. (77) demonstrated that TPS-A is an inhibitor of wild-type BCRP. At relatively high concentrations (up to 50 µM), TPS-A was able to restore cell sensitivity toward mitoxantrone, without inducing noticeable toxicity.

The structural difference between FTC and TPS-A is essentially in the noncohering diketopiperazine C ring in TPS-A, allowing alternative conformation. It could also be hypothesized that rings A, B, and D, as well as the C3 side chain, are necessary for BCRP inhibition. Modifications of the E-ring by acyclic constituents in FTC derivative are not associated to a loss of activity on BCRP.

10.4.8. Taxanes and BCRP Inhibition

Paclitaxel is a medicinal drug discovered from the bark of the Pacific yew tree in the 1970s. This drug binds to β-tubulin and inhibits its depolymerization. Structure-activity relationship studies have shown that the presence of the C-13 side chain is fundamental for the activity. Brooks et al. (78) have pro-
posed a series of noncytotoxic taxoids where the C-13 side chain is replaced by an acetylxy group. Structural diversity was obtained by branching different substituents on the hydroxyl groups at positions 1, 2, 7, 10, and 14. It was reported that the taxane-based agent tRA98006 is active against BCRP<sup>R482</sup>, BCRP<sup>R482T</sup>, and BCR<sup>832G</sup> (52) (Fig. 10.8). Interestingly, this compound is the one having a basic amino group, which reinforces the argument related to the importance of a basic nitrogen among inhibitors of BCRP. In such situations, it is useful to check the activity of the substituent having the basic nitrogen before branching it to the taxane backbone. It is important to note that reported taxanes are not selective for a specific transporter. However, in hematological diseases, such as acute leukemia, it is important to have compounds able to modulate several ABC proteins. Some advantages are the greatest absorption following oral administration and the penetration in “protected tissue” such as brain and testis.

10.5. CONCLUSIONS AND PERSPECTIVES

Any attempt to draw a global quantitative structure–activity relationship is rendered complex by the variety of cellular tests conducted during the biological evaluation. Indeed, a large number of cells from different tissues and from different origins (man and animal) have been used. In addition, the BCRP substrates used may vary from one study to another (79).

However, a qualitative structure–activity relationship may be drawn. Globally, the most reported inhibitors fulfill Lipinski’s rule. The average molecular weight of inhibitors ranges from 350 to 550 g/mol. The major structures present less than five hydrogen-bond donors and five hydrogen-bond acceptors. The common entity shared by major specific inhibitors of BCRP is a substituted polyaromatic entity. An indole ring or its equivalent is frequently met. The presence of at least one nitrogen atom is a frequent structural element. The latter can be a tertiary amine or a tertiary amide. A piperazine ring might be a privileged entity to ensure the presence of nitrogen atoms. One important feature is the hydrophobic characteristic of BCRP inhibitors.
An important element which deserves to be explored is the flavonoid family. Some flavonoid analogs, without bearing any nitrogen atom, still behave as potent inhibitors. Hence, the introduction of nitrogen-bearing substituents could be a valuable approach to obtaining more potent derivatives.

As discussed above, a lot of inhibitors of BCRP have been developed with more or less specificity against this protein to overcome drug resistance. The major goal is to increase the intracellular drug and to allow the latter to meet the target. Difficulties encountered for the *in vivo* use are the same as for P-gp. BCRP is expressed in the BBB, on detoxification organs, and the employment of inhibitors can greatly modify drug absorption, distribution, and the cellular effect. Some of the inhibitors are themselves anticancer drugs. A better knowledge of the pharmacokinetics data would help physicians to initiate phase I and phase II trial studies. Much data are needed to evaluate myelosuppression of the use of MDR inhibitors since it was demonstrated that P-gp and BCRP are expressed on stem cells.

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11

STRATEGIES TO OVERCOME DRUG RESISTANCE IN ACUTE AND CHRONIC LEUKEMIAS

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ABC Transporters and Multidrug Resistance, Edited by Ahcène Boumendjel, Jean Boutonnat and Jacques Robert
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11.1. INTRODUCTION

The efficacy of acute and chronic leukemia treatment is precluded in many patients by the resistance of part of their leukemic cells to the cytotoxic drugs, together with a lack of appropriate immune response. When a complete response is obtained, patients often relapse through expansion of those cells that have resisted initial treatment. Mechanisms by which leukemic cells resist to drugs thus remain a major issue in hematological malignancy treatment.

P-glycoprotein (P-gp; P for permeability) was identified more than 30 years ago as a plasma membrane protein that reduced drug permeability (1). P-gp was associated in the early 1980s to resistance of mammalian cells to diverse antineoplastic drugs (2) and was subsequently correlated with a poor outcome in acute myeloid leukemia (AML) (3). This latter observation provided the evidence for testing pharmacologic strategies aiming to neutralize this adenosine triphosphate (ATP)-dependent efflux pump in clinical trials.

P-gp, an ATP-binding cassette (ABC) transporter, belongs to a family of 49 evolutionarily conserved, transmembrane proteins involved in the transport of a variety of substrates through extra- or intracellular membranes. Mammalian ABC genes are divided into seven subfamilies (ABCA to ABCG). Besides P-gp, also known as ABCB1 (or MDR1), other ABC transporters proposed to mediate resistance of leukemic cells to a wide variety of structurally unrelated chemotherapeutical compounds including ABCC1 (multidrug resistance protein, MRP1), ABCG2 (breast cancer resistance protein, BCRP), and ABCA3. The responsibility of each of these transporters in leukemic drug resistance is only partly defined (4).

It is now clear that ATP-dependent redistribution of drugs is only one of the mechanisms by which leukemic cells escape drug-induced cell death. Leukemic cells can develop other multidrug resistance (MDR) mechanisms such as overexpression of glutathione-S transferase P1.1 isoform, deregulation of cell death pathways, and the poorly understood tumor dormancy, or resistance to specific agents including nucleoside analogs through defaults in cellular metabolism and small molecule kinase inhibitors through mutation of the targeted enzyme (Fig. 11.1). Strategies have been imagined to overcome these resistance mechanisms and some of them have now been tested in clinics. This chapter summarizes these different resistance mechanisms and the clinical studies performed or envisioned in an attempt to reverse them.

11.2. MDR

11.2.1. Prognostic Impact of ABCB1 Phenotype in Acute Leukemias

The most studied resistance mechanism in acute leukemias is the so-called “MDR,” which is the consequence of an increased expression of the mdr1
Drug resistance mechanisms in leukemic cells include poorly specific multidrug resistance mechanisms (a) and those that are specific to a family of compounds (b). Multidrug resistance includes enhanced drug efflux by ATP-binding cassette proteins such as ABCB1 (P-gp/MDR1), increased detoxification by cellular enzymes such as glutathion S-transferase P1.1, defective pathways to death (mostly by apoptosis), and less well-defined cell adhesion-mediated drug resistance (also known as CAM-DR) and tumor dormancy. More specific mechanisms are those that prevent nucleoside analog cell entry and cellular metabolism and amplification or mutations of specific cellular targets such as topoisomerase or kinases, mainly tyrosine kinases.

The gene encodes the 170-kDa P-gp or ABCB1 (Fig. 11.2), an ATP-dependent transmembrane pump that facilitates the cellular efflux of various substances. When overexpressed in leukemic cells, ABCB1 reduces intracellular accumulation of anthracyclines, amsacrine, and epipodophyllotoxins. The protein also specifically interferes with various forms of caspase-dependent apoptosis (5–7).

ABCB1 phenotype can be detected in clinical specimens through various methods that include molecular techniques such as reverse transcriptase-polymerase chain reaction (RT-PCR), immunodetection of the protein, and functional assays employing fluorophore substrates (8). Whatever the method used, ABCB1 phenotype is an independent prognostic variable for induction failure in AML and displays close linkage with other well-recognized prognostic factors in AML such as age, expression of CD34 antigen, unfavorable karyotype, and secondary leukemia (9–14). ABCB1 phenotype is a more dis-
criminant negative prognostic factor in previously untreated populations with a high prevalence of constitutive expression, such as chemotherapy-naive, elderly patients with secondary AML, than in patients with relapsed disease in whom alternate transporters have emerged such as ABCG2 and ABCC1 (Fig. 11.2) (15, 16).

ABCB1 phenotype also has a negative prognostic impact in acute lymphoblastic leukemia (ALL). As in AMLs, the incidence of this phenotype increases with age at ALL diagnosis. Its prognostic impact, which is consistently found in adult patients, is more controversial in pediatric patients in whom it could depend on the therapeutic strategy (16).

ABCB1-mediated efflux of cytotoxic molecules can be reversed by noncytotoxic molecules that competitively bind to ABCB1 within its substrate channel and sterically interfere with the binding of cytotoxic substrates (17).

Preclinical experiments in which drug-sensitive cancer cells were treated with MDR-related cytotoxic drugs together with ABCB1 modulators suggested that these modulators could prevent the emergence of drug resistance. This later observation provided a rationale for considering MDR modulation early in the course of the disease (18).

11.2.2. Clinical Trials Targeting ABCB1

The first identified modulators of ABCB1-mediated resistance were pharmaceuticals already in use for other treatments. They were structurally diverse.
but demonstrated in all cases the lipophilicity required to penetrate plasma membrane and diffuse passively into the cytoplasm. Their efficacy was shown to depend on extent of protein binding, site of ABCB1 interaction, and binding affinity of both the modulator and the cytotoxic drug.

Verapamil was the first molecule demonstrated to reverse drug resistance in an ABCB1-expressing cell line (19). A series of first-generation modulators was subsequently identified by in vitro assays. These modulators included calcium channel blockers, antiestrogens, immunosuppressive drugs such as cyclosporine A, protein kinase inhibitors, quinine, and derivatives, monoclonal antibodies, and others (20). The pharmacologic profile of most of these modulators was inadequate for clinical studies due to variability in absorption, excessive binding to plasma proteins, or unacceptable toxicity at plasma concentrations required for effective ABCB1 modulation.

Quinine and cyclosporine A emerged from phase I/II studies testing these first-generation modulators (21, 22). The two molecules could be administered at sufficient doses to yield pharmacologically active serum concentrations, as confirmed by ex vivo assays, without excessive toxicity (both induced hyperbilirubinemia and prolonged myelosuppression in a dose-dependent manner). Cyclosporine A and, to a lesser extent, quinine, were observed to delay elimination of the targeted cytotoxic drug with wide interpatient variation, and this effect did not compromise the administration of conventional doses of the cytotoxic drug. More recently, a phase II trial suggested that tetrandrine, a potent inhibitor of ABCB1 efflux pump, could be efficient in poor-risk AML when combined with daunorubicin, etoposide, and cytarabine (23).

Three randomized phase III studies testing quinine and two other testing cyclosporine A as chemosensitizers have now been reported in AML patients. Quinine trials were completed by the Groupe Ouest Est Leucemies Aigues Myeloblastiques and the Groupe Français des Myelodysplasies. In the first one that involved 315 patients, addition of quinine to mitoxantrone and intermediate-dose cytarabine increased the complete response rate in ABCB1-positive AMLs (including refractory and relapsed de novo or secondary AMLs) without being statistically significant (24). In a series of 131 chemotherapy-naive patients with high-risk myelodysplastic syndrome (MDS) or related leukemia, quinine improved the response rate to mitoxantrone and intermediate-dose cytarabine and the median survival specifically in ABCB1 positive patients (25). In a series of 425 younger patients with de novo AML, quinine demonstrated a selective benefit in the small group of patients whose blast cells effluxed rhodamine efficiently. In these patients, quinine improved the response rate and extended disease-free survival and four-year survival (26). Altogether, these studies demonstrated a selective benefit of quinine in AML patients whose blast cells demonstrated ABCB1-mediated drug efflux.

Ciclosporine A studies were performed by the Medical Research Council (27) and the Southwest Oncology Group (28). The first one did not demonstrate any benefit, due to a cyclosporine A dose (5 mg/kg/d) that proved insufficient to efficiently block ABCB1. The second was performed in patients with
secondary, refractory, or relapsed AML and high-grade MDS, and tests showed more elevated doses of ciclosporine A (16 mg/kg/d). When combined with daunorubicin and high-dose cytarabine during induction and consolidation, ciclosporine A reduced the frequency of resistant disease and the need for second induction. The chemosensitizer selectively improved disease-free and overall survival in patients with ABCB1-positive leukemia.

A new generation of modulators with greater potency, specificity, and binding affinity was developed. PSC833 (Valspodaar®, Novartis), a cyclosporin-D analog with 10-fold greater potency for ABCB1 blockade than ciclosporin A, was the only one that completed phase III trials in AML patients (29). Like ciclosporine A, PSC833 inhibited cytochrome P450 isoenzyme 3A4 (CYP3A4), which delayed hepatic elimination of natural product-derived antineoplastic drugs. Based on this observation, the phase III trials were conducted with reduced doses of the cytotoxic drug(s) to be modulated. Two of them were closed prematurely, due to the lack of improvement of complete response (30) and excessive mortality associated with induction treatment in the experimental arm (31), respectively. The two other trials failed to demonstrate an improved complete response rate or disease-free survival (32). Wide interpatient variation in the pharmacokinetic interaction between PSC833 and the targeted antineoplastic drug could account for this failure (18).

A third-generation of highly specific modulators was developed using quantitative structure-activity relationships and combinatorial chemistry techniques (33–35). Those in clinical development include inaquidar (R101933), tariquidar (XR9576; Xenova), zosuquidar (LY335979; Eli Lilly), and ONT-093. These agents display minimal activity against other members of the ABC transporter family, lack interaction with CYP3A4, and act as noncompetitive ABCB1 antagonists (33). Their preferred site for ABCB1 interaction remains unclear, but these molecules demonstrate greater affinity for the efflux pump than transported substrates, and their activity is sustained. Following promising results of a phase II study (35), the Eastern Cooperative Oncology Group (ECOG) has initiated a double-blind, phase III randomized trial testing of zosuquidar in combination with daunorubicin in older patients with advanced AML or high-grade MDS.

The last generation of drug resistance modifiers is emerging from efforts to delineate structural interactions with either ABCB1 or the transcriptional regulators of the corresponding gene (mdr1). Two farnesyl protein transferase inhibitors (SCH66336 or lonafarnib, Schering-Plough; and R115777 or tipifarnib (Zarnestra; Johnson & Johnson)) directly interact with the ATP-binding site, thus impeding ATP utilization, which offers the prospect to disrupt multiple mechanisms of cell defense (36). Other therapeutic strategies to counter ABCB1-mediated resistance include the use of ABCB1-derived synthetic peptides to elicit a serologic response that specifically impedes ABCB1 transport function (37) and the induction of a WT1-specific immune response that may extend to ABCB1-expressing cells as mdr1 gene expression is coordinately regulated with the WT-1 gene (38).
11.2.3. Therapeutic Strategies Targeting ABCC1

Together with ABCB1, the multidrug resistance-associated protein 1 (MRP1, ABCC1) is a transporter involved in MDR (39) (Fig. 11.2). This transmembrane protein functions mainly as a (co-) transporter of amphipathic organic anions. While it transports hydrophobic drugs conjugated to the anionic tripeptide glutathione, the efficient extrusion of nonconjugated drugs by this protein depends on a normal cellular supply of glutathione. ABCC1 has broad substrate specificities, including anthracyclines, vinca alkaloids, epipodophytoxins, mitoxantrones, methotrexate, and to a lesser extent, taxanes (39). The expression of ABCC1 was identified in acute leukemia cells, but its prognostic impact remains a controversial issue (40–44). An increased expression of ABCC1 has been reported in chronic lymphocytic leukemia and prolymphocytic leukemia cells (45).

The discovery and development of ABCC1 inhibitors with high efficacies and appreciable safety have been much more difficult than to ABCB1, most probably because ABCC1 is an anionic transporter. Probenecid is anionic in nature and is an ABCC1 inhibitor. However, the development of this compound as a clinical ABCC1 modulator was limited by its clinical toxicity at requested doses (46). Actually, an anionic molecule enters an intact cell poorly, so that the effective intracellular concentration of an anionic ABCC1 inhibitor is difficult to be attained. Sulindac was identified as a competitive substrate for ABCC1, and a phase I trial of epirubicin in combination with escalating oral doses has identified the maximum tolerated dose (47), but the clinical benefit of such a modulator in patients with acute or chronic leukemia remains to be explored.

Several ABCB1 inhibitors were found to cross-react with ABCC1, among which, VX-710 (a pipecolinate derivative) and MS-209 (a quinoline derivative) are of particular interest. Dibenzocyclooctadiene lignans also inhibit both ABCB1 and ABCC1 (48). Schisandrin B, the most abundant dibenzocyclooctadiene lignan present in *Schisandra chinensis*, demonstrated significantly stronger potency than probenecid in ABCC1 inhibition (49). Since the clinical MDR appears to be multifactorial, modulators with broad specificity could be preferable to the combination of several specific modulators to reduce the drug-drug interaction and cumulative toxicities.

11.2.4. ABC Proteins and Leukemic Stem Cells

Normal hematopoietic stem cells are characterized by their ability to efflux fluorescent dyes (rhodamine-123; Hoechst 33342) and express high levels of a variety of ABC transporters. The current knowledge supports a role for these transporters in protecting stem cells from genetic damage of naturally occurring xenobiotics (50).

CD34 + 38- cell hematopoietic stem cells, committed progenitors, or even differentiated cells may become leukemia-initiating cells through the activa-
tion of oncogenic pathways that reestablish self-renewal capacities. In AML, cells with leukemic stem-cell characteristics (capacity for engraftment and self-renewal potential) were identified by using the nonobese diabetic/severe combined immunodeficient mouse model (51–52). A similar role for a leukemic stem cell has been suggested for chronic myelogenous leukemia (CML) (53), ALL (54), and MDS (55).

Normal stem cells express high levels of ABC transporters (50). If the expression of these transporters is maintained at these levels in leukemic stem cells, chemotherapeutic drugs produce clinical improvement and responses but are unlikely to be curative. In AML, the ABC-transporter gene expression profile of CD34 + 38- cells is largely conserved, but many of these genes are expressed at a distinct level when compared to a normal counterpart (56). For example, ABCB1 is the major molecular determinant of the mitoxantrone and rhodamine-123 “dull” phenotype of CD34 + 38- cells in normal bone marrow, but ABCB1 expression and ABCB1-mediated drug efflux are decreased in CD34 + 38- leukemic cells compared to their normal counterparts, and ABCB1 modulation abrogates mitoxantrone efflux in normal but not leukemic CD34 + 38- cells (57). Together with a series of other in vitro data, these observations indicate that ABCB1 modulation may preferentially target normal stem cells rather than leukemic stem cells, which could account for both the increased myelosuppression and the poor results of ABCB1 modulation on long-term disease outcome in clinical trials.

ABCG2 (BRCP) is the other important determinant of the rhodamine-dull phenotype of hematopoietic stem cells (HSCs) (Fig. 11.2) (58, 59). ABCG2 is expressed in leukemic CD34 + 38- stem cells and contributes to mitoxantrone efflux in these cells, but its expression and function do not differ from those measured in normal CD34 + 38- cells. Selective blockage of ABCG2 by the fumitremorgin C (FTC) analog Ko143, a potent ABCG2 inhibitor, results in increased intracellular mitoxantrone accumulation in leukemic CD34 + 38- cells in most patients but does not increase their sensitivity to chemotherapeutic agents (60). Simultaneous modulation of several transporters, including ABCB1, ABCC1, and ABCG2 was suggested to be required to sufficiently increase drug accumulation and eradicate CD34 + 38- stem cells in AML (50). The improved clinical outcome in a trial using cyclosporine as a P-gp inhibitor in AML, in contrast to the other poor results of selective ABCB1 modulation on long-term disease outcome, could be in line with this assumption since cyclosporine is a more promiscuous ABC-transporter inhibitor with effects on both ABCB1 and ABCG2 (28).

11.2.5. MDR Mediated by Detoxification Enzymes

Glutathione-S-transferases (GSTs) are a complex multigene superfamily of phase II detoxification enzymes that catalyze the conjugation of glutathione (GSH) to a variety of exogenous and endogenous electrophilic compounds. These enzymes form three main families: membrane-bound microsomal, mito-
Chondrial, and cytosolic. The latter exist as monomers and are catalytically active in a homo- or heterodimeric state (61, 62). The cytosolic family is further divided into seven classes: alpha, mu, omega, pi, sigma, theta, and zeta.

Chemical carcinogens react with DNA after metabolic activation by hydrolysis, reduction, or oxidation. The results of this interaction are mutations and, eventually, the initiation of cancer. Substrates for the GST enzymes are not only environmental pollutants, such as benzo[a]pyrene and other polyaromatic hydrocarbons, but also anticancer drugs, including alkylating agents, anthracyclines, and cyclophosphamide metabolites. Actually, many cancer drugs that decompose to produce electrophilic species can be detoxified via glutathione metabolism (61).

For example, GST-P1.1 (GST-π) is the main isoenzyme in lymphoid tissues, and its expression correlates with the efficacy of chemotherapy in hematological malignancies as well as in solid tumors (Fig. 11.3). The GST-P1-1 gene, located at 11q13, is coamplified with the cyclin D1 gene (in the same amplicon) in mantle cell lymphoma, and this overexpression could account for the drug resistance of this specific lymphoma (63). Overexpression of GST-P1.1 was also identified as an independent prognostic factor in diffuse large B-cell lymphomas (64).

![Stressful stimulus inducing ROS production](image)

**FIGURE 11.3.** Multidrug resistance mediated by glutathion S-transferase P1.1. Interaction with JNK. GST-π can inhibit JNK phosphorylation in nonstressed conditions by sequestering JNK/c-Jun. Stressful stimuli such as exposure to a cytotoxic drug induce oligomerization of GST-π through alteration of the redox potential, which dissociates the GST-π-JNK complex and permits JNK activation, c-Jun phosphorylation, and downstream events such as apoptosis. Overexpression of GST-π prevents the activation of this pathway.

ROS = radical oxygen species.
Aberrant expression of GST isozymes was initially correlated with tumor cell resistance towards alkylating agents (65). However, GST isozyme transfections provided disparate and sometimes controversial results. It appeared that, in addition to their well-characterized catalytic activity, certain GST isozymes regulated mitogen-activated protein (MAP) kinases or facilitated the addition of glutathione to cysteine residues in target proteins (S-glutathionylation). These multiple functionalities provided a rationale as to why many of the drugs that appeared to be sensitive to GST expression were neither subject to conjugation with GSH, nor substrates for GSTs (66). Many anticancer agents such as cisplatin induce apoptosis via activation of the MAP kinase pathway (67), specifically via c-Jun N-terminal kinase 1 (JNK) and p38 (68); and elevated levels of GST are associated with an increased resistance to apoptosis initiated by these agents (69, 70). Since GST-P1.1 is an inhibitor of the MAP kinase pathway via interaction with JNK (Fig. 11.3) and GST Mu (71) is an inhibitor of apoptosis via interaction with ASK1 (apoptosis signal-regulating kinase) (Fig. 11.4) (72), novel small molecule therapeutics targeting GST isoforms have been developed, and several compounds are now in late-stage clinical testing.

The first clinical modulatory study was performed with ethacrynic acid (EA), an approved drug otherwise used as a diuretic. The therapeutic value of EA as a chemosensitizer was demonstrated in patients (73, 74), but its clinical utility was limited both by its diuretic properties and its lack of isozyme

![Diagram](image-url)

**FIGURE 11.4.** Multidrug resistance mediated by glutathion S-transferase M. Interaction with apoptosis signal-regulating kinase 1 Kinase (ASK1). GST-M1 binds to and inhibits the mitogen-activated protein (MAP) kinase kinase kinase ASK1. Upon stressful stimuli, radical oxygen species are produced and induce GST-M1 oligomerization. In turn, ASK1 is released, oligomerizes, is activated through autophosphorylation, and activates downstream kinases such as JNK and p38 pathways to trigger cell death by apoptosis.

ROS = radical oxygen species.
specificity (75, 76). New GSH conjugates and peptidomimetics working as competitive inhibitors of GSH binding site were developed, including a peptide moiety that either enhanced the GST isozyme selectivity or increased the drug stability. The peptidomimetic TLK199 was identified as a low micromolar inhibitor of GST (77, 78) that also inhibits ABCC1 (79). TLK199 disrupts interactions between GSTP1-1 and JNK, which could explain the small myeloproliferative effect of the molecule detected in rodents (80). Telik Inc is developing TLK199 (Telintra) as an inhibitor of GSTP1-1 for the prevention of myelosuppression in MDS. Intravenous treatment was associated with improvement in all three types of blood cells in patients with all types of MDS, including those in intermediate- and high-risk groups. A new, oral formulation of the drug is currently tested in MDS patients (81). Another less developed inhibitor is NBDHEX, a 7-nitro-2,1,3-benzoxadiazole derivative that demonstrated strong cytotoxic activity toward cancer cells and low toxicity in mice (82).

Another approach used the synthesis of prodrugs to be converted to active molecules upon activation by GST. For example, cis-3-(9H-purin-6-ylthio) acrylic acid is a prodrug of 6-mercaptopurine that requires GSH conjugation and subsequent metabolism for activation (83), but its development was precluded by the limited efficacy of the parental molecule. The GSTP1-1 activated prodrug Telcyta (TLK286; Canfosfamide HCl) is a first-in-class investigational cancer cell-activated chemotherapeutic. It is presently in late-stage clinical development. Phase I clinical trials showed minor drug-related side effects that included fatigue, nausea, vomiting, and hematuria (84), and clinical benefits have now been reported in solid tumors (85, 86). More recently, sulfonylhydrazine prodrugs, such as KS119, that utilize the reductive environment of hypoxic tumors to generate cytotoxic species were developed (87).

A novel class of GST-P1.1 inhibitor acting through nitric oxide (NO) release has been developed recently. Unlike other NO donors, they do not react with GSH to release NO in the absence of GST. NO-releasing GST inhibitors inhibit GST-P1.1 while increasing arsenic and cisplatin toxicity in previously resistant tumor cells (88). This GST-P1.1 inhibition also leads to an enhanced activation of JNK and extracellular related kinase (ERK) (89).

Members of the GST family (GSTM1, GSTP1, and GSTT1) exhibit genetic polymorphism in their population distribution. Deletion of the GSTM1 and GSTT1 genes results in a “null” genotype characterized by a general deficit in enzymatic activity. Individuals homozygous for these deletions are thought to be at increased risk for acute leukemias as a consequence of a decreased capacity to detoxify possible carcinogens. A recent meta-analysis of 30 case-control studies suggests that GSTM1 and GSTT1, but not GSTP1 polymorphisms, are associated with a modest increase in the risk of ALL. GSTM1 and GSTT1 deletions could also negatively affect the outcome after induction therapy in AML as default in detoxification of antileukemic drugs would increase their systemic toxicity (67). In contrast to this lower survival rate, patients null for GSTM1 or GSTT1 showed a twofold reduction in cancer relapse during remission (90).
Tumor dormancy is a condition where small numbers of tumor cells persist in the host for months or years but do not proliferate. These cells may be responsible for late clinical relapse in hematologic malignancies and late developing metastases in solid tumors (91). Most studies of tumor dormancy have been performed in hematological tumors. For example, residual leukemic cells were detected by the polymerase chain reaction (PCR) in patients with CML in whom interferon-alpha had induced a durable cytogenetic remission (92); for example, residual disease was detected in progenitor cells derived from patients in continuous cytogenetic remission for several years. The presence of a minority of Ph-positive CML progenitor cells for a very long period of time was still compatible with durable remission, confirming that a situation of tumor dormancy had been induced.

Tumor dormancy appears as a specific form of tumor cell resistance. The question remains opened of whether it will be better to induce and/or maintain dormancy of tumor cells, or to induce cell death in residual dormant cells by targeting their survival and drug resistance mechanisms. Whichever the strategy, there is a need for a better detection of dormant disease and identification of the mechanism of tumor cell dormancy. Various mechanisms have been identified, including immunosurveillance, angiogenic dormancy, and cell cycle arrest, each of them suggesting distinct therapeutic interventions (Fig. 11.5) (91).

Cytotoxic CD8+ T lymphocytes could maintain proliferating tumor cells at a subclinical number through death induction whereas dormant tumor cells

**FIGURE 11.5.** Mechanisms involved in tumor cell dormancy. Persistence of a small number of tumor cells in the host for months or years without proliferation defines this condition that may be a specific form of tumor (stem?) cell resistance. Mechanisms involved include the inhibition of immune response, the lack of angiogenic switch, cell cycle arrest, and protective interaction with surrounding cells forming a “niche.”
would escape this immunosurveillance, either by downregulation of specific tumor-associated antigens or by expression of costimulatory molecules that induce apoptosis of CD8+ T cells (93). In a mouse model of AML, dormant tumor cells were shown to resist to apoptosis induced by cytotoxic T cells through a progressive decrease of suppressor of cytokine signaling 1 (SOCS1) gene expression due to methylation of the corresponding gene. Demethylation of SOCS1 gene, SOCS1 gene transfer, and JAK2 inhibitors all restored the dormant cell sensitivity to cytotoxic T cells. Dormant tumor cells were also resistant to apoptosis induced by irradiation, cytarabine, or imatinib mesylate through autocrine overproduction of interleukine-3 (94). These observations suggest several potential ways of therapeutic eradication of dormant cells.

Failure to induce an angiogenic response is another mechanism that may be responsible for the dormant state in some tumors. Upregulation of angiogenic factors such as vascular endothelial growth factor (VEGF) and basic (b) FGF in poorly tumorigenic cancer cell lines can reverse their engraftment potential and lead to progressively growing tumors. In this situation, the escape of tumors from dormancy depend on the so-called angiogenic switch, a discrete event that can be triggered by various signals including genetic mutations, hypoxia, and other metabolic stress, mechanical stress, and the immune/inflammatory response (95, 96).

In hematological malignancies, there are reciprocal interactions between leukemic cells and cells of the bone marrow microenvironment in which specific niches may provide a sanctuary for subpopulations of leukemic cells to evade chemotherapy-induced death, to acquire a drug-resistant phenotype, and to become quiescent. It is assumed that dormant disease is chemotherapy-resistant because dormant cells are not dividing. Induction of p21 or p27 in colon cancer cells causes G1 arrest and doxorubicin resistance in vitro. In vivo studies of green fluorescent protein-tagged disseminated breast cancer cells showed that they are growth arrested and resistant to doxorubicin. In addition, active mechanisms might protect dormant cells from chemotherapy. In squamous carcinoma cells in which a low ERK:p38 activity ratio induces dormancy, activation and induction of the eIF2α kinase RNA-dependent protein kinase-like endoplasmic reticulum kinase (EIF2AK3, also known as PERK) and the chaperone protein HSPA5, respectively, protected dormant cells from chemotherapy independently of proliferation. HSPA5 did so by inhibiting BAX (BCL2-associated protein X) activation (97–99).

11.3. RESISTANCE TO SPECIFIC DRUGS

11.3.1. Resistance to Nucleoside Analogs and Nucleobases

The anticancer nucleosides include analogs of physiological pyrimidine and purine nucleosides and nucleobases. The two primary purine nucleosides are deoxyadenosine derivatives that are used alone or in combination for the treatment of specific diseases, that is, fludarabine for chronic lymphocytic
leukemia and cladribine for hairy-cell leukemias. Among pyrimidine analogs, cytarabine and gemcitabine are used in the treatment of hematological malignant diseases whereas fluoropyrimidines are used to treat solid tumors. The nucleobases mercaptopurine and thioguanine are analogs of hypoxanthine and guanine, respectively, and remain commonly used in treating leukemias. The mechanisms of resistance to deoxynucleoside analogs include alterations in production and intracellular accumulation of their active forms, qualitative and quantitative alterations of drug-interacting proteins required for the cytotoxic effect, and alterations in DNA repair and cell death machineries (100).

Fludarabine, unlike cladribine, is administered as the soluble 5′-monophosphate form (fludarabine monophosphate) and dephosphorylated by serum phosphatases and the membrane-bound 5′-nucleotidase, CD73, before transport into the cell (101). Attempts to improve the efficacy of deoxynucleoside analogs first include their conjugation to other molecules that would improve their pharmacokinetics without affecting their activity, for example, cytarabine ocfosfate (102) and l-valyl-arac (103) have been developed recently for oral administration. In addition, high levels of deaminases in plasma can preclude intratumoral accumulation of active deoxynucleoside analogs (Fig. 11.6), and addition of a biologically inactive entity to the amino-group that is the substrate for deaminases might increase the stability of the deoxynucleoside analogs in plasma and their accumulation in tumor cells (104).

Most of the nucleoside analogs are hydrophilic molecules that require specialized nucleoside transporters to enter cells (Fig. 11.6). Nucleoside transporters include equilibrative (ENT) and concentrative (CNT) nucleoside transport-facilitating proteins. The members of each family differ in substrate specificity and sensitivity to inhibition by different molecules such as dilazep, dipyridamole, and 6-[(4-nitrobenzyl)thio]-9-β-d-ribofuranosylpurine. The abundance and tissue distribution of these transporters contributes to cellular specificity and sensitivity to nucleoside analogs (105).

Intracellular penetration of cytarabine depends on the plasma concentration. The human ENT1 is the rate-limiting factor in cytarabine uptake when administered at conventional doses (plasma concentration 0.5–1.0 µM) (106, 107), whereas simple diffusion rates exceed those of pump-mediated transport when the drug is administered at high doses (plasma concentration >10 µmol/L (108). The use of high-dose nucleoside analogs is one of the strategies designed to improve their antitumor efficacy (108) whereas the pharmacological manipulation of nucleoside transporters has not yet reached the clinical level (108).

Inside the cells, deoxynucleoside analogs are activated by intracellular metabolic steps to triphosphate derivatives. First, they are phosphorylated by deoxynucleoside kinases (four identified in humans), including deoxycytidine kinase, the rate-limiting step in intracellular anabolism of cytarabine (conversion to arabinosyl cytidine monophosphate [CMP]). Then, monophosphorylated deoxynucleoside analogs are further phosphorylated into di- and tri-phosphorylated forms by nucleoside monophosphate kinases and nucleoside diphosphate kinases (100) (Fig. 11.6). Different combinations of nucleoside analogs or other cytotoxic drugs could potentiate nucleoside analog
intracellular metabolism; for example, the combination of nelarabine and fludarabine is effective and well tolerated in fludarabine-refractory indolent diseases (109), and etoposide stimulates deoxycytidine kinase in vitro (110). It was suggested also that the use of pronucleotides, that is, monophosphorylated deoxynucleoside analogs carrying a biolabile protection of the phosphate moiety that would enter the cells by diffusion and be activated in the cytoplasm by nonspecific esterases, could bypass the deoxycytidine-kinase-monophosphorylation step (111). However, these molecules have not shown important antitumor activity in animal models.

The latest designed deoxycytidine analogs include gemcitabine (112), clofarabine (recently approved for the treatment of ALL) (113), troxacitabine

**FIGURE 11.6.** Resistance to nucleoside analogs. High levels of deaminases in the plasma can preclude intratumoral accumulation of active deoxynucleoside analogs (dNA). Most of these dNA are hydrophilic molecules that require specialized nucleoside transporters (NT) to enter cells. Inside the cells, dNA are phosphorylated by deoxynucleoside kinases. Then, monophosphorylated deoxynucleoside analogs are further phosphorylated into di- and tri-phosphorylated forms by nucleoside monophosphate and diphosphate kinases. Intracellular 5'-nucleotidases can dephosphorylate the monophosphorylated dNA. The tri-phosphorylated forms of dNA are incorporated into DNA or interfere with enzymes involved in synthesis of nucleic acids, such as ribonucleotide reductase.
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(promise results in AML and MDS in phase II trials (114), and tezacitabine (115). Gemcitabine is more lipophilic than cytarabine and is a better substrate for nucleoside membrane transporters whereas troxacitabine is a poor substrate for human nucleoside transporters and enters cells mainly by diffusion. Gemcitabine demonstrates the greatest affinity for deoxycytidine kinase. In addition, after incorporation of gemcitabine into nucleic acids, an additional natural nucleotide is added, preventing DNA repair by base-pair excision. Gemcitabine activity is self-potentiating as intracellular concentrations of normal deoxynucleotide triphosphates (particularly deoxycytidine triphosphate [dCTP]) decrease, which potentiates the drug efficacy through various mechanisms.

Deoxynucleosides and monophosphorylated deoxynucleosides can also be inactivated by intracellular deaminases, such as cytidine deaminase (CDA) and deoxycytidilate deaminase (dCMP-DA). AraC and gemcitabine are substrates of CDA and the deaminating reaction results in the production of the corresponding inactive uracil derivatives araU and dFdU. Finally, cytoplasmic 5’-nucleotidases can dephosphorylate the monophosphorylated deoxynucleoside analogs (Fig. 11.6). More recent compounds are insensitive to these degradative enzymes, for example, fludarabine, cladribine, troxacitabine, and high doses of gemcitabine are resistant to degradation by adenosine deaminase and cytidine deaminase (116).

The active forms of these drugs exert cytotoxic activity by being incorporated into and altering the DNA and RNA macromolecules themselves or by interfering with enzymes involved in synthesis of nucleic acids, such as DNA polymerases and ribonucleotide reductase (Fig. 11.6) (117). These actions result in inhibition of DNA synthesis and apoptotic cell death. The role of ribonucleotide reductase was shown to be central in the cellular response to gemcitabine (118). Some of these agents are cytotoxic to resting cells, probably through inhibition of cellular DNA repair, and their active triphosphate metabolites could interact with the pro-apoptotic factor Apaf-1 to activate the caspase cascade (119). Cladribine has also been shown to cause direct or indirect alterations in mitochondrial function (120, 121). Again, potentiation of these last steps of the cytotoxic effect of these drugs could be achieved through combination strategies, for example, an antibody targeting a stem-cell factor (anti-SCF) was shown to strongly sensitize AML cells to death induced by low-dose cytarabine (122).

The potential of nucleoside analogs to be incorporated into nucleic acids by the DNA repair machinery makes them interesting candidates for combination with DNA-damaging agents. Once incorporated, nucleoside analogs are fairly resistant to repair excision and cause irreversible damage that is recognized by the cell. Inhibition of DNA repair by nucleoside analogs also increases accumulation of DNA lesions induced by DNA-damaging agents and slows their removal. This synergistic effect may explain why the combination of fludarabine and cyclophosphamide has become the gold standard for treatment of chronic lymphocytic leukemia (123).
11.3.2. Resistance to Tyrosine Kinase Inhibitors (TKIs)

Imatinib, a 2-phenylaminopyrimidine derivative, was the first small-molecule TKI to be introduced into clinical practice. This compound targets the constitutively active tyrosine kinase, BCR-ABL, that is observed in CML cells as a consequence of the translocation of chromosomes 9 and 22 (Ph chromosome). Imatinib occupies the site in which ATP binds the ABL-kinase domain, which prevents a change in conformation to the active state of the molecule, with the subsequent death of target cells (124).

Imatinib is tremendously successful as a front-line therapy in chronic phase CML, with 80% to 90% of patients remaining progression free for more than 5 years. The initial goal of the treatment is the attainment of complete hematological response (normal peripheral-blood count; less than 5% of blasts in the bone marrow), usually before the 12th week of treatment. The next level is the complete cytogenetic remission, usually within 12 months. The last level is molecular response monitored by RT-PCR (sensitivity of $10^{-5}$) whose assessment requires serial monitoring (125).

The majority of patients with CML receiving imatinib retain residual leukemic cells that are detectable by molecular techniques (92). In addition, any response observed in patients in the more advanced stages of CML (accelerated and blastic phases) are typically short-lived. Lastly, patients treated with imatinib may eventually develop resistance, particularly those treated in the accelerated or blastic phases (125).

Mechanisms of resistance to imatinib include increased plasma protein binding, increased drug efflux, the appearance of BCR-ABL mutants (point mutations in the ABL-kinase domain) that have low affinity for imatinib, the appearance of BCR-ABL independent proliferation signals, and the amplification of the BCR-ABL gene.

Imatinib is given orally and plasma concentration varies because the molecule is neutralized by the cytochrome P450 isoenzyme 4A (CYP3A4) (126). It is essential to monitor imatinib plasma levels (they may be in excess of 1 µmol/L) as subtherapeutic dosing favors the selection of a resistant clone. Imatinib is 89%–96% bound to protein, predominantly to albumin, but also to alpha-1 acid glycoprotein (AGP). Varying concentrations of AGP could interfere with measurements of plasma concentrations as non-protein-bound imatinib is the only form that is available for cellular uptake (127, 128).

Inhibition of drug influx due to a polymorphism of polyspecific organic cation transporter 1 (OCT-1) could affect imatinib entry into cells, but a role for OCT-1 in clinical resistance is unclear (129). Excessive drug efflux by ABC transporters is also a controversial issue. ABCB1 expression confers resistance to imatinib in cell lines studied in vitro, but ABCB1-mediated efflux of imatinib is less efficient than efflux of other anticancer drugs. Imatinib can be effluxed also by ABCG2 (130), which influences its bioavailability and pharmacokinetics (131). As in AML, redundant imatinib resistance mechanisms...
may exist in CML stem cells. Therefore, expectations about the efficacy of ABC transporter inhibition as a strategy to eradicate CML stem cells in chronic phase of the disease remain limited. The use of a combination of TKIs that have overlapping molecular targets, but that do not necessarily share the same transport mechanisms, could overcome the drug transport alterations.

Overexpression of BCR-ABL due to gene amplification (likely to be related to genetic instability) is the most frequent abnormality identified in cell lines engineered to develop resistance, but point mutations in the ABL-kinase domain are more frequently identified in patients, possibly because overexpression of BCR-ABL is harmful to the cell (132). The first identified mutation was located in the ATP-binding pocket and resulted in a single amino acid change at position 315 (T315I) (133). Threonine 315 forms a crucial hydrogen bond with imatinib, and the absence of an oxygen atom in the substituted isoleucine prevents bond formation. Up to now, more than 50 mutants have been described, some at a higher frequency than others (the mutations responsible for 66% of reported cases occur at seven sites only) (125). Several groups have shown that the presence of mutations can antedate treatment with imatinib, a finding that suggests that the mutation can occur in a proliferating stem cell early in the disease course, but offers no survival advantage until exposure in vivo to imatinib. The significance of developing a mutation is now very unclear as the development of a mutation in a patient with otherwise stable disease might not be associated, at least in the short term, with any change in disease status or response to treatment (125).

Dasatinib (Sprycel; Bristol-Myers Squibb) (134, 135) is a next-generation kinase inhibitor that binds to both Src and to multiple conformations of BCR-ABL. It is capable of blocking several BCR-ABL mutants that are resistant to imatinib. Clinical trials have shown that dasatinib is effective in maintaining patients in a chronic phase and can return a percentage of patients with advanced phase to chronic phase. The imatinib-related compound nilotinib (Tasigna; Novartis) is also highly efficacious in many patients with imatinib resistant CML (135). A potential limitation of these compounds, particularly of dasatinib, is that their increased potency may be associated with untoward off-target toxicities, which probably relate to their inhibitory activity against a broader range of protein kinases than imatinib.

The T315I mutation poses a therapeutic challenge as not only does it mediate complete resistance to imatinib, but also to many of the next generation of ABL kinase inhibitors, including dasatinib and nilotinib (135). Several novel compounds inhibit the kinase activity of BCR-ABL through mechanisms of action other than interference with the ATP binding site of the kinase while preserving their specificity for BCR-ABL (136).

Even in the best responses, TKI do not eradicate all leukemic cells. Leukemic stem cells could remain resistant to imatinib by virtue of their quiescent or dormant status (91, 137), of inherent differences in the balance of drug influx or drug efflux in primitive cells compared with their more mature counterparts (53), of overexpression of BCR-ABL by gene amplification or other mecha-
nisms, of decreased protein degradation, or of inaccessibility or insensitivity of the target protein to the inhibitor, perhaps by favoring the active conformation or the development of ABL-kinase mutations (132, 137). The most promising approach to eradicate the residual leukemic cells would seem to be the combination of a TKI with another agent, for example, a signal-transduction inhibitor (e.g., a farnesyl transferase inhibitor) (138), inducing the cell cycle (e.g., by growth factors) (136) or immunological methods, such as antigen-specific T cells (139).

In addition, new molecules are currently tested, such as those targeting the Src family of kinases (136). Kinases of this family, more specifically Lyn, Hck, and Gfr, are involved in the pathophysiology of BCR-ABL1–positive B-ALL. The conformation of the kinase domain of ABL closely resembles that of the inactive Src kinases, and a series of molecules has been identified that demonstrate overlapping activity against both ABL and SFKs and enhanced activity against imatinib-resistant BCR-ABL kinase mutant isoforms. Two of them have been introduced in clinics. Bosutinib (also known as SKI 606), whose dose-limiting toxicity is a severe rash, is currently being tested in patients with CML who have become resistant to imatinib or the second-generation TKIs, nilotinib and dasatinib (140). INNO-406 (also known as NS-187), which is a potent inhibitor of ABL and Lyn kinase, is being currently tested in a phase I trial (141). Preclinical studies suggest that the molecule could be efficient in treating central nervous system localization of BCR-ABL1–positive leukemias (6%–12% of patients) (136).

The BCR-ABL1 T315I mutation can be detected in 10%–20% of patients after failure of imatinib therapy and confers resistance to all currently approved agents for the treatment of CML, including nilotinib and dasatinib. A first strategy pursued in an attempt to overcome T315I-mediated resistance is the development of small molecules targeting BCR-ABL motifs outside the ATP-binding domain of BCR-ABL. One such agent, ON012380, blocks the substrate binding site of ABL (142) whereas another one, GNF-2, blocks the autoregulatory myristate binding cleft of BCR-ABL located at the N-terminus, which results in stabilization of the protein in an inactive state with an exceptional selectivity (143). Another strategy is the modeling of molecules amenable to the accommodation of structural constraints imposed by this mutant. Third, some molecules designed to target other kinases such as the human Aurora kinases (144) and the p38 MAPK (also known as MAPK14) (136) have shown off-target effects that include the inhibition of BCR-ABL T315I kinase. Lastly, homoharringtonine has recently been shown to inhibit the proliferation of imatinib-resistant cells expressing the T315I mutation, both in vitro and in vivo, which provided the rationale for an ongoing Phase II trial (145).

Other tyrosine kinases are now targeted in hematological malignancies. The emergence of drug resistance will be addressed for each of the small molecule inhibitors developed. Based on the mutation of the conserved gatekeeper threonine residue (T315I) identified in BCR-ABL, structurally related mutations have been described in other kinases such as KIT (T670I) in GIST,
EGFR (T790M) in NSCLC, and FIP1L1-PDGFR α (T674I) in hypereosinophilic syndrome, and compounds are being investigated as potential inhibitors of these gatekeeper mutants (146).

To date, ABL kinase inhibition has not yielded cures in patients with CML, and the general consensus is that combination approaches will be required to eradicate BCR-ABL1-positive leukemic clones. Although the clinical relevance of the novel BCR-ABL kinase inhibitors has yet to be proved, it is tempting to speculate that the combination of potent dual ABL and SFK inhibitors (e.g., bosutinib) with TKIs that target BCR-ABL motifs other than the kinase domain (e.g., GNF-2), might provide a powerful strategy to simultaneously inhibit multiple nonoverlapping BCR-ABL-related elements. The clinical development of these combinations must be preceded by a systematic effort to track their effects on molecular targets in vitro.

11.4. CONCLUSIONS

MDR in leukemic cells can also be mediated by defects in the core regulators of the cell death pathways, mainly those leading to apoptosis. Drugable targets have been identified in the mitochondrial and the death receptor pathways of caspase activation, for example, inhibition of Bcl-2 and IAPs and activation of TRAIL receptors (147, 148). Smac mimetics simultaneously target the two pathways. These XIAP antagonists that relieve caspase-9 inhibition in the intrinsic cell death pathway also promote cIAP degradation, especially that of cIAP1, which activates the extrinsic pathway. Smac mimetics stimulate cIAP autoubiquitination and degradation, leading to NF-κB activation and TNFα secretion. The autocrine TNFα signaling in turn induces caspase-8 activation and cancer cell death (149). Several molecules targeting cell death regulators have now entered clinical trials, including antisense oligonucleotides, natural products, and synthetic chemicals targeting Bcl-2, antisense oligonucleotides targeting XIAP, and an agonist antibody against TRAIL receptor DR4 (Mapatumumab) (150). Early phase clinical trials testing Bcl-2 antisense have demonstrated that the molecule was safe, but phase III studies demonstrated limited efficacy. Currently, ongoing trials testing small molecule Bcl-2 inhibitors such as Obatoclax (151) and ABT-737 (152–154) will be informative as to whether these other inhibition strategies are more effective in treating leukemias.

It has been demonstrated in several hematological malignancies that cell-cell or cell-matrix adhesion processes were also involved in chemoresistance. The integrin which mediates adhesion of hematopoietic cells onto the extracellular matrix (fibronectin) or stromal cells has recently been involved in cell adhesion-mediated drug resistance (CAM-DR) of AML in vitro and in vivo (155). In addition, the phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B, PKB)/mammalian Target Of Rapamycin (mTOR) signaling pathway is frequently activated in blast cells from patients with AML (156). The
conclusive activation of this signal transduction cascade could contribute to drug-resistance of leukemic cells and may represent a target for innovative therapeutic approaches. Several pharmacological inhibitors that block this pathway will probably be tested in the future, either as apoptosis inducers or chemosensitizers (157, 158).

It was suggested recently that, depending on the death pathways activated, drug-induced apoptosis of malignant cells could stimulate an antitumor immune response or not. For example, exposure of cancer cells to anthracyclines or ionizing radiation triggered a death that produced a potent immune response in vivo whereas other tested DNA damaging agents did not. The anthracycline effect was mediated by exposure at the cell surface of calreticulin, a calcium-binding protein that is localized in the lumen of the endoplasmic reticulum in untreated cells. Its exposure occurs before the first morphological signs of apoptosis and involves the phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α). The potent antitumor immune response is mediated by the rapid recognition and phagocytosis of apoptotic tumor cells by dendritic cells, leading to cytotoxic T-cell response (159). Other drugs can induce immunogenic cell death through distinct mechanisms that include induction of heat shock proteins at the plasma membrane or release of inflammatory mediators or late-stage degradation products (160).

The ubiquitin-proteasome pathway is vital in the degradation of normal proteins involved in cell cycle progression, proliferation, and apoptosis and abnormal proteins that result from oxidative damage and mutations. Transformed cells can be significantly more sensitive to proteasome inhibition than normal cells (161), and proteasome inhibitors were proposed as chemosensitizers. Bortezomib (PS-341), a dipeptide boronic acid analog that is the best-known proteasome inhibitor exhibits substantial potency against multiple myeloma and non-Hodgkin lymphoma. In some cases, drug resistance could be overcome when proteasome inhibitors are combined with traditional chemotherapeutics (162). Plant polyphenols that include genistein, a soybean isoflavone, curcumin (diferuloylmethane) derived from the rhizome of an East Indian plant, and polyphenolic catechins found in green tea could synergize with classical anticancer agents in a variety of drug-resistant human cancers (163). Their in vivo efficacy remains to be demonstrated.

A long time has passed since drug resistance of leukemic cells was summarized by P-gp overexpression. Improving knowledge in drug resistance mechanisms, including those common to structurally distinct anticancer molecules and more specific mechanisms of resistance to a given drug or family of compounds, has suggested a number of strategies to improve the efficacy of current therapeutic approaches. We have learned from previous studies that simultaneous modulation of several ABC transporters was probably better than specific modulation of one of them to eradicate leukemic stem cells (50) and that nucleoside analogs can be very potent when combined with DNA-damaging agents (123). Current results with Abl inhibitors indicate that the combination of a potent tyrosine kinase inhibitor targeting both Abl and the
Src kinases with another tyrosine kinase inhibitor that targets Bcr-Abl motifs other than the kinase domain could possibly eliminate the residual disease in CML (136). Many other efficient combinations have been identified in vitro, for example, the combination of a small molecule tyrosine kinase inhibitor with a Bcl-2 antagonist (153) or rapamycin (164). Epigenetic approaches including histone deacetylase inhibitors and DNA-hypomethylating agents that promote transcription of silenced genes (165) and new kinase inhibitors targeting fms-like tyrosine kinase 3 (166) or ras (167) are currently developed either alone or in combination with other molecules (168). Each of these approaches may encounter resistance mechanisms while reversing resistance to some other compounds. In the coming years, the leukemia cure will probably need to select an appropriate combination of molecules based on a careful analysis of the characteristics of leukemic cells, with a specific attention paid to those identified as leukemic stem cells.

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MULTIDRUG RESISTANCE REVERSAL IN SOLID TUMORS

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12.1. INTRODUCTION

The first demonstration that verapamil, a calcium channel inhibitor, could reverse multidrug resistance (MDR) in vitro was made as early as 1981 by the group of Tsuruo (1). Rapidly, a number of scientists have confirmed this observation and identified, essentially on in vitro models, many other compounds with the same properties (see Part IV, Chapter 8). Within a few years, all of the compounds we now classify as “first-generation reversing agents” had been discovered, and several of their analogs had also been tested on the same models. This generated an important hope of being able to take advantage of these compounds for the reversal of drug resistance in the clinical setting. Several clinical trials were rapidly designed for the combination therapy of hematological malignancies (see Part IV, Chapter 11) and solid tumors. Without anticipating the conclusions of this chapter, one can say that the disappointment was at the same magnitude as the hopes that had been put into resistance reversal. We will describe in this chapter the clinical trials that were undertaken with that aim, and we will try to analyze the reasons for this failure.

12.2. CLINICAL TRIALS AIMED AT MDR REVERSAL IN SOLID TUMORS

Several dozens of compounds aimed at MDR reversal have been brought to clinical trials, but the development for most of them has been discontinued, either for lack of activity or due to toxicity. We will not review here all of these clinical trials but will rather focus on the compounds that are still under clinical evaluation in solid tumors, with the exception of the two historical leaders, verapamil and cyclosporine A, which deserve some analysis. Excellent reviews have been regularly published on this subject, to which the reader is referred (2–5).

12.2.1. Verapamil and Derivatives

As early as 1987 the first clinical trial of MDR reversal with verapamil was published (6). It concerned eight ovarian cancer patients treated with doxorubicin; the trial had to be discontinued because of the significant cardiac toxicity of verapamil, and no conclusions could be drawn, except that analogs devoid of proper pharmacological properties had to be developed. Whereas the proof-of-concept of MDR reversal with verapamil was brought in hematological malignancies (non-Hodgkin’s lymphoma and myeloma, see Part IV, Chapter 11), no such evidence was obtained in solid tumors, possibly because only low doses of verapamil were used by fear of cardiac toxicity (7–9). In a study on nonsmall cell lung cancer (10), encouraging results were obtained but were never confirmed. Similarly, Cairo et al. (11) have shown interesting preliminary results in pediatric malignancies when combining verapamil to
vinblastine and etoposide, but this remains an orphan study. More recently, a randomized study performed in anthracycline-resistant metastatic breast cancers treated with vindesine, combined or not with verapamil, revealed a significantly higher survival of patients receiving verapamil (12).

Since verapamil is a racemic mixture of two enantiomers whose activity on MDR is similar whereas only the S-form is theoretically active on calcium channels (13), the R-form (dexverapamil) was developed as an MDR-reversing agent. A phase I study combining dexverapamil with doxorubicin (14) has shown the feasibility of this approach, which has encouraged the implementation of phase II trials, especially in breast cancer (15, 16), colorectal cancer (17), and renal cancer (18). Only some responses were obtained in the first trial (15), and the drug was not developed further because of some cardiac toxicity that was observed at high dexverapamil dosage.

12.2.2. Cyclosporin A and Derivatives

Like verapamil, cyclosporine A entered very early into clinical trials of MDR reversal. There again, the inherent pharmacological properties of this potent immunosuppressor were hardly compatible for combination with anticancer drugs. In addition, it became rapidly obvious that cyclosporine A exerted a major pharmacokinetic effect, with an almost doubling of the AUC (area under the curve) of the anticancer drug. The positive effects that were noticed could, therefore, be related either to a pharmacodynamic effect (inhibition of P-glycoprotein at the membrane level of the cancer cell) or to a pharmacokinetic effect (higher plasma concentrations allowing higher cancer cell uptake). Whereas the proof-of-concept of cyclosporine A activity was brought in hematological malignancies, no evidence of recruitment of new responders was brought in colorectal (19) or renal (20) carcinomas.

As for verapamil, an analog devoid of the immunosuppressive properties of cyclosporine A was developed and extensively studied, PSC833, now known as valspodar (21). This compound also exerts a major effect on the pharmacokinetic behavior of all the anticancer drugs tested (doxorubicin, paclitaxel, etoposide, mitoxantrone). Reducing the dose of valspodar for the clinical trials of MDR reversal was proposed during the development of this reverter but did not attract clinicians to enter patients into such clinical trials. Several phase I, dose-finding studies were undertaken, combining valspodar with paclitaxel (22–24), vinblastine (25), or doxorubicin and paclitaxel (26). They have shown the feasibility of the reversal approach and demonstrated that in vivo inhibition of P-glycoprotein function actually occurred.

Few phase II studies have been conducted with valspodar outside the field of hematology. In metastatic breast cancer, the combination of valspodar with paclitaxel was not clearly more active than single agent paclitaxel (27). No activity at all was seen in renal cancer when valspodar was combined with vinblastine (28). In ovarian cancer, two phase II studies combining valspodar with paclitaxel (29) or with doxorubicin and cisplatin (30) were published.
They show clear evidence of valspodar recruitment of new responders to chemotherapy in situations where resistance was clearly documented. However, in a phase III study comparing paclitaxel and carboplatin combined or not with valspodar, the addition of valspodar did not improve time to progression or overall survival and was more toxic in patients with advanced ovarian cancer (31).

12.2.3. Quinolines

Quinidine entered very early into clinical trials for the reversal of anthracycline resistance in resistant metastatic breast cancer (32), but no significant difference emerged when compared to the anthracycline alone. The important cardiac toxicity of quinidine outside its use in cardiology did not encourage the development of other clinical trials. Using the same approach as for verapamil and cyclosporine A, structural analogs devoid of cardiac toxicity were designed for further experimentation in MDR reversal. Quinine is a stereoisomer of quinidine, also largely used in the clinics. It is much less toxic than quinidine and has also been used in clinical trials of MDR reversal. There again, proof-of-concept studies revealed potential reversing activity in hematological malignancies, but that was never tested in solid tumors. The situation is the same for cinchonine, the demethoxylated derivative of quinine.

An original quinoline compound, MS-209 or dofequidar, specifically designed for MDR reversal and belonging as such to the third generation of MDR reverter agents, has recently entered clinical trials. In a phase I study, Diéras et al. (33) have shown the feasibility of its combination with docetaxel in advanced breast cancer. Recently, in a randomized phase III study including 221 breast cancer patients, Saeki et al. (34) detected, in the dofequidar arm, a 24.6% relative improvement, a 10.5% absolute increase in overall response rate \((p = 0.077)\), and a trend for prolonged progression-free survival \((366 \text{ vs. } 241 \text{ d}; p = 0.145)\). The survival advantage was significant in premenopausal patients who had no prior therapy and were stage IV at diagnosis with an intact primary tumor. This appears to be one of the most positive results of MDR reversal in this disease.

12.2.4. Agents Specifically Designed for MDR Reversal

After the successive failures of the compounds of the first and second generation of researchers, researchers of the pharmaceutical industry tried to design novel reversing agents based on a concept different from analog development, taking advantage of the understanding of the molecular features that systematically characterized P-glycoprotein-interfering molecules (35, 36). Using this approach, several compounds emerged and were progressively brought up to clinical trials after \textit{in vitro} and \textit{in vivo} evidence of highly potent MDR-reversing properties, active in the nanomolar range, whereas verapamil was active at concentrations higher than 10\(\mu\)M and cyclosporine or valspodar at 1\(\mu\)M. Four
CLINICAL TRIALS AIMED AT MDR REVERSAL IN SOLID TUMORS

such compounds are still under evaluation in this respect: biricodar, elacridar, zosuquidar, and tariquidar. Results of phase II studies have not all been published and are eagerly awaited by the scientific community.

Biricodar (VX-710) is a piperidine carboxylate that was first explored in two phase I trials in combination with doxorubicin (37) or paclitaxel (38). These phase I studies have shown no interference with doxorubicin pharmacokinetics but a significant reduction in paclitaxel clearance. Increased hepatic uptake and retention of $^{99m}$Tc-sestamibi in the presence of biricodar has encouraged further development. A series of phase II studies in a variety of solid tumors have shown a potential interest in the combination of biricodar with doxorubicin in soft-tissue sarcomas (39) and with paclitaxel in advanced breast cancer (40), but no evidence of activity was detected in ovarian cancer (41), prostate hormone-refractory cancer (42), and nonsmall lung cancer (43).

Elacridar (GF-120918) is an acridone carboxamide specifically designed for MDR reversal and that been shown to be active also on an ATP-binding cassette (ABC) pump distinct from P-glycoprotein and called BCRP or ABCG2. Phase I studies revealed no interaction with the pharmacokinetics of doxorubicin (44, 45) and have prepared the way for clinical evaluation of this combination in solid tumors. The main interest in this compound may well come from its activity on intestinal ABC proteins, since it has the potential to increase the uptake of orally administered anticancer drugs such as topotecan (46, 47) and paclitaxel (48).

Zosuquidar (LY-335979) is a difluorocyclopropyl dibenzosuberane derivative that has shown no interference with doxorubicin (49, 50), docetaxel (51), or vinorelbine (52) pharmacokinetics in phase I clinical trials, which allows its evaluation in combination with full doses of anticancer agents. No proof of activity has yet been obtained in solid tumors in a phase II setting.

Tariquidar (XR-9576) is an anthranlyc acid derivative also devoid of pharmacokinetic interactions with the cytotoxin (53). Phase I studies have demonstrated an effect of this drug on functional parameters related to P-glycoprotein inhibition (53, 54). In a phase II study on metastatic breast cancer patients (55), tariquidar showed limited clinical activity to restore sensitivity to anthracycline or taxane chemotherapy.

12.2.5. Other Approaches

In addition to the classical approach of MDR reversal with “small molecules,” numerous other ways have been explored, such as the use of P-glycoprotein targeted antibodies such as UIC2 (56) or the use of antisense strategies targeting the MDRI messenger RNA (57). More recently, the development of transcriptional regulators (58) and of interfen RNAs (59) appears promising. However, as long as we do not know whether the MDR reversal strategy is worthy of development, these approaches appear even less realistic than the “small molecule” approach. The encapsulation of anticancer drugs in liposomes (60) or nanospheres (61) has also been claimed to be able to circumvent
MDR. Among the various formulations of liposomal anthracyclines that have been studied and even marketed, none appear able to recruit responders outside the usual field of anthracycline activity. An original approach was suggested by Ludwig et al. (62) who have shown that some drugs could be specifically more active on MDR cells than on cells devoid of P-glycoprotein overexpression. It would be tempting to develop this approach in the clinical setting.

Finally, lonidamine, a compound affecting energy metabolism by inhibiting mitochondrial hexokinase and the electron transport chain, has been developed in an attempt to restore drug accumulation in MDR cells by energy deprivation (63). Some responders can be recruited by combining lonidamine to anthracycline-based chemotherapy in metastatic breast (64) or nonsmall-cell lung cancer (65), and an increase in patients' survival has also been mentioned. As pointed out in 2003, the total number of trials reported in the literature and the length of follow-up are still insufficient to draw firm conclusions (66), but large studies combining lonidamine with drugs extruded by adenosine triphosphate (ATP)-requiring transporters remain warranted.

12.3. MDR REVERSAL IN SOLID TUMORS: THE REASONS FOR A FAILURE

At every step in the first part of this review, negative results were far more numerous than positive results. Twenty years after the first clinical trial of MDR reversal, the initial questions remain: Why have we not succeeded? Will it work someday? At least three conditions must be fulfilled for the success of this approach: (i) The tumors to be treated must be resistant to chemotherapy, at least in a significant part, through the MDR mechanism that is targeted; (ii) The inhibition of P-glycoprotein (or another pump) should be feasible in tumor cells in vivo without deleterious effects in normal tissues expressing the pump; and (iii) The compounds used as MDR reversing agents should not have toxicity preventing a safe usage; it should be within the limits of the toxicity acceptable for anticancer treatments.

12.3.1. Tumors Should Be Resistant through the Mechanism Targeted

The proof of clinical resistance was not definitively provided in the first phase II studies that were designed for MDR reversal. It is well known that resuming a treatment after a delay of several months may recruit new responses to this treatment. There should be a clear documentation of ongoing clinical resistance for including patients in such phase II studies in order to determine whether the adjunction of a reverter modifies tumor response. In addition, inappropriate selection or absence of selection of patients for targeted inhibition of drug resistance mechanisms might well have been the most important cause of failure in clinical assessment of efficacy of MDR inhibitors. For
example, no improvement of chemotherapy can be expected for nonsmall-cell lung carcinoma by P-glycoprotein modulators without assessment of tumor P-glycoprotein, since this protein is not expressed in most of these tumors. The development of targeted therapies has been possible, due to the identification of the target in the tumor to be treated. The same attitude should prevail for the development of these targeted modulators. Unfortunately, the diagnosis of MDR remains difficult and interlaboratory differences still prevent any consistent view of the effect of these drugs on the MDR phenotype. Therefore, it appears impossible to interpret correctly the data from the clinical trials in the absence of a credible determination of the MDR phenotype of the tumor treated. From a theoretical point of view, neither immunohistochemistry nor quantitative PCR assay of MDR1 gene expression would be satisfactory, since only evaluation of drug accumulation and/or efflux may reflect the actual function of the P-glycoprotein in cancer cells. This can be easily carried out for circulating cells (hematological malignancies) but not for solid tumors.

When it is clear that an MDR-reversing agent is being tested on a truly drug-resistant cancer expressing the target mechanism (most often P-glycoprotein), the question arises as to whether this mechanism is unique and responsible for the drug resistance of the tumor. This may not be always the case. Tumors may be resistant by additive contributions of several ABC transporters. Under these conditions, drug resistance reversing agents should be only tried for cancers for which resistance is actually mediated by P-glycoprotein. Defining the tumor type for drug development should, therefore, be the most important step for this type of approach. In this respect, hematological malignancies certainly appear as privileged for MDR reversal, since this mechanism is likely to be preponderant in non-Hodgkin’s lymphoma as in acute myeloid leukemias.

12.3.2. Inhibition of P-glycoprotein Should Not Have a Deleterious Effect on Normal Tissues

The efficacy of reversing agents has been generally well documented on in vitro models, but these models do not mimic the in vivo requirements. For instance, protein binding is quite different, and the fraction of unbound drug is not the same in vitro and in vivo. It is obvious that we lack in vivo models of MDR. Some MDR tumors have been developed for growth in nude mice (67) and have allowed the demonstration that only a small number of the MDR reverters that work in vitro can significantly reverse MDR in vivo. Such a demonstration is relatively difficult to accomplish for large-scale screening of potential reverters because of the delays in tumor growth and in establishing schedules for treatment in the absence and presence of the reversing agent. However, this step is necessarily required between the in vitro screening steps and the introduction of the MDR reverter in the clinical setting. Another useful in vivo model for large-scale screening was proposed by the group of Gottesman in the 1990s. Galski et al. (68) had produced a strain of transgenic
mice overexpressing the human MDR1 gene in their bone marrow. This model allowed evaluation of the effect of a reverter by restoration of the hematological toxicity of the cytotoxin without requiring a tumor xenograft. However, this strain progressively lost transgene expression and has never allowed the identification of new reverters active in vivo. An absence of deleterious effects of the reversing agent on normal tissues expressing P-glycoprotein must be demonstrated. P-glycoprotein is present in the endothelial cells of vessels present in the central nervous system and the testis, with a clear role of protection of these organs from the compounds present in the bloodstream (69). In these conditions, it could be dangerous to administer a potentially neurotoxic drug combined with a strong inhibitor of P-glycoprotein. In addition, such an inhibition of hepatic P-glycoprotein may lead to a major alteration of anticancer drug disposition, with pharmacokinetic changes that may increase the general toxicity of the treatment. There is, therefore, a relatively narrow window for the use of MDR-reversing agents, the most active ones on tumor cells being also those that increase most of the general toxicity of the anticancer agent.

12.3.3. Reversing Agents Should Not Have an Intrinsic Toxicity That Prevents Safe Usage

This important condition was put at the top level by pharmaceutical companies that are developing MDR-reversing agents. This explains the successive steps that were followed and the three generations of compounds that were developed. The first step was the use of compounds already used clinically for other specific therapeutic applications. Obviously, these compounds had an intrinsic toxicity because they were pharmacologically active, and this hindered the use of effective MDR reversal doses of these compounds. This was the case for verapamil, cyclosporine A, and quinidine, which cannot be used safely at the dosages required for MDR reversal. The second step was to identify analogs of those compounds that were devoid of the pharmacological properties of the original molecule. This approach was quite interesting and should have led to the identification of clinically useful compounds. However, because no toxicity was accepted for these compounds in the clinical trials, the stringent conditions that followed have prevented the complete development of such drugs. The third step consisted of the design of novel molecules on the basis of structural features (lipophilicity, positive charge at neutral pH, presence of aromatic rings). There again, the challenge of a complete absence of proper toxicity has prevented the full evaluation of compounds presenting major MDR-reversal properties. The requirements of high efficacy and total lack of toxicity may have been set at too high a level for a positive identification of clinically useful compounds. Dealing with a disease for which the positive effect of a drug or a combination of drugs is evaluated in months of survival should have led to the acceptance of some toxicity balanced against the gain in survival. As a consequence, despite the fact that the “proof of
principle” has been given in several instances at the phase II level for several drugs, very few phase III trials have been conducted, and not a single compound has been approved for routine use or is close to such an approval. This is unfortunate because it appears that it would have been possible to bring valuable compounds to the clinics if a better understanding among clinicians, pharmacologists, and the pharmaceutical industry had existed.

12.4. CONCLUSIONS

The major hopes that were put into MDR reversal as a therapeutic strategy in the 1990s have not been materialized. Certainly, one of the major causes lies in the fact that P-glycoprotein-mediated MDR is only one of the mechanisms used by the cancer cell to thwart the effects of anticancer drugs, especially in solid tumors. However, the clinical trials have not generally been carried out with efficient designs, which allowed the trials to reach unambiguous conclusions. The perfect MDR reverter probably does not exist, and neither does the perfect anticancer drug. Selecting appropriate malignancies expressing P-glycoprotein as a major cause of resistance, appropriate anticancer drugs behaving as major substrates for P-glycoprotein, and appropriate reversing agents at their optimal dosage would be required for the demonstration that MDR reversal is a valid approach.

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PART V

BIOLOGICAL AND CLINICAL ASPECTS OF MULTIDRUG RESISTANCE: THE ROLE OF THE TRANSPORTERS AT THE MAIN PROTECTION BARRIERS (ABCB1, ABCC1, ABCC2, ABCG2) ON THE BIOAVAILABILITY OF MANY TYPES OF DRUGS AND MEDICATIONS
13

ABC SUPERFAMILY TRANSPORTERS AT THE HUMAN BLOOD–BRAIN BARRIER

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13.1. INTRODUCTION

The delivery of drugs in the brain remains a challenging question for treating brain diseases. One of the main obstacles is the drugs’ weak permeability across the main physiological interface separating the brain tissue from the bloodstream, that is, the blood–brain barrier (BBB). As a consequence, the BBB is the major limiting step to drug distribution in brain tissues. One of the reasons for poor drug permeability across the BBB is related to the dense expression of several ATP-binding cassette (ABC) proteins that confer their common multidrug resistance (MDR) properties at this physiological barrier. Brain diseases may affect the BBB permeability properties. For example, brain parenchyma invasion by tumor tissue associated with angiogenesis renders heterogeneous, at the anatomic level, the exchange properties between blood and tumor brain tissues and modifies the regulation of the molecular targets involved in the pathways of ABC transporters. After a brief review of normal and pathological BBB properties, we will address the expression and role of the ABC transporters presently identified in the human normal and diseased brain and will conclude by addressing the role of the ABC transporters involved in the efficacy of the drugs used to treat brain diseases.

13.2. THE BBB IN A NORMAL PHYSIOLOGICAL STATE

The BBB is formed by the endothelial cells of the brain capillaries, which represent approximately 95% of the total area of the barriers between blood and brain (1). Another barrier is the blood–cerebrospinal fluid barrier that lies at the choroid plexuses floating in the brain ventricles. It is formed by a single layer of epithelial cells that separates the plexus blood from the cerebrospinal fluid (CSF); this barrier mainly contributes to the formation of CSF and governs the exchange of water, ions, and substances between the two fluids (2).

The BBB is formed by a complex architecture of vascular and perivascular cells that constitutes a functional unit called the neurovascular unit. The blood capillaries of the central nervous system of vertebrates are enveloped by a perivascular sheath of glial cells, mainly astrocytes, which forms a discontinuous sheath around the vascular walls. While the astrocytes themselves do not form the barrier, they play an important role in the development and maintenance of the BBB by releasing factors that can modulate BBB functions (3). The endothelial cells are also separated from the astrocyte foot processes by a basement membrane and by pericytes that lie peri-endothelially on the abluminal side of the capillaries and cover around 20%–30% of the microvasculature circumference (Fig. 13.1) (4). This neurovascular unit is the main interface controlling the uptake of drugs into the brain parenchyma, and, hence, it is the target to overcome for delivery of drugs to the brain (5).

It has long been held that the restrictive permeability of the BBB to solutes is principally due to the presence of complex tight junctions, in addition to the
adherent junctions, between adjacent endothelial cells of the brain microvasculature. This complex molecular network, the absence of fenestrations in the capillary wall, and the sparseness of the pinocytic vesicular traffic together make the brain inaccessible for the flux of hydrophilic compounds across the BBB. The remaining transeellular pathways are responsible for the direct permeation through the endothelial cell membranes of diffusible low-molecular-weight and lipophilic solutes and active carrier-mediated transporters that may or may not be expressed symmetrically on the plasma membranes of the endothelial cells. Receptor- or adsorptive-mediated transcytoses may be other ways for some macromolecules, proteins, and peptides to enter the brain parenchyma (Fig. 13.1) (6).

It is now accepted that the BBB is not only a physical barrier but also involves protein-mediated transport activities. Carrier-mediated transport across the BBB is used by many hydrophilic nutrients, including glucose,
amino acids, nucleosides, and monocarboxylic acids (5). Less attention has been paid to the efflux properties of the BBB, that is, carrier-mediated transport can prevent drugs from entering the brain by exporting them from the brain endothelial cell membranes to the blood compartment or by facilitating their removal from the brain interstitial fluid to the blood.

The carrier-mediated efflux of drugs at the BBB is probably the most important discovery that has occurred in the past decade of BBB research. The weak permeability of several classes of anticancer drugs such as vinca-alkaloids, anthracyclines, camptothecins, and taxane derivatives across the BBB was evidenced in the 1990s, when the role of an efflux pump at the luminal side of the BBB, P-glycoprotein (P-gp), was evidenced. All these anticancer agents are transported out of the brain by P-gp or by other ABC transporters, and it is now accepted that the physicochemical properties of these compounds, such as their hydrophilicity or molecular weight, are not the only reasons why they do not readily enter the brain.

### 13.3. ABC TRANSPORTERS OF THE BBB

P-gp is the first human ABC transporter to be cloned and is the best-known transmembrane member of the ABC superfamily. Among the 48 human ABC proteins, only a few are implicated in the so-called MDR phenotype (7, 8). P-gp was first shown to be implicated in this phenomenon in 1976 by Juliano and Ling (9), but little or no attention was paid to the impact of ABC transporters in pharmacology and toxicology until the pioneering studies of Cordon-Cardo et al. (10) and Thiebault et al. (11) in 1989. They showed that P-gp is present in a very wide range of normal human tissues, including the endothelial cells of the BBB.

Members of three of the seven ABC protein subfamilies (B, C, and G) are presently known to act as drug transporters at the levels of the BBB and the cells of the brain parenchyma (12). The ABCB subfamily is represented by P-gp (ABCB1), the ABCC subfamily by several multidrug resistance-associated proteins (MRPs), and the ABCG subfamily by the breast cancer resistance protein (BCRP or ABCG2).

No consensual characterization of the ABC proteins at the BBB has yet been published, and some proposed localizations remain uncertain because of great differences between the results of experimental studies. Some studies have investigated gene transcripts while others have assayed the proteins themselves in various tissues, including isolated brain capillaries, immortalized cells, or primary cultures of brain microvessel endothelial cells from several species. Because three types of cells contribute to the BBB, the endothelial cells, the glial cells, and the pericytes, the use of isolated brain capillaries cannot ensure that the detected transcript or protein belongs only to the endothelial cell unit; it could belong to the pericytes or glial end feet that cannot be completely removed during the preparation of brain capillaries.
Cultures of endothelial cells do not suffer from this contamination, but the culture conditions, with or without glial cells, are known to frequently influence the activity of these genes. The polarity of the brain capillary endothelial cells is also critical. Hence, the finding of an ABC mRNA or protein does not reveal which membrane, luminal or abluminal or both, contains the ABC transporter. The most common methods used for immunohistochemistry with light and electron microscopy can produce many artifacts due to sample preparation or the use of unspecific antibodies. Only sophisticated experiments based on the biochemical isolation of the luminal or abluminal membrane or on \textit{in situ} immunofluorescence studies can provide the precise location of the ABC protein at the BBB. Some of these methods are combined with \textit{in vitro} or \textit{in vivo} functional experiments on a wide range of substrates and inhibitors of ABC transporters. However, the specificities of these substrates and inhibitors frequently overlap between ABCs and other families of drug transporters, sometimes leading to confusing observations. Mice whose genes that encode several ABCs have been knocked out have proved to be very powerful tools for investigating the presence and function of ABC transporters at both barriers. Nevertheless, data obtained by comparing wild-type and ABC knockout mice cannot simply be extrapolated to other animal species or to humans.

Therefore, the recent development of quantitative liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) methods for membrane protein measurement has opened the access to a quantitative atlas of ABC membrane transporter proteins at the mouse and human BBBs (13). This quantitative proteomic analysis will help to clarify the unresolved question on the functional expression of ABC transporters at the BBB among species. A preliminary result compares the quantitative levels of the ABC proteins found in human isolated brain microvessel preparation to the mRNA levels. ABC protein and mRNA profiles closely correlate, and these combined data suggest that ABCB1, ABCG2, ABCC4, and ABCC5 are the main ABC proteins present in the BBB in both rodents and humans (Fig. 13.2).

13.3.1. P-gp or ABCB1

\textbf{P-gp at the Luminal Membrane of Brain Capillary Endothelial Cells} While humans have only one gene (\textit{MDR1}, now called \textit{ABCB1}) that encodes the drug transporter P-gp, rodents have two genes, \textit{mdr1a} and \textit{mdr1b}, that encode closely related P-gps with overlapping substrate specificities (14). Nevertheless, \textit{mdr1a} is the major gene expressed in the luminal membrane of brain capillary endothelial cells of rodents, while \textit{mdr1b} is the main gene expressed in the brain parenchyma.

In 1997, Partridge et al. (15) gave rise to some uncertainty as to the exact location of P-gp at the BBB as they asserted that they had found the protein on the astrocyte processes and not on the membranes of the brain capillary endothelial cells. Several studies published before and after that statement showed that P-gp lies in the brain capillary endothelial cells at the luminal
membrane. P-gp mRNAs have been detected by real time–polymerase chain reaction (RT-PCR) in the rat brain, and by RT-PCR and Western blotting in bovine, porcine, and rodent primary cultures of endothelial cells (16–18). Most light and electron microscopy immunochemical experiments using several anti-P-gp antibodies indicate that P-gp (mdr1a and MDR1) is inserted into the luminal membrane of the cerebral endothelial cells of microvessels and not into the abluminal side (19, 20). An elegant study by Beaulieu et al. (21) showed that P-gp was concentrated on the luminal side of membranes purified from rat brain capillary endothelial cells by density gradient centrifugation. This preparation contained a much higher concentration of P-gp than did the rest of the brain tissue. Thus there is little doubt that P-gp lies on the luminal side of the BBB in both humans and animals.

Nevertheless, recent investigations examined the cellular/subcellular distribution of P-gp in situ in rat and human brain tissues using immunogold cytochemistry at the electron microscopy level. P-gp was found as localized on both the luminal and abluminal membranes of capillary endothelial cells as well as on adjacent pericytes and astrocytes (22).

**P-gp as a Gatekeeper in the BBB** Schinkel et al. were the first to suggest that P-gp acted as a gatekeeper in the BBB (23). The strength of this metaphor has received considerable support over the past 13 years. The study of mice in which the mdr1a gene or the mdr1b gene is disrupted or in which both the mdr1a and mdr1b genes are disrupted has helped demonstrate that P-gp in the BBB limits the entry of many drugs into the brain (24). The most convincing
phenotype has been found in mdr1a knockout mice that have no P-gp in their BBB and are 100 times more sensitive than wild-type mice to the neurotoxic pesticide ivermectin, which was initially sprayed on these mice to treat a mite infestation (23). The result well illustrates that P-gp is a first line of defense at the luminal side of the BBB against xenobiotics entering the brain. This transport barrier was also shown in central nervous system pharmacokinetic studies in which the brain uptake of many drugs including anticancer drugs was limited by P-gp. For example, the blood plasma concentrations of vinblastine are about twofold higher in P-gp knockout mice than in control mice, and the vinblastine concentration in the brains of the deficient mice was 20 times higher than in control mice (25). Many other P-gp substrates, including colchicine, digoxin, loperamide, and imatinib (26), are similarly accumulated in the brains of P-gp-deficient mice (24). Similar effects have been reported for other species and experimental models using chemical substrates and inhibitors of P-gp. The in situ perfusion in the brains of mice or rats, which allows the measurement of the brain uptake clearance when the drug first contacts the luminal surface of the brain microvasculature, has also helped quantify the efflux activity of P-gp (27). Finally, positron emission tomography imaging with [11C] radiolabeled substrates of P-gp, like verapamil, colchicine, or loperamide, has provided an elegant and direct visualization of the protective role provided by P-gp at the BBB (28).

Saturation of P-gp-Mediated Transport in the BBB Most in vitro and in vivo studies have been designed to show how P-gp impedes drug accumulation in the brain. We have used in situ brain perfusion in mice and rats to assess the risk of P-gp saturation by various substrates. The uptake clearance by the brain of some drugs, like colchicine and morphine, is concentration independent up to a concentration of 2mM, whereas the systemic concentrations of these drugs cannot be greater than micromolar even in acute poisoning (29). In contrast, vinblastine is transported across the BBB by a concentration-dependent P-gp-mediated efflux with an IC50 of 71µM in rats and 56µM in mice (29, 30). However, there is little risk of vinblastine saturating its own P-gp transport at the BBB, as the plasma concentrations of vinblastine never exceed nanomolar values. In contrast TXD258, a chemotherapeutic taxane, inhibits its own P-gp-mediated efflux at the BBB in vivo and in situ with an IC50 of around 13µM, which corresponds to a blood exposure that is high enough to be therapeutically useful. This saturation could have several advantages, such as overcoming P-gp-mediated efflux, but the nonlinear brain accumulation could increase the risk of toxicity (31). These differences in the transport capacities of the four cited compounds may well be due to the multiple transport sites of P-gp, which can endow the molecule with a low or high transport capacity, depending on the substrate. This capacity-limited transport at the BBB implies that drug–drug interactions at P-gp may be not only competitive but also noncompetitive because of the communication between the various drug-binding sites on P-gp. For example,
verapamil can bind to P-gp together with vinblastine, but to a different site, to produce negative allosteric modulation (32). The mutual effects of pairs of substrates on P-gp transport have been widely studied to investigate the topology of the “binding” domain of this protein (33, 34). However, few in vivo experimental or clinical studies have been conducted to assess the clinical risk of drug–drug interaction. We also examined the interaction of vinblastine with compounds like verapamil, quinidine, progesterone, or valsponar (a cyclosporine analogue that binds to P-gp sites other than the vinblastine site) using in situ mouse brain perfusion. While progesterone interacts moderately with vinblastine, valsponar inhibits the P-gp transport of vinblastine in an “all-or-none” fashion, suggesting that drug–drug interactions by some pairs of compounds may have very acute effects (29).

The interaction of loperamide, an antidiarrheal drug that is transported by P-gp, with quinidine has been evaluated in healthy volunteers. Loperamide had several side effects on the central nervous system, including respiratory depression, demonstrating that the inhibition of the BBB P-gp by quinidine allowed loperamide to be transported across the BBB, although blood plasma concentration of loperamide remained unchanged (35).

**P-gp and Variable Responses of the Central Nervous System to Drugs**

There is always a risk of interaction between P-gp-mediated drugs at the BBB. Any resulting modulation of P-gp transport activity may also give rise to variations in the response of the central nervous system to drugs, both between individuals and within the same individual, depending on the other drugs being administered. The synthesis of the P-gp transporter may also be altered by enhanced or inhibited gene transcription and translation. These variable concentrations of P-gp transporters in the brain may dramatically modify the pharmacological activity of certain P-gp substrates. One such is the dopamine antagonist domperidone, which produces an antiemetic effect only in P-gp-competent mice due to its selective peripheral activity. The antipsychotic effect of domperidone becomes its main effect when it is given to mice lacking P-gp, indicating its distribution and activity in the central nervous system (36). Similarly, the antinociceptive effect of morphine and other opioids is increased in mice lacking P-gp (37). These data suggest that a genetic lack of P-gp at the BBB may have dramatic consequences due to the overexposure of the brain parenchyma to drugs.

**13.3.2. BCRP/Mitoxantrone (MXR) or ABCG2**

The ABCG subfamily is composed of six half-transporters, and the ABCG2 gene confers resistance to several anticancer drugs including MXR. This MDR was first identified in human breast tumor cell lines (38) and is also present in normal tissues involved in drug absorption (small intestine, colon), excretion (canalicular membrane of hepatocytes), and distribution (placental barrier and BBB). ABCG2 is believed to function as a homodimer in the cell
membrane and can carry out the polarized transport of several substrates, such as daunorubicin, doxorubicin, etoposide, topotecan, and camptothecins in the same way as P-gp (39).

**BCRP at the Luminal Side of the BBB** BCRP has been found in the luminal membranes of brain capillary endothelial cells in brain samples from both humans and pigs (40, 41). There appears to be more BCRP than P-gp in porcine brain endothelial cells, according to the concentrations of their mRNAs, but functional studies have not shown that this has a marked influence on brain transport. This raises doubts about how efficiently the molecule undergoes homodimerization in the cell membrane (42). Zhang et al. (43) confirmed that the ABCG2 mRNA and protein product are both present in human brain endothelial cells in vitro; their concentrations in human brain vessels are similar to those of ABCB1 (P-gp) mRNA and protein and are much higher than in the whole brain tissue. They also found that the ABCG2 was upregulated in the brain vessels of patients with multiform glioblastoma, suggesting that the transporter may limit the access of chemotherapeutics to brain tumors. More recently, BCRP has been found on the luminal side of rat brain capillaries, and ABCG2 gene transcription was increased by culturing these endothelial cells together with astrocytes (44). BCRP mRNA and protein have also been found in the endothelium of the mouse brain. We found that the abcg2 mRNA level was 700 times higher in the brain microvessels than in the murine cortex, and we also found a similar pattern with human samples (45).

**The Transport Activity of BCRP at the BBB** BCRP has been found in a wide range of animal species, but there is still a lack of knowledge of its transport activity at the BBB. The *in situ* brain transport of the substrates of P-gp and BCRP, prazosin and MXR, was 2.1- and 3.0-fold greater in mdr1a (-/-) mice than in controls, suggesting that BCRP affects their transport at the mouse BBB (45). The impact of BCRP, by impeding drug transport across the BBB or by acting as a neuroprotector like P-gp, remains to be ascertained. The involvement on BCRP substrate absorption by the gut and distribution to the brain was elegantly shown in studies on bcrp (-/-) mice. These mice develop phototoxic lesions when the skin of their ears is exposed to light. This phenotype is caused by pheophorbide, a chlorophyll breakdown product present in the ingested food; it is not absorbed by the gut of wild-type mice but is absorbed by abcg2 knockout mice at the gut and brain levels, allowing the development of these phototoxic lesions (46).

BCRP seems to protect the brain parenchyma from multiple toxic xenobiotics such as the carcinogenic agents PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine), N-hydroxy PhIP, Me IQx (2-amino-3,8-dimethylimidazo [4,5-f] quinoxaline), dantrolene and drugs like prazosin (47), and purine nucleotide analogues like cladribine, puromycin, and 9-(2-[phosphonomethoxy]ethyl)-adenine (PMEA) (48). A recent study using the
triple knockout *mdr1a/b* (-/-) *abcg2* (-/-) mice clearly suggests the effect of BCRP in limiting the brain distribution of topotecan. Moreover, topotecan brain penetration is dependent on both P-gp and BCRP, which are acting in concert to limit its penetration (49).

### 13.3.3. MRPs or ABCCs

Although P-gp and BCRP are considered to be the major drug export mechanisms at the BBB, MDR phenotypes that are not P-gp mediated can also be associated with several MRPs or ABCCs. The MRP subfamily of proteins was first described in 1992 when Cole et al. cloned the *ABCC1* gene (50). Other MRPs followed in 1996, and several among the 12 members of the ABCC subfamily have now been found at the BBB. Although several molecules are substrates of ABCC1, ABCC2, ABCC3, and P-gp under unconjugated cationic (vincristine, doxorubicin) or neutral (etoposide) form, MRPs preferentially transport anions (like many phase II metabolites of drugs) conjugated to glutathione (GSH), glucuronate, or sulphate. The ABCC4 and ABCC5 proteins mainly confer resistance to cyclic nucleosides and purine analogues. They transport substrates by a different mechanism from P-gp; there may even be multiple mechanisms that include cotransport with GSH. All these proteins are concentrated in specific surface areas of polarized cells like the epithelial cells of the gut and kidney, and probably also in the brain microvessel endothelial cells or in the different cell partners of the neurovascular unit.

**MRPs at the BBB** The distributions of MRPs within the brain are still not clearly understood and appear more complex and controversial because of the existence of several different homologues of each MRP. RT-PCR has been used to demonstrate the presence of MRP1, MRP4, MRP5, and MRP6 both in primary cultures of bovine brain microvessel endothelial cells (BBMEC) and in a capillary-enriched fraction of bovine brain homogenate (51). This study did not detect MRP2 by RT-PCR or Western blotting, but a low concentration of MRP3 was found in the BBMEC and not in the capillary-enriched fraction. A more recent Western blotting study by the same group, using separated luminal and abluminal membranes of cells from the BBMEC, showed that Mrp1 and Mrp5 were concentrated in the luminal membrane, and Mrp4 was concentrated equally on both plasma membranes. This study also indicated that the distribution of MRPs in the endothelial cells forming the BBB was different from the one usually found in polarized epithelial cells (52).

This raises the question of whether all these MRPs are functional at the BBB, as other studies have not always confirmed the presence of all these MRPs. For example, immunohistochemical studies with two commonly used antibodies to MRP1 found no immunolabeling on human brain slices, and Northern blotting found no MRP1 mRNAs in porcine capillaries (53). No significant MRP1 protein or *ABCC1* gene expression was detected in isolated...
human brain capillaries by immunohistochemistry or by RT-PCR (54). Similarly, none was found in isolated porcine capillaries by Western blotting (55) and very recently by using quantitative proteomic analysis (13). However, MRP1 mRNAs have been found in the human brain by RNase protection assay and in the rat brain by RT-PCR (16). These differences might be due to the low rate synthesis of MRP1 and other MRPs, and their genes could be upregulated by cell culture, rendering their expression more detectable, depending on the sensitivity and specificity of the assay used.

**The Transport Activity of MRPs at the BBB** The *in vivo* function of MRP1 and its luminal or abluminal location still need to be determined. We measured an MRP1-mediated efflux across the luminal side of the BBB using *mrp1* (-/-) and wild-type mice and *in situ* brain perfusion of several known MRP1 substrates (56). The brain transport of etoposide, 17β-estradiol-17β-D-glucuronide, vincristine, and doxorubicin was similar in both strains of mice, indicating that MRP1 is not functional at the luminal side of the mouse BBB when substrates are circulating in the cerebral bloodstream. MRP1 does not seem to be a gatekeeper in the BBB in the same way as P-gp.

In contrast to these studies, MRP2 has been visualized by immunohistochemistry and confocal microscopy in the luminal membranes of the endothelial cells from isolated brain capillaries of fish, rats, and pigs (57) but has not been detected by quantitative LC/MS/MS (13). The expression of the other MRPs detected in cellular BBB models is still unclear, except for MRP4 and MRP5. The development of *abcc4*-deficient mice has shown that there is enhanced accumulation of the anticancer drug topotecan, an MRP4 substrate, in brain parenchyma and CSF (58). Immunocytochemical analyses showed that MRP4 is concentrated at the luminal membrane of the brain capillary endothelial cells and at the basolateral membrane of the choroid plexus. This dual distribution of MRP4 indicates that MRP4 may protect the brain from cytotoxins at both BBBS. However, a more recent study did not confirm the influence of MRP4 on topotecan brain uptake in mice. In contrast, the transport of purine nucleoside analogues like 9-(2-phosphonyl-methoxyethyl) adenine (PMEA) and cladribine to the brain is influenced by MRP4, which could limit the transport of several purine analogues across the BBB (48).

A immunohistochemical study by Nies et al. (59) found different intensities of MRP1, MRP4, and MRP5 in the luminal side of the BBB in perilesional samples of an adult human brain. There was weak immunostaining for MRP1 at the BBB in the luminal membrane of human endothelial cells, suggesting that MRP1 function at the BBB may be limited. The same study found no MRP2 in brain tissue, after using three different specific antibodies. This is in contrast to the findings of Potschka et al. (60). Moreover, MRP6 mRNA was below detectability. Finally, the high concentrations of MRP4 and MRP5 mRNAs were associated with a relatively weak immunostaining, indicating that the mRNA and protein concentrations are not correlated and that
posttranslational regulation may be involved. This last study highlights the differences in MRP synthesis and distribution at the BBB between species and between normal and diseased brains.

13.4. ABC GENETIC POLYMORPHISMS AT THE BBB

13.4.1. P-gp and ABCB1 Polymorphisms

There has been considerable interest in ABCB1 polymorphisms in recent years because of the importance of P-gp in drug metabolism and toxicology. A total of 29 single-nucleotide polymorphisms (SNPs) have been reported in the ABCB1 gene to date (61). SNPs in exons 26 (C3435T), 21 (G2677T/A), and 12 (C1236T) are the most frequent genotype variations, and the allele frequencies of the three variant sites differ markedly between populations of different ethnic origin. The effect of ABCB1 polymorphisms on the distribution and pharmacokinetic parameters of drugs such as digoxin, fexofenadine, cyclosporine, and tacrolimus remains very controversial, but this seems to involve mainly their absorption by the gut (61). The C3435T SNP is most frequently associated with nortriptyline-induced postural hypotension in patients with major depression who are being treated with nortriptyline and fluoxetine (56). However, other studies have shown no association between antidepressant-induced respiratory depression and the ABCB1 C3435T variation (62). These studies are considerably hampered by the lack of ABCB1 haplotype analysis. A recent study by Yi et al. (63) examined the three most frequent SNPs on exons 12, 21, and 26 and showed that the disposition of fexofenadine varied considerably among healthy Korean male volunteers. These subjects were allocated to one of six groups, depending on their drug pharmacokinetics. ABCB1 polymorphism may also influence the susceptibility of an individual to diseases. The exon 26 allele is significantly more frequent in subjects with drug-resistant epilepsy, and the same exon seems to protect patients with parkinsonism, especially those with a history of exposure to pesticides (which are frequently substrates of P-gp) (64, 65). Additional studies that focus on haplotypes, environmental factors, patient size, and selection are needed to demonstrate just how ABCB1 polymorphisms influence drug transport across the BBB.

13.4.2. ABCG2 Polymorphisms

The transport activity of BCRP can also be influenced by single amino acid mutations in the protein. For example, the arginine at position 482 confers resistance to MXR and irinotecan; replacing this arginine by threonine or glycine results in additional BCRP efflux transport substrates (66). Other polymorphisms have been characterized. The replacement of the lysine at position 141 by glutamine results in low protein concentration and poor drug
resistance. The frequency of this variation varies widely between ethnic groups (67). Various SNPs are being intensively evaluated, as are the details of BCRP homodimerization. Nevertheless, additional studies are needed to evaluate the role of SNP in the transport of drugs across the BBB.

13.5. REGULATION PATHWAYS OF ABC PROTEINS AT THE BBB

P-gp activity in the BBB can be modulated. Hartz et al. (68) described that the polypeptide hormone endothelin-1 (ET-1) rapidly and reversibly decreased P-gp-mediated transport in rat brain capillaries. The activation by ET-1 or ET(_B) receptor agonists, which are immunolocalized to both the luminal and abluminal surfaces of the capillary endothelium, is associated with a linear signaling pathway involving nitric oxide synthase and protein kinase C activation (68). P-gp activity can also be altered by any modulation of gene transcription. Modulation of the transcription of ABCBI gene expression can result from exposure to xenobiotics and mediators of inflammation as well as cell stress. The pregnane X receptor (PXR) controls the transcription of ABCBI and is activated by several steroids, dietary compounds, toxicants, and a number of currently used drugs. Whereas Bauer et al. (69) detected no PXR in whole brain homogenates, they did find PXR mRNA in isolated rat brain capillaries and confirmed its expression by immunostaining. They also exposed rat brain capillaries to steroids and found increased P-gp mRNA and protein, together with greater efflux transport of P-gp substrates. This study was the first to provide evidence for PXR gene expression at the BBB and its regulation by nuclear receptors of xenobiotic transporters. PXR regulates several genes encoding phase I and II metabolism enzymes and transporters, such as the ABC proteins MRP2 and MRP3 in the liver and intestine. This raises the question of whether there is a coordinated defense system at the BBB. It may have at least three lines of defense that can be activated in response to xenobiotics. P-gp could be the first line of defense, reducing the uptake of substrates; phase I and II enzymes could be the second, producing metabolites, while the third line of defense could consist of other transporters, like MRPs, excreting the metabolites.

13.6. ABC TRANSPORTERS AT THE BBB IN DISEASE STATES

As described earlier, under physiological conditions the BBB is relatively impermeable. In pathological conditions, tight junction disorganization and BBB breakdown are frequently reported. A large number of humoral agents that are released in many neurologic conditions such as HIV-associated dementia, multiple sclerosis, Alzheimer’s disease, hypoxic-ischemic insults, septic encephalopathy, and brain tumor participates in the BBB breakdown. These proinflammatory conditions lead to the release of reactive oxygen
species, nitric oxide, cytokines including interferon-γ, tumor necrosis factor-α (TNF-α), and interleukin-3 (IL-3), excitatory amino acids, ET-1, and a number of neurotoxins and neurotropins. Some of these agents are produced by the activated endothelium itself, astrocytes, and microglia or by infiltrated activated macrophages (70). Many of these inflammatory mediators are capable of upregulating ABC genes.

P-gp expression in the neovasculature of tumors metastatic to the brain is similar to P-gp expression in the neovasculature of the primary extracranial tumor. In contrast, gliomas have higher P-gp expression in their neovasculature, similar to the high expression of P-gp in normal brain vasculature. This leads to increased tissue concentrations of the P-gp substrate paclitaxel in metastatic brain tumors compared with gliomas (71). This was also confirmed in the study of Fine et al. (72), who found higher paclitaxel deposition in the periphery of metastatic brain tumors, corroborating several reports of decreased P-gp expression in metastatic versus primary brain tumors.

In Alzheimer disease, the deposition and accumulation of β-amyloid peptides (Aβ) in cerebral vessels result in cerebral amyloid angiopathy and permeability changes at the BBB. Moreover Aβ accumulation plays an important role in inducing strong upregulation of many inflammatory genes and the ABCB1 and ABCG2 genes (73).

Hypoxic environment and oxidative challenge in the endothelial cells during brain stroke and neurodegenerative diseases are also associated with upregulation of P-gp, BCRP, and MRP4. All these data indicate that several brain diseases can affect the function of the main ABC transporters expressed at the BBB. They also suggest that the variability in central nervous system drug effects that are frequently observed in patients might be dependent on the regulation of the ABC proteins at the BBB.

### 13.7. CONCLUSIONS

Presently we have no undisputed model of the distributions of ABC transporters at the BBB. However, we can attempt to combine the abundant and varied data obtained from both in vitro (isolated brain capillaries or endothelial cells) and in situ/in vivo studies, measurements of mRNAs and/or proteins and their distributions, and from a large number of functional studies. Doing so results in the distribution of ABC transporters at the BBB as shown in Fig. 13.3.

There is good evidence for the presence and the transport activity of P-gp and BCRP in the luminal, blood-facing membranes of the endothelial cells in all species. The protection that P-gp and BCRP afford against several drugs and xenobiotics clearly shows that these two ABC transporters are critical players in the BBB function. This explains why P-gp and BCRP together can be called a “gatekeeper” or “a first line of defense.” Experimental and clinical studies have now demonstrated that P-gp and BCRP modulate the uptake of a large number of anticancer drugs and other drugs by the brain, thus influencing their concentrations within the brain parenchyma and their activity in the
central nervous system, including their side effects. The inhibition of P-gp or BCRP at the BBB by specific inhibitors (or due to competition with other drugs) may result in variations in the response of the central nervous system to drugs. The evidence for the presence of MRPs at the luminal or abluminal sides of the BBB is much more speculative. There are still large differences between species, and most of the proposed models of the distribution of MRPs are supported by the findings of the gene and/or the protein, but not by the demonstration of \textit{in vivo} transport activity. MRP4 and MRP5 are thought to be present in the luminal membranes of the endothelial cells, while the presence of MRP1, MRP2, MRP3, and MRP6 remain questionable and not functionally demonstrated.

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REFERENCES


14

THE ROLE OF ABC TRANSPORTERS AT THE INTESTINAL BARRIER

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14.1. INTRODUCTION

In general, the oral administration of drugs is convenient and practical for patients and is preferred for many reasons. Patients are able to take the oral medication themselves; there is no need for expensive and frequent hospitalization or the discomfort of an injection, which is also associated with the risk of infection (1, 2). Additionally, chronic exposure following repeated oral administration can have clinical benefits over intermittent therapy. However, oral administration of drugs may lead to limited and variable oral bioavailability (1, 3, 4). There are a number of important factors that can explain the variable and/or low oral bioavailability of drugs (5), including (i) physicochemical properties of the drug (e.g., lipophilicity, solubility); (ii) pharmaceutical factors (e.g., dosage form); and (iii) physiological factors (e.g., gastric emptying rate, gastric and intestinal pH, blood flow to the intestine, metabolic enzyme activity) (6, 7). Also the expression and activity of ATP-binding cassette (ABC) drug transporters (ABC transporters) and the metabolic cytochrome P450 enzymes expressed in the gastrointestinal (GI) tract have proven to limit oral bioavailability (6, 8). The combined activity of drug transporters and metabolic enzymes may explain the low and variable bioavailability of a range of drugs (9). In this chapter, we will focus on the ABC transporters localized at the intestinal barriers. These transporters play a pronounced role in the pharmacokinetics (i.e., absorption, tissue distribution, and hepatobiliary, intestinal, and/or renal clearance) of a broad range of drugs, toxins, and endogenous compounds and their metabolites. These transporters extrude drugs, xenobiotics, and metabolites from the intestine, in an active adenosine triphosphate (ATP)-dependent manner, and thereby prevent drug and/or xenobiotic absorption into the blood or lymph circulation and protect the body against acute and chronic toxicity (10, 11).

14.2. ABC DRUG TRANSPORTERS AT THE INTESTINAL BARRIER

14.2.1. Mechanisms of Transport through the Intestinal Epithelium

The human small intestine is approximately 6-m long and has an inner diameter of 2.5–3.0 cm. It is divided into three structural parts: the duodenum, the jejunum, and the ileum, which comprise 5%, 50%, and 45% of the length, respectively. The small intestine represents the principal site of absorption for any ingested compound, whether dietary, therapeutic, or toxic. Drug absorption occurs predominantly on the outer surface of the GI epithelium (specifically, enterocytes). The highly differentiated villi of these cells have an absorptive function (Fig. 14.1). The role of the colon in drug absorption is limited, even though it has the capacity to be an absorption site for certain types of drugs.
In the small intestine, different forms of drug transport can be distinguished: paracellular (between cells) and transcellular (across cells) transport (12). Paracellular permeation is only possible for small molecules and occurs through the gaps (tight junction) in the epithelial membrane (Fig. 14.1). However, absorption via this route is generally low since intercellular tight junctions restrict free transepithelial movement between epithelial cells. Transcellular transport from lumen to blood requires uptake across the apical membrane followed by transport across the cytosol, the exit across the basolateral membrane and into the blood compartment. Transcellular transport can be divided into passive diffusion, endocytosis, and carrier-mediated transport. Passive diffusion is the route available for most drugs entering the systemic circulation and depends largely on the physicochemical properties of a drug, such as lipophilicity (Fig. 14.1). The lipophilicity factor is commonly used to characterize the solubility of a drug in cell membranes. Endocytosis is a process by which a particle enters into a cell without passing through the cell membrane. The transcellular absorption or efflux of hydrophilic drugs, toxins, or
metabolites can be facilitated via specific carrier-mediated transport (Fig. 14.1). The solute carrier ligand family of transporters does not require ATP and transports the drugs according to their concentration gradient; the ABC transporter family, in contrast, acts in an ATP-dependent manner and can work against a steep concentration gradient. These transport mechanisms are involved in the uptake of nutrients, for example, amino acids and vitamins. In addition, drugs or toxic compounds that cross the apical membrane and are substrates for apical transporters are extruded back into the lumen and thereby limit the uptake (13). Three major subfamilies of ATP-dependent energy transporters, the ABC transporters, such as P-glycoprotein (P-gp), the multidrug resistance proteins (MRPs), and the breast cancer resistance protein (BCRP), are expressed at the apical or basolateral surface of epithelial cells lining the intestine (Fig. 14.1). These ABC transporters are known to be involved in the uptake and elimination of a wide range of structurally different drugs, carcinogens, and other toxins. Below, the expression levels of the different ABC transporters present on the three structural parts of the small intestines, that is, the duodenum, jejunum, and ileum, are summarized.

14.2.2. ABC Transporters Expressed at the Intestinal Barrier

Different families of ABC drug transporting proteins have been described as residing within intestinal tissues, including ABCB1 (MDR1, P-gp), ABCC1-6 (multidrug resistance protein 1-6), and ABCG2 (BCRP). Available data demonstrate that the expression levels of ABC transporters vary over the total length of the human GI tract. P-gp is expressed in the apical membrane of epithelial cells, such as enterocytes, and P-gp expression gradually increases from the stomach and the duodenum to the colon (Fig. 14.2). Its messenger RNA (mRNA) level in colon tissue is similar to that in ileum tissue, which is approximately sixfold higher than in the duodenum (14–16). Moreover, characterization of the regional intestinal kinetics of drug efflux in rat and human intestine revealed that the magnitude of P-gp-mediated efflux correlated with the expression levels of P-gp. The efflux ratios (B-to-A permeability/A-to-B permeability; B and A denote basolateral and apical membranes, respectively) in the ileum are typically higher than in other regions of the intestine (17).

Besides P-gp, other ABC transporters such as MRP2 and BCRP are also expressed at the apical surface of epithelial cells throughout the small intestine and colon. MRP2 expression is highest in the duodenum and subsequently decreases toward the terminal ileum and the colon (Fig. 14.2) (18, 19). BCRP mRNA expression is also maximal in the duodenum and decreases continuously into the direction of the rectum (20). Surprisingly, BCRP and MRP2 are more abundant in the jejunum than MDR1 transcripts. Thus, the expression of a number of efflux transporters in the jejunum is equal to or even higher than MDR1, suggesting significant roles for these proteins (in particular BCRP and MRP2) in intestinal drug efflux (21). In contrast with these apically localized transporters, MRP1 and MRP3 are expressed at the basolateral surface.
of gastrointestinal cells in the small intestine and in the colon (22, 23). MRP3 expression is higher in the duodenum, ileum, and colon relative to the jejunum (Fig. 14.2). Zimmermann et al. (18) demonstrated that MRP3, compared with MRP1–5 and MDR1, was the most abundantly expressed MRP in the duodenum and in all segments of the colon, and that MDR1 showed the highest level of expression in the terminal ileum. The differences in ABC transporter expression levels in the intestinal tissues between studies could be due to variability between patients and between the small patient groups included. Also genetic polymorphisms in the ABC drug transporter genes could play roles in the drug transporter function and could influence the bioavailability of drugs and toxins.

The expression levels of the individual ABC transporters over the total length of the GI tract, as described above, is expected to have an impact on the site of absorption of drugs. A study in humans with cyclosporin A (CsA), an immunosuppressant drug, provides a good example of the impact of P-gp
THE ROLE OF ABC TRANSPORTERS AT THE INTESTINAL BARRIER

on oral absorption. CsA transport has been shown to be impaired by P-gp in a variety of P-gp-containing in vitro systems (24). The influence of uneven distribution of P-gp in the intestine was demonstrated in a clinical study with CsA (25, 26). CsA was given to 10 volunteers at different parts of the GI tract (stomach, jejunum/ileum, and colon). The absorption profile of CsA was in rank order stomach > jejunum/ileum > colon in accordance with the expression of P-gp. Furthermore, Lown et al. (27) demonstrated that 30% of the variability in oral $C_{\text{max}}$ and 17% of the variability in oral clearance of CsA in humans can be explained by the interindividual variation in intestinal P-gp levels. Therefore, P-gp not only limits the absorption of CsA but also contributes to the interindividual variation in absorption and consequently to the variability in systemic exposure after oral administration.

In conclusion, preclinical and clinical studies clearly demonstrate that ABC transport-mediated intestinal efflux limits absorption of substrate drugs, can result in variable pharmacokinetics, and has a major influence on drug disposition of orally administered compounds.

14.3. IMPACT OF SMALL INTESTINAL ABC TRANSPORTERS ON ORAL ABSORPTION OF DRUGS

14.3.1. P-gp-Mediated Transport

P-gp is probably the best known secretory drug transporter in the gut. It can transport a variety of drugs of many therapeutic classes with diverse structures and pharmacological activities. The number of substrates and inhibitors for P-gp is continuously increasing and includes anticancer agents, antibiotics, antivirals, calcium channel blockers, and immune suppressive agents (Table 14.1). Naturally occurring substrates for P-gp include biologically active compounds in the normal diet, such as plant chemicals. These compounds act with P-gp as part of detoxification and excretion pathways.

The first evidence of the involvement of intestinal P-gp in drug absorption was obtained in vitro with human colonic adenocarcinoma (Caco-2) cells, in which P-gp was highly expressed at the apical domain, cultured on porous supporting materials. In these cells, the intestinal B-to-A secretory transport of the anticancer drug vinblastine was 10-fold higher than the A-to-B absorptive transport (28). Moreover, the secretory transport of vinblastine could be reduced significantly when P-gp was inhibited by verapamil. Using similar in vitro approaches, numerous studies have been performed in which P-gp secreted several other drugs (29–31). In fact, Caco-2 cell monolayers have been widely used as in vitro models of human intestinal mucosa to evaluate intestinal drug absorption of investigational new drugs (32). However, Caco-2 cells are not the ideal tools for high-throughput screening because it is time consuming to culture and to maintain these cells. Another major disadvantage is that Caco-2 cells have a relatively low expression of other transporters, for
<table>
<thead>
<tr>
<th>Oral Drugs</th>
<th>Inhibitor</th>
<th>Effect Measured</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paclitaxel</td>
<td>—</td>
<td>↑ oral bioavailability in P-gp knockout mice</td>
<td>(6)</td>
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<tr>
<td>Paclitaxel</td>
<td>PSC 833</td>
<td>↑ oral bioavailability in wild-type mice by P-gp inhibition</td>
<td>(44)</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>Cyclosporin A</td>
<td>↑ oral bioavailability in wild-type mice by P-gp inhibition</td>
<td>(45)</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>GF120918</td>
<td>↑ oral bioavailability in wild-type mice by P-gp inhibition</td>
<td>(47)</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>—</td>
<td>↑ oral bioavailability in P-gp knockout mice</td>
<td>(42)</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>Cyclosporin A</td>
<td>↑ oral bioavailability in wild-type mice by P-gp inhibition</td>
<td>(42)</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>Ritonavir</td>
<td>↑ oral bioavailability in wild-type mice by P-gp/CYP3A4 inhibition</td>
<td>(42)</td>
</tr>
<tr>
<td>Etoposide</td>
<td>—</td>
<td>↑ oral bioavailability in P-gp knockout mice</td>
<td>(41)</td>
</tr>
<tr>
<td>Etoposide</td>
<td>GF120918</td>
<td>↑ plasma levels by inhibition of P-gp</td>
<td>(41)</td>
</tr>
<tr>
<td>Indinavir</td>
<td>—</td>
<td>↑ oral bioavailability in P-gp knockout mice</td>
<td>(31)</td>
</tr>
<tr>
<td>Nelfinavir</td>
<td>—</td>
<td>↑ oral bioavailability in P-gp knockout mice</td>
<td>(31)</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>—</td>
<td>↑ oral bioavailability in P-gp knockout mice</td>
<td>(31)</td>
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<tr>
<td>Digoxin</td>
<td>—</td>
<td>P-gp contributed to direct elimination of digoxin.</td>
<td>(40)</td>
</tr>
<tr>
<td>Talinolol</td>
<td>Verapamil</td>
<td>↑ oral bioavailability in wild-type rats by P-gp inhibition</td>
<td>(112)</td>
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<tr>
<td>Topotecan</td>
<td>—</td>
<td>↑ oral bioavailability in BCRP knockout mice</td>
<td>(60)</td>
</tr>
<tr>
<td>Topotecan</td>
<td>GF120918</td>
<td>↑ oral bioavailability in P-gp knockout mice</td>
<td>(58)</td>
</tr>
<tr>
<td>Irinotecan</td>
<td>Gefitinib</td>
<td>↑ oral bioavailability in wild-type mice by BCRP inhibition</td>
<td>(63)</td>
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### TABLE 14.1. Continued

<table>
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<tr>
<td>PhIP</td>
<td>—</td>
<td>↑ oral bioavailability in BCRP knockout mice</td>
<td>(67)</td>
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<td>PhIP</td>
<td>—</td>
<td>↑ oral bioavailability in MRP2-deficient rats</td>
<td>(77)</td>
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<tr>
<td>Methotrexate</td>
<td>Pantoprazole</td>
<td>↓ clearance in wild-type mice by BCRP inhibition</td>
<td>(61)</td>
</tr>
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</table>

**Clinical Studies**

<table>
<thead>
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<th>Inhibitor</th>
<th>Effect Measured</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Digoxin</td>
<td>Quinidine</td>
<td>↑ oral bioavailability in humans by P-gp inhibition</td>
<td>(98)</td>
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<tr>
<td>Digoxin</td>
<td>Talinolol</td>
<td>↑ oral bioavailability in humans by P-gp inhibition</td>
<td>(113)</td>
</tr>
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<td>Paclitaxel</td>
<td>Cyclosporin A</td>
<td>↑ oral bioavailability in humans by P-gp inhibition</td>
<td>(100)</td>
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<td>Paclitaxel</td>
<td>GF120918</td>
<td>↑ oral bioavailability in humans by P-gp inhibition</td>
<td>(108)</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>Cyclosporin A</td>
<td>↑ oral bioavailability in humans by P-gp inhibition</td>
<td>(107)</td>
</tr>
<tr>
<td>Topotecan</td>
<td>GF120918</td>
<td>↑ oral bioavailability in humans by P-gp inhibition</td>
<td>(109, 110)</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>Omeprazole/</td>
<td>↓ clearance in humans by BCRP and/or other</td>
<td>(62)</td>
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<td></td>
<td>lansoprazole</td>
<td>transporter inhibition</td>
<td></td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>—</td>
<td>Correlation between oral exposure and P-gp expression/interindividual variation</td>
<td>(26, 27)</td>
</tr>
<tr>
<td>Talinolol</td>
<td>—</td>
<td>Correlation between oral exposure and P-gp expression</td>
<td>(114, 115)</td>
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<tr>
<td>Tacrolimus</td>
<td>—</td>
<td>↓ absorption by intestinal P-gp</td>
<td>(43)</td>
</tr>
<tr>
<td>Digoxin</td>
<td>—</td>
<td>↓ absorption by intestinal P-gp</td>
<td>(116)</td>
</tr>
<tr>
<td>Fexofenadine</td>
<td>—</td>
<td>↓ absorption by intestinal P-gp</td>
<td>(117)</td>
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Oral bioavailability indicates apparent oral bioavailability under all circumstances.
example, BCRP, while this transporter is highly abundant in the jejunum where it plays an important role in drug efflux from the systemic circulation into the feces (21, 33). This will probably result in an underestimation of the importance of BCRP-mediated transport of orally applied drugs. Madin–Darby canine kidney II (MDCKII) epithelial cells or porcine kidney epithelial cells (LLC-PK1), transfected with various ABC drug transporters, have been helpful in demonstrating drug transporter activity. The relatively short culture time required for these cells makes the LLC-PK1 and MDCKII cells excellent tools for identifying drug transporter activity. However, these cells are not able to express differentiation features characteristic of mature intestinal cells.

Direct evidence supporting a role for P-gp in limiting intestinal absorption was derived from in vivo studies with P-gp knockout mice (34). P-gp knockout mice were developed by Schinkel et al. (35) to investigate P-gp function in vivo. Mice have two mdr1 genes, mdr1a and mdr1b, which together appear to perform the same function as human MDR1/P-gp. Knockout mice were generated for both genes, mdr1a<sup>-/-</sup> and mdr1b<sup>-/-</sup>, as well as for mdr1a/1b<sup>-/-</sup>; and all display normal viability, fertility, and lifespan, with no obvious physiological abnormalities (35, 36). A good example of the contribution of P-gp to the disposition of paclitaxel, an anticancer agent, was a study in P-gp knockout and wild-type (WT) mice (6). When paclitaxel was administered orally to WT mice, the bioavailability was very low (<10%). The systemic exposure to paclitaxel was two- and sixfold higher in the P-gp knockout versus WT mice after intravenous (i.v.) and oral dosing, respectively. Consequently, the apparent oral bioavailability of paclitaxel increased from 11% in the WT to 35% in the P-gp knockout mice. These results indicated that the oral absorption of paclitaxel was effectively limited by P-gp-mediated efflux from the intestinal epithelial cells back into the lumen. The bioavailability did not increase to 100%, probably due to other drug transporters or first-pass intestinal/hepatic extraction by metabolizing enzymes (6, 37, 38). Besides these examples, intestinal P-gp-mediated efflux of a wide range of other drugs, such as docetaxel, vinblastine, etoposide, digoxin, indinavir, saquinavir, tacrolimus, nelfinavir, and talinolol, had been demonstrated in the P-gp knockout mice (Table 14.1) (31, 39–43).

Based on this observation, several studies have been initiated with P-gp inhibitors in combination with paclitaxel in order to enhance oral bioavailability (Table 14.1). Studies in mice revealed that coadministration of PSC 833, a CsA analog and potent P-gp inhibitor, and paclitaxel resulted in a 10-fold increase in systemic exposure to paclitaxel (44). A similar study that has shown comparable effects was performed with CsA and paclitaxel (45). Additionally, it was noted that the plasma levels of paclitaxel obtained in WT mice cotreated with CsA were even higher than those obtained in P-gp knockout mice that were treated with the same dose of oral paclitaxel alone. This can be explained by decreased elimination of paclitaxel by inhibition of the metabolic enzyme cytochrome P450 3A (CYP3A) (45, 46). However, blockade of other yet unidentified drug transporters or drug-eliminating pathways cannot be ruled out. Because the use of CsA for long-term oral dosing may be complicated by
potential immunosuppressive effects, an alternative, nonimmunosuppressive P-gp blocker, GF120918 (elacridar), was explored to enhance the oral bioavailability of paclitaxel. Bardelmeijer et al. demonstrated that elacridar significantly increased the oral bioavailability of paclitaxel (47). The oral bioavailability of paclitaxel in WT mice increased from 8.5% to 40.0%, and the pharmacokinetics of paclitaxel in WT mice receiving elacridar was similar to that found in P-gp knockout mice. Thus, elacridar effectively blocks P-gp in the intestine and most likely does not interfere with other pathways involved in paclitaxel uptake or elimination. Of note, it was demonstrated that elacridar is also an effective inhibitor of the ABC drug transporter BCRP (48).

Studies in mice were also performed with the anticancer agent docetaxel, another P-gp substrate. These studies confirmed that P-gp also plays an important role in the low bioavailability of docetaxel (42). In addition, coadministration of the HIV protease inhibitor, ritonavir, an effective inhibitor of CYP3A4 with minor P-gp-inhibiting properties, was tested in combination with docetaxel in mice (42). An increase was shown in the apparent bioavailability of docetaxel from 4% to 183%; thus, extensive first-pass metabolism and inhibition of the elimination may largely contribute to the low bioavailability of oral docetaxel in mice. In addition, van Herwaarden et al. (49) showed in vivo that expression of CYP3A4 in the intestine dramatically decreased absorption of docetaxel into the bloodstream. Inhibiting P-gp as well as CYP3A4 could be a successful strategy for increasing the systemic exposure to oral docetaxel. These findings may guide the clinical development of combinations of a poorly absorbed anticancer drug plus a boosting agent, such as CsA or ritonavir. Clinical examples will be described in Section 14.4.

Besides inhibitors of ABC transporters, clinical and preclinical findings reveal that the expression of P-gp is also inducible. Expression levels of P-gp (as well as other ABC transporters and drug-metabolizing enzymes) appear to be regulated by nuclear receptors like the pregnane X receptor, constitutive androstane receptor, and vitamin D binding receptor (50). Recent in vitro studies demonstrated that several drugs, including rifampicin, paclitaxel, and reserpine, can induce CYP3A4 and MDR1 gene expression through these mechanisms and possibly can influence the absorption of drugs (51, 52). However, thus far, only rifampicin has been documented to significantly induce intestinal P-gp in humans. In duodenal biopsies performed in healthy volunteers after rifampicin administration, P-gp was induced 3.5-fold (53). Similar interactions with rifampicin have been reported for talinolol (54), fexofenadine (55), and CsA (56). For the other inducers, only in vitro data are available, thus raising doubts whether results obtained in cell lines can be extrapolated to the human in vivo situation.

### 14.3.2. BCRP-Mediated Transport

The ABC half-transporter BCRP is expressed abundantly at the apical membrane of the small intestine, in particular the jejunum, where it limits drug absorption and/or facilitates secretion of clinically important drugs back into
the gut lumen (57). This has been well demonstrated in several studies. In vitro studies reveal that the anticancer drug topotecan, and other camptothecin-derived topoisomerase I inhibitors, is efficiently transported by BCRP and has a low affinity for P-gp (58, 59). Jonker et al. (60) compared the oral bioavailability of topotecan in BCRP knockout and WT mice and found that the systemic exposure of orally administered topotecan is about sixfold higher in BCRP knockout mice than in control mice. Thus, BCRP appears to be a major determinant of the bioavailability of topotecan following oral administration. In P-gp knockout mice, topotecan was coadministered with and without elacridar, an inhibitor of BCRP and P-gp (Fig. 14.3). Topotecan showed decreased plasma clearance, decreased hepatobiliary excretion, and increased reuptake in the small intestine (58), indicating that most likely BCRP mediates these processes.

Furthermore, the mechanism of the pharmacokinetic interaction between the antifolate drug methotrexate (MTX) and the benzimidazole drugs, for example, pantoprazole and omeprazole, observed in patients, was investigated in vitro and in vivo (61). In vitro inhibition of BCRP-mediated transport of MTX was reached at clinically relevant concentrations of benzimidazoles. In addition, the in vitro results also revealed that benzimidazoles are actively transported by BCRP themselves. In vivo data showed that pantoprazole significantly reduced the clearance of MTX by 1.8-fold in WT mice, and reduced it to similar levels as in BCRP knockout mice (61). The interaction between MTX and benzimidazoles is not frequently observed in patients. Increased serum levels were only reported in cancer patients receiving high-dose MTX and omeprazole (62). Breedveld et al. showed that BCRP plays a significant role in the systemic clearance of high-dose MTX (500 mg/kg) but has a limited role in the systemic clearance of low-dose MTX (5 mg/kg) (61).

**FIGURE 14.3.** The effect of elacridar (GF120918) on the oral bioavailability of topotecan. (a) Plasma topotecan concentration versus time curve in P-gp knockout mice pretreated with GF120918 or vehicle (control) (reprinted with permission). (b) Plasma concentration versus time curve of total topotecan in cohort of eight patients. Patients received single topotecan or in combination with GF120918 (reprinted with permission).
Recently, other preclinical studies demonstrated that the oral bioavailability of the anticancer drug irinotecan could also be improved by inhibition of BCRP upon coadministration of the epidermal growth factor receptor (EGFR) inhibitor gefitinib (Iressa®) (63). SN38, which is the active metabolite of irinotecan, is a BCRP substrate drug (48, 64) and gefitinib reverses the BCRP-mediated resistance to SN38 (63, 65). Gefitinib effectively inhibits BCRP, although it is not a substrate for BCRP in vitro (63). At high concentrations, gefitinib also inhibits P-gp (66). Additionally, coadministration of oral gefitinib and oral irinotecan in non-tumor-bearing severe combined immunodeficiency (SCID)−/− mice resulted in a 63% increase in the oral bioavailability of irinotecan compared to irinotecan administration alone (63). Whether the increased oral bioavailability is completely due to inhibition of BCRP is not clear. Indeed, gefitinib may also, at least partially, increase the oral bioavailability of irinotecan by inhibiting the metabolism of irinotecan in the mouse intestine. If gefitinib or other BCRP inhibiting drugs are used clinically in combination with substrate drugs such as irinotecan or topotecan, careful—drug-drug interaction studies need to be performed. Another example of limiting drug entry by BCRP into the body is that BCRP also restricts exposure to dietary carcinogens such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). Pharmacokinetic studies by van Herwaarden et al. (67) demonstrated that the systemic exposure of oral and i.v. administration of PhIP was 2.9- and 2.2-fold higher in BCRP knockout mice than in WT mice, respectively. In mice with cannulated gall bladder, both hepatobiliary and direct intestinal excretion of PhIP were largely reduced in BCRP knockout mice compared with WT mice. The data suggest that BCRP effectively restricts the exposure of mice to ingested PhIP by decreasing its uptake from the small intestine and by mediating hepatobiliary and intestinal elimination. The importance of BCRP as a detoxification efflux transporter in the small intestine was also highlighted by Jonker et al. (60), who found that BCRP knockout mice are prone to developing phototoxic lesions on light-exposed areas of the skin when a diet containing large amounts of chlorophyll was given. Further studies showed that BCRP efficiently limits the uptake of the chlorophyll-breakdown product phaeophorbide A, and the deficiency of BCRP increased the exposure of mice to phaeophorbide A, leading to the high risk of protoporphyria and diet-dependent phototoxicity. This illustrates the importance of drug transporters in the protection of toxicity from normal food constituents. The preclinical data and known overlapping substrate specificity of P-gp (68) and BCRP suggest that these transporters could play a similar role in regulating absorption and disposition of substrate drugs.

14.3.3. MRP-Mediated Transport

Like P-gp and BCRP, MRPs are members of the ABC drug transporter super family and have the capacity to mediate transmembrane transport of many (conjugated) drugs and other compounds. Unfortunately, little is known about
the MRP-mediated transport process occurring at the apical or basolateral membrane of the intestinal epithelium. Thus far, it is known that MRP1, MRP3, and MRP5 are localized at the basolateral side and MRP2 and MRP4 at the apical side of the membranes in the small intestines.

**MRP1** MRP1 is localized primarily at the basolateral membrane of the crypt cells in mouse and human small intestine. The physiological role of MRP1 is probably protection against toxic substrates by extruding them from the cells into the blood (69). Studies in a range of cancer-cell lines demonstrated the ability of MRP1 to confer resistance to cytotoxic drugs including daunorubicin, doxorubicin, and vincristine (70–73). Additionally, MRP1 was also shown to transport heavy metal anions, cytotoxic peptides, hydrophobic drugs, and other compounds that are conjugated or complexed to the anionic tripeptide glutathione (GSH), to glucuronic acid, or to sulfate (74). Studies have shown that efficient transport of several nonanionic anticancer drugs by MRP1 is dependent upon normal cellular supply of GSH. Transport can be facilitated either by cotransport with GSH or by conjugation to GSH by glutathione-S-transferase followed by transportation by MRP1. However, the exact role of MRP1 in intestinal drug transport has not yet been clearly established, and no efficient and selective MRP1 inhibitors are known.

**MRP2** Like P-gp, MRP2 is located at the apical membrane of epithelial cells of the small intestine increasing from crypt to villus (75). Compounds transported by MRP2 show a great similarity with MRP1 substrates; however, there is not a complete overlap in substrates. MRP2 plays a functional role in the intestinal secretion of many drugs, for example, both conjugated and unconjugated anionic compounds, and in the cotransport of weakly basic drugs with GSH and/or metabolites from the small intestine (73). Experiments with MRP2-deficient rats indicate that the protein plays a role in reducing the oral availability and the biliary and intestinal excretion of the food-derived carcinogen PhIP (76). Thus, components of our daily diet are also substrates for MRP2 besides BCRP. Using rat intestine from MRP2-deficient and WT animals, MRP2 was shown to mediate luminal excretion of the metabolite 2,4-dinitrophenyl-S-glutathione (DNP-SG) following administration of its parent compound 1-chloro-2,4-dinitrobenzene (CDNB) (77). CDNB molecules are rapidly taken up by somatic cells and are conjugated with cellular reduced GSH to form DNP-SG, a MRP2 substrate. Collectively, it was suggested that MRP2 is involved in the intestinal excretion of GSH conjugates. Furthermore, Fromm et al. (13) described a variable interindividual upregulation of MRP2 by rifampicin in the apical membrane of enterocytes in the small intestine of 16 individuals. Tamoxifen influenced expression of the protein, thereby influencing the bioavailability, activity, and toxicity of the substrates as well in vitro and in vivo (78, 79). However, not only rifampicin or tamoxifen but also other compounds such as cisplatin and dexamethasone have been...
reported to affect MRP2 expression \textit{in vitro} \cite{80, 81}. MRP2 upregulation may influence the acquisition of multidrug resistance during chemotherapy \cite{82}.

It is clear that MRP2 has some degree of overlapping substrate specificity with P-gp, for example, paclitaxel and doxorubicin \cite{83, 84}. The colocalization of MRP2 and P-gp at the apical membrane sites important to drug disposition (i.e., intestine, liver, and kidney) presents a barrier to drugs. Both transporters, for example, were shown to mediate the blood-to-lumen secretion of the fluoroquinolone antibiotic grepafloxacin by rat intestine \cite{85}, as well as human intestinal Caco-2 cell monolayers \cite{86} and MDCKII cell monolayers stably transfected with either P-gp or MRP2 \cite{87}. Therefore, it is suggested that MRP2 plays a significant role in mediating drug detoxification and in limiting the oral absorption of their ligands by extruding them back into the intestinal lumen. In contrast with P-gp, clinical investigations regarding the influence of MRP2 inhibition, for example, by the MRP2 inhibitor MK571 \cite{88} on the oral bioavailability of substrates are lacking. An explanation could be that anionic MRP2 substrates serve as competitive inhibitors when applied in cellular \textit{in vitro} systems; and, furthermore, most MRP2 substrates are also transported by other transporters such as OATP2 \cite{89}.

\textbf{MRP3} Similar to MRP2, MRP3 expression increases from the crypt to the villus tip \cite{90}. Like MRP1, MRP3 is localized to the basolateral membrane \cite{91}. MRP3 shares a considerable overlap in substrate specificity with MRP2, transporting a wide range of bile salts, nonconjugated organic anions, and glucuronide conjugates \cite{90}. The affinity of bile salts, together with their pattern of expression in the intestine, suggests a possible role for MRP3 in mediating bile salt reabsorption as part of the enterohepatic recirculation. Although MRP3 has not been studied as extensively as MRP1 and MRP2, it has several interesting properties. It can confer resistance to anticancer drugs such as etoposide, teniposide and MTX \cite{92}. Also, it increases efflux of toxic compounds from cells \cite{93}. Further research to elucidate the physiological function of MRP3 is warranted.

\textbf{MRP4} MRP4 has also been detected at low levels at the apical membrane of the jejunum. However, the exact role of MRP4 in intestinal drug transport has not yet been clearly established. MRP4 might limit the intestinal absorption of nucleoside phosphonate analogs, thereby contributing to their low oral bioavailability \cite{94}.

\textbf{MRP5} MRP5 is also expressed at the intestinal barrier, including the colon \cite{18, 95}. Although the normal subcellular distribution of MRP5 is presently unclear, human MRP5 is routed to the basolateral membrane when stably transfected into MDCKII cells. There are no reports at present that MRP5 plays a role in intestinal absorption or disposition of substrate drugs.
**MRP6** MRP6 shows low expression in the duodenum and colon (96). Recent work using MRP6-transfected Chinese hamster ovary cells has indicated that MRP6 can mediate the transport of several cytotoxic agents that are also substrate for MRP1–3 (97). This suggests a role for MRP6 in drug transport in tissues, including the intestinal epithelium; however, thus far, there are no reports available about the possible role of MRP6 in intestinal drug absorption.

### 14.4. CLINICAL EXAMPLES OF MODULATION OF THE ACTIVITY OF INTESTINAL TRANSPORTERS TO INCREASE SYSTEMIC EXPOSURE OF ORALLY ADMINISTERED DRUGS

In view of the importance of intestinal transporters in the absorption of drugs, efforts have been made to identify chemical inhibitors of ABC efflux transporters. These inhibitors temporarily reduce the efflux activities of the transporters and thereby can increase the oral bioavailability of some poorly absorbed drugs. Based on the preclinical results described in section 14.3, numerous clinical proof-of-concept studies in humans have been initiated (Table 14.1) to evaluate the feasibility and the safety of the coadministration of a substrate drug and an ABC transport inhibitor.

The first example is the quinidine–digoxin interaction. Because digoxin is a P-gp substrate that is not metabolized (98, 99), it has become a well-established model substrate to determine P-gp transporter activity. The absolute bioavailability of digoxin was increased in the presence of an oral dose of the P-gp inhibitor quinidine. This suggests that quinidine increased the bioavailability of digoxin by inhibiting P-gp efflux into the intestine and possibly in the hepatobiliary excretion route.

In a clinical phase I study, five cancer patients received a safe oral dose of paclitaxel (60 mg/m²), and nine other patients received oral paclitaxel combined with a single oral dose of the P-gp and CYP3A inhibitor CsA (15 mg/kg) (100). CsA increased the systemic exposure to oral paclitaxel eight-fold, and the apparent bioavailability from 4% without CsA to 47% with CsA. In addition to P-gp inhibition, drug exposure was further improved by the concomitant inhibition of metabolism by CsA (101). At the highest dose level of oral paclitaxel (300 mg/m²) in combination with CsA (15 mg/kg), the total fecal excretion was 76%, 61% of which was the parent drug. The high percentage of unabsorbed drug can be explained by the presence of the cosolvent Cremophor EL, which resulted in the entrapment of paclitaxel (102). Different oral formulations are currently being tested (103). To date, several hundreds of patients have received oral paclitaxel in combination with CsA at different doses and schedules. Three phase II studies were performed to investigate the activity and safety of repeated oral administration, in nonsmall-cell lung cancer, advanced gastric cancer, and breast cancer (104–106). These studies
show encouraging results, which may ultimately lead to application of oral paclitaxel as standard therapy in these types of cancer.

Similar results were obtained in another clinical study with docetaxel (107). Patients received one course of oral docetaxel (75 mg/kg) with or without a single dose of CsA (15 mg/kg). Pharmacokinetic results showed that coadministration of oral CsA resulted in a 7.3-fold increase of the systemic exposure to docetaxel. The apparent bioavailability of oral docetaxel in cancer patients increased from 8% without CsA to 88% in combination with CsA. Inhibition of metabolism as well as by P-gp inhibition in the GI tract by CsA can explain this increase, but the magnitude of both mechanisms cannot be determined exactly. Furthermore, in another phase I study, patients received elacridar prior to oral paclitaxel (108). The increase in systemic exposure to paclitaxel was of the same magnitude as in combination with CsA.

Coadministration of elacridar also resulted in a significant increase in the systemic exposure of oral topotecan in patients (109) (Fig. 14.3b). Previous studies reported low bioavailability (30.0% ± 7.7%) and moderate interpatient variability of the i.v. formulation of topotecan administered orally (110, 111). Elacridar increased the oral bioavailability of topotecan from 40.0% to 97.1% in eight patients, and the plasma area under the curve (AUC) increased significantly from 32.4 ± 9.6% to 78.7 ± 20.6 µg·h/L. The interpatient variability of the oral bioavailability of topotecan decreased from 17% without elacridar to 11% with elacridar. Because topotecan is a selective substrate for BCRP and has low affinity for P-gp, these results suggest that the interaction between elacridar and topotecan is due to inhibition of BCRP-mediated intestinal absorption. These encouraging results may have clinical implications for the oral application of topotecan and drugs with low oral bioavailability due to affinity of BCRP (9). It is not yet known whether the selection of a dual P-gp and BCRP inhibitor is better than the selection of a selective P-gp or BCRP inhibitor. This will depend on the affinity of the substrate drug for P-gp and/or BCRP and on the toxicity profile of the combination of inhibitor and anticancer drug. Thus far, phase I clinical studies have shown that dual inhibitors such as elacridar can be administered to patients with only minimal side effects. Clinical trials with third-generation modulators of P-gp (e.g., biricodar, zosuquidar, and laniquidar) specifically developed for multidrug resistance (MDR) reversal are ongoing. The results will give insight into the possible clinical feasibility of this strategy.

14.5. CONCLUSIONS

During the past two decades, significant progress has been made in understanding the pharmacological and physiological role of ABC drug efflux transporters. Although these transporters were once thought to be relevant only in making cancer cells resistant to anticancer drugs, it is now clear that they have a pronounced role in the pharmacokinetics (i.e., absorption, tissue distribution,
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THE ROLE OF ABC TRANSPORTERS AT THE INTESTINAL BARRIER


15

GENETIC POLYMORPHISMS IN ABC TRANSPORTERS

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15.1. INTRODUCTION

For decades, the scientific community has recognized the importance of ATP-binding cassette (ABC) transporters in the development of multidrug resistance (1). More recently, the role of ABC transporters in determining the pharmacokinetic and pharmacodynamic properties of numerous drugs has been described (2). Interindividual variation in ABC transporter expression and function has been documented in solid tumors and hematological malignancies (3–6) and in normal tissues (7–9). One potential source of interindividual variability is genetic variation. The introduction of high-throughput
DNA sequencing technologies has enabled studies of the effects of genetic variation on ABC transporter expression and function. The ultimate goal of understanding the functional and clinical significance of genetic variation in ABC transporters is to be able to predict an individual’s resistance to a drug or patient-specific pharmacokinetic and pharmacodynamic properties. This in turn would provide clinicians with the means to tailor drug therapy for optimal response and toxicity profiles.

Both forward (phenotype-to-genotype) and reverse (genotype-to-phenotype) genetic methods have been used to study variations in ABC transporter functions. Forward genetic studies have identified critical roles for ABC transporters in diseases ranging from cystic fibrosis (CF) to adrenal leukodystrophy (10). There are now numerous examples where dysfunctional ABC transporters result in severe chronic effects, underlining the role of these proteins in the transport of endogenous compounds and in maintaining homeostasis of physiological processes. Genetic polymorphisms in ABC transporters have been associated with disease, and in some cases, the mechanism through which the polymorphism is leading to transporter dysfunction and disease has been elucidated.

Genetic polymorphisms in ABC transporters can have a multitude of effects. The severity of the effect depends on a number of factors, including where a polymorphism is located in the gene (exon, intron, regulatory region), if it changes an amino acid (i.e., is non-synonymous), and the significance of that amino acid change. Initial pharmacogenetic studies focused on changes in protein function caused by nonsynonymous single-nucleotide polymorphisms (SNPs) since these were often fairly easy to explain mechanistically. For example, a SNP in exon 4 of ABCG2 encodes a premature stop codon at residue 126 (376T > C, Q126stop); this SNP abrogates mitoxantrone resistance protein (MXR) - mediated methotrexate transport (11). In another example, the Q141K polymorphism substantially decreases MXR ATPase activity, thereby rendering the protein incapable of transport (12).

Polymorphisms located throughout the gene may also influence transporter expression at both the mRNA and protein levels. SNPs found in regulatory regions such as the promoter may impact transcriptional regulation, as is the case with the -14C > T polymorphism in the gene encoding the cholesterol transporter ABCA1, in which the T allele was found to have higher transcriptional activity in vitro (13). Splice sites are another regulatory factor that may be affected by SNPs; deletions in ABCA4 have been associated with alternative splicing, resulting in expression of shortened mRNA transcripts and defective retinoid transport (14). A synonymous polymorphism in ABCB1 has been found to cause allele specific expression, resulting in genotype-dependent expression of ABCB1 mRNA in the human liver (15).

In some cases, polymorphisms have been associated with clinical phenotypes, although the molecular mechanism that causes this phenotype has not been identified. Indeed, these associated polymorphisms may not be causative of the phenotype but may exist in linkage disequilibrium with the causative SNP or SNPs. One way to circumvent such misleading SNP associations is to
perform haplotype association studies rather than to focus on a single SNP (16). It has become clear that determining why a SNP in an ABC transporter leads to a clinical phenotype, whether it be a disease state, a pharmacokinetic parameter (e.g., plasma drug concentration), or a pharmacodynamic parameter (e.g., drug resistance), is a challenging and time-consuming process, albeit one that is crucial to advancing our understanding of ABC transporter biology.

15.2. MUTATIONS AND POLYMORPHISMS LEADING TO DISEASE

The role of genetic variation in ABC transporters in the phenotypes of several inherited and acquired diseases is clearly defined. One well-studied disease is progressive familial intrahepatic cholestasis (PFIC), in which bile flow is impaired. Families with a certain type of PFIC have heterogeneity on chromosome 2. Detailed mapping demonstrated the existence of mutations in ABCB11, which encodes the bile salt export pump (BSEP) (17). BSEP is localized to the canalicular membrane of hepatocytes and plays a crucial role in bile production (18). Immunohistochemistry has shown that liver specimens from PFIC patients with mutations in ABCB11 have decreased protein expression (19). \textit{In vitro}, PFIC-associated ABCB11 mutations impair the trafficking and/or transport function of BSEP (20–23). Case reports continue to be published describing novel ABCB11 mutations in PFIC patients.

BSEP maintains bile acid homeostasis along with other transporters such as MRP2 (ABCC2), which is also expressed on the canalicular membrane of hepatocytes. MRP2 effluxes anionic conjugates such as reduced glutathione, creating a driving force for bile flow (18). Interestingly, the role of MRP2 in an inherited disease called Dubin–Johnson syndrome (DJS) was uncovered through rat studies in which a strain deficient in the canalicular multispecific organic anion transporter (cMOAT) presented with impaired hepatobiliary transport of a number of compounds (24, 25). The human ortholog of cMOAT, ABCC2, was screened for mutations in DJS patients (26, 27). The presence of mutations in ABCC2 (e.g., premature stop codon) confirmed that reduced expression and/or function of this transporter leads to DJS (27). As with PFIC, the process of identifying and functionally characterizing the ABCC2 mutations that cause DJS is ongoing.

Another rare disorder whose pathophysiology involves mutations in transporter genes is pseudoxanthoma elasticum (PXE). Individuals with PXE experience mineralization of elastic fibers in the skin, eye, and cardiovascular system. Genetic analysis of PXE families narrowed the causative region to approximately 500 kb on chromosome 16 (28) and mutations in one of the genes in this region, ABCC6, were found in PXE patients (29, 30). In cell culture, PXE-associated ABCC6 mutations abolished transport activity (31). The endogenous substrate of MRP6 remains unknown, and it is unclear why mutations in ABCC6 cause PXE (32).

Genetic variation in another ABC transporter gene, ABCC7, causes a more common inherited disorder, CF. An old folk saying that “a child that tastes
salty when kissed will soon die” is now thought to refer to the abnormally high salt concentration in the sweat of children with CF (33); CF was later associated with decreased chloride ion conductance across the membranes of epithelial cells (34). Reverse genetic approaches were used to identify, clone, and characterize the CF gene, now designated cystic fibrosis transmembrane conductance regulator or \textit{ABCC7} (34), which encodes a chloride ion channel (35). The deletion of a single amino acid, the phenylalanine at position 508, located in the first nucleotide-binding domain, was commonly found in patients with CF (34). Over a thousand additional mutations in \textit{ABCC7} have since been associated with CF (10). The discovery of these mutations has allowed genetic testing for CF in neonates, with screening being mandatory in a number of states in the United States (36).

15.3. POLYMORPHISMS AFFECTING DRUG PHARMACOKINETICS

Polymorphisms and rare mutations in ABC transporter genes have been implicated in a number of serious disorders, as reviewed above. However, changes in these genes may not lead to an obvious phenotype, as demonstrated with the creation of the \textit{mdr1a} knockout mouse. The knockout mice appeared physiologically normal until substrate drugs were administered, at which time significant alterations in pharmacokinetic parameters were observed (37). In the late 1990s, researchers began examining the effects of genetic polymorphisms on the pharmacokinetics of ABC transporter substrate drugs in humans, hoping that these polymorphisms would help to explain the interindividual variability in dosing requirements and pharmacological response. A summary of the most commonly genotyped polymorphisms in xenobiotic ABC transporters is provided in Table 15.1.

\textit{ABCB1} was one of the first genes to be fully sequenced in coding and regulatory regions, and several groups published reports describing the extent and nature of sequence variation in \textit{ABCB1} (38–40). The effects of \textit{ABCB1} SNPs on the pharmacokinetics of certain drugs with narrow therapeutic ranges, such as the cardiac glycoside digoxin, were of interest because of their clinical relevance. The first report to investigate \textit{ABCB1} pharmacogenetics in relation to digoxin pharmacokinetics was published in 2000. The findings of this study indicated that the \textit{ABCB1} 3435C > T synonymous SNP was correlated with lower duodenal P-glycoprotein expression and higher digoxin plasma levels (40). Subsequently, \textit{ABCB1} 2677G > T, a non-synonymous polymorphism that frequently occurs in a haplotype with the 3435C > T SNP, was shown to increase digoxin transport in vitro (38), in contrast to the in vivo results (40). The \textit{ABCB1} 2677T and 3435T alleles have also been associated with increased digoxin plasma levels (41–43), suggesting reduced \textit{ABCB1}/P-glycoprotein expression and/or function. In contrast, this same SNP has been associated with decreased digoxin plasma levels and increased duodenal \textit{ABCB1} mRNA expression (44), suggesting increased \textit{ABCB1}/P-glycoprotein expression and/
or function. Other groups found no effect of \textit{ABCB1} SNPs on digoxin pharmacokinetics (45, 46). Such discordant results from investigations of a single drug appear to be the common scenario for association of \textit{ABCB1} SNPs with pharmacokinetic parameters.

Pharmacogenetic studies of fexofenadine pharmacokinetics are also conflicting, with \textit{ABCB1} 2677T/3435T-containing haplotypes correlating with increased (47) or decreased plasma levels (38), or having no effect (48). The immunosuppressant cyclosporin A, often administered following organ transplantation, is also a substrate of \textit{P}-glycoprotein. Some reports suggest that transplant patients with the 3435T allele or haplotypes containing that allele had higher dose-adjusted cyclosporin A plasma levels (49–52), while others noted the opposite (53, 54) or found no effect (55, 56). The reasons for these conflicting results may be due to differences in phenotyping methods, study design (e.g., controlling for population admixture or ethnicity), degree of statistical power, or some sort of underlying linkage disequilibrium that has not been taken into account.

Polymorphisms in the hepatic organic anion transporter \textit{ABCC2} have been shown to cause the genetic disorder DJS (described above), but more recently, they have been associated with interindividual differences in irinotecan pharmacokinetics. The active metabolite of irinotecan, SN-38, is glucuronidated by uridine diphosphate glucuronosyltransferases, and SNPs in \textit{UGT1A1} have been associated with the rate of SN-38 glucuronidation (57) and irinotecan toxicity (58). However, both irinotecan and SN-38 are transported from hepatocytes into the bile by MRP2 (59, 60). A haplotype consisting of six SNPs in \textit{ABCC2}, designated \textit{ABCC2}*2, was significantly associated with lower

<table>
<thead>
<tr>
<th>Gene</th>
<th>rs no.</th>
<th>nt Change</th>
<th>AA Change</th>
<th>Minor Allele Frequency</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Caucasian</td>
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<tr>
<td>\textit{ABCB1}</td>
<td>3213619</td>
<td>–129T &gt; C</td>
<td>Asn21Asp</td>
<td>0.051</td>
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<td></td>
<td>9282564</td>
<td>61A &gt; G</td>
<td>Ser400Asn</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>2229109</td>
<td>1199G &gt; A</td>
<td>–</td>
<td>0.459</td>
</tr>
<tr>
<td></td>
<td>1128503</td>
<td>1236C &gt; T</td>
<td>Ala893Ser</td>
<td>0.464</td>
</tr>
<tr>
<td></td>
<td>2032582</td>
<td>2677G &gt; T</td>
<td>Ala893Thr</td>
<td>0.036</td>
</tr>
<tr>
<td></td>
<td>1045642</td>
<td>3435C &gt; T</td>
<td>–</td>
<td>0.561</td>
</tr>
<tr>
<td>\textit{ABCC2}</td>
<td>717620</td>
<td>–24C &gt; T</td>
<td>Val417Ile</td>
<td>0.195</td>
</tr>
<tr>
<td></td>
<td>2273697</td>
<td>1249G &gt; A</td>
<td>Val141Ile</td>
<td>0.170</td>
</tr>
<tr>
<td></td>
<td>3740066</td>
<td>3972C &gt; T</td>
<td>–</td>
<td>0.383</td>
</tr>
<tr>
<td>\textit{ABCG2}</td>
<td>2231137</td>
<td>34G &gt; A</td>
<td>Val12Met</td>
<td>0.066</td>
</tr>
<tr>
<td></td>
<td>2231142</td>
<td>421C &gt; A</td>
<td>Gln141Lys</td>
<td>0.081</td>
</tr>
</tbody>
</table>
irinotecan clearance and an increased risk of irinotecan-induced diarrhea, suggesting that impaired MRP2 biliary transport function and elevated irinotecan plasma levels lead to toxicity (61).

MXR has also been implicated in the efflux of SN-38 into the bile, and the effect of the ABCG2 421C > A SNP on irinotecan pharmacokinetics has been investigated, since this SNP has been shown to decrease ABCG2 expression *in vitro* (62). Several studies did not find statistically significant associations between the ABCG2 421A allele and SN-38 plasma levels (63–65), although one group reported that carriers of the 421A allele had elevated plasma concentrations of topotecan, another topoisomerase I inhibitor, and anticancer drug (66). The example of irinotecan pharmacogenetics underscores the importance of considering polymorphisms in multiple genes in a pharmacokinetic pathway (Fig. 15.1), including metabolizing enzymes and drug transporters; such pathway genotyping may become crucial in the clinical application of pharmacogenomics.

**FIGURE 15.1.** Irinotecan response pathway. The schematic illustrates a model human liver cell showing blood, bile, and intestinal compartments, indicating tissue-specific involvement of genes in the irinotecan pathway. This schematic is taken from http://www.pharmgkb.org.
In those cases where the proteins in a pharmacokinetic pathway are unknown, animal models can help elucidate which proteins may be important. For example, the creation of the Abcc2 knockout mouse facilitated the identification of MRP2 as an important protein in determining pravastatin disposition (67). Subsequently, a number of researchers investigated the effects of ABCC2 polymorphisms on pravastatin pharmacokinetics. An association between the ABCC2 1446C > G SNP and reduced pravastatin exposure has been reported by some but not all studies (68, 69). It should be noted that polymorphisms in SLCO1B1, which encodes for an uptake transporter, have also been correlated with pravastatin disposition and activity (68, 70), again demonstrating the importance of considering the entire pharmacokinetic pathway of a drug rather than focusing on a single component.

15.4. POLYMORPHISMS AND DRUG/TOXIN RESISTANCE

Even when the pharmacokinetic pathway of a drug is well established, the proteins involved in the pharmacodynamics or pharmacologic activity of a drug may be unknown, and often the pharmacokinetic and pharmacodynamic pathways do not overlap. In some cases, the plasma levels of a drug have no effect on its activity because its site of action is physiologically protected. For example, antiepileptic drugs act in the brain, but because of the existence of the blood–brain barrier, plasma levels do not reflect drug levels in the brain. It follows that the xenobiotic transporters localized to the blood–brain barrier may therefore play an important role in the pharmacodynamics of antiepileptic drugs, as well as in the development of drug-resistant epilepsy. Similar hypotheses regarding a role for ABC transporters in drug and toxin resistance have been proposed in Parkinson’s disease, inflammatory bowel disease, cancer, and AIDS (Table 15.2).

The first investigation into ABCB1 pharmacogenetics and drug-resistant epilepsy was published in 2003, when it was reported that patients with drug-resistant epilepsy were less likely to have the 3435TT genotype (71). This finding agreed with an earlier report that lymphocytes from individuals with the TT genotype accumulated more rhodamine 123 (a P-glycoprotein substrate) than did carriers of the C allele (72), indicating that the TT genotype conferred a reduced P-glycoprotein function; and it is consistent with a decreased incidence of drug resistance compared to the 3435CC genotype. Similar findings of a decreased frequency of the ABCB1 3435T allele in patients with drug-resistant epilepsy have been described (73–75), although reports of the opposite effect have also appeared (76, 77). However, a similar number of studies found no association between ABCB1 3435C > T genotype and drug-resistant epilepsy (78–83). It has been suggested that one or more intronic SNPs in ABCB1, which are in tight linkage disequilibrium with the 3435C > T polymorphism, may be responsible for multidrug-resistant epilepsy;
<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism</th>
<th>Population</th>
<th>Outcome Marker</th>
<th>Finding</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCBI</td>
<td>3435C &gt; T</td>
<td>200 drug-resistant and 155 drug-sensitive epileptic patients</td>
<td>Refractory epilepsy</td>
<td>CC genotype associated with refractory epilepsy</td>
<td>(71)</td>
</tr>
<tr>
<td>ABCBI</td>
<td>1236C &gt; T</td>
<td>210 epileptic patients</td>
<td>Refractory epilepsy</td>
<td>1236C/2677G/3435C haplotype increased risk of drug resistance</td>
<td>(73)</td>
</tr>
<tr>
<td>ABCBI</td>
<td>3435C &gt; T</td>
<td>27 drug-sensitive and 63 drug-resistant epileptic patients</td>
<td>Refractory epilepsy</td>
<td>CC genotype associated with refractory epilepsy</td>
<td>(74)</td>
</tr>
<tr>
<td>ABCBI</td>
<td>1236C &gt; T</td>
<td>114 drug-resistant and 213 drug-sensitive epileptic patients</td>
<td>Refractory epilepsy</td>
<td>3435CC genotype associated with refractory epilepsy; 2677G&gt;T, 3435C&gt;T and interaction predict resistance</td>
<td>(75)</td>
</tr>
<tr>
<td>ABCBI</td>
<td>3435C &gt; T</td>
<td>221 drug-resistant, 297 drug-sensitive, and 228 drug-active epileptic patients</td>
<td>Refractory epilepsy</td>
<td>TT genotype associated with refractory epilepsy</td>
<td>(76)</td>
</tr>
<tr>
<td></td>
<td>-129T &gt; C</td>
<td>126 drug-resistant and 84 drug-sensitive epileptic patients</td>
<td>Refractory epilepsy</td>
<td>2677TT and 3435TT genotypes associated with refractory epilepsy</td>
<td>(77)</td>
</tr>
<tr>
<td>ABCBI</td>
<td>3435C &gt; T</td>
<td>401 drug-resistant and 208 drug-sensitive epileptic patients</td>
<td>Refractory epilepsy</td>
<td>No statistical association</td>
<td>(78)</td>
</tr>
<tr>
<td>ABCBI</td>
<td>3435C &gt; T</td>
<td>230 drug-resistant and 170 drug-sensitive epileptic patients</td>
<td>Refractory epilepsy</td>
<td>No statistical association</td>
<td>(79)</td>
</tr>
<tr>
<td>ABCC1</td>
<td>3435C &gt; T</td>
<td>63 drug-resistant, 108 drug-sensitive epileptic patients</td>
<td>Refractory epilepsy</td>
<td>No statistical association (80)</td>
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<tr>
<td>ABCC1</td>
<td>1236C &gt; T 2677G &gt; T/A 3435C &gt; T tSNPs</td>
<td>503 epileptic patients</td>
<td>Time to first seizure, time to 12-month remission, time to withdrawal</td>
<td>No statistical association (81)</td>
<td></td>
</tr>
<tr>
<td>ABCC1</td>
<td>1236C &gt; T 2677G &gt; T/A 3435C &gt; T tSNPs</td>
<td>73 drug-resistant and 76 drug-sensitive epileptic patients</td>
<td>Refractory epilepsy</td>
<td>No statistical association (82)</td>
<td></td>
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<tr>
<td>ABCC1</td>
<td>3435C &gt; T tSNPs</td>
<td>242 drug-resistant and 198 drug-sensitive epileptic patients</td>
<td>Refractory epilepsy</td>
<td>No statistical association (83)</td>
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<tr>
<td>ABCC1</td>
<td>2677G &gt; T/A 3435C &gt; T tSNPs</td>
<td>440 drug-resistant and 196 drug-sensitive epileptic patients</td>
<td>Refractory epilepsy</td>
<td>3435 allele and intronic SNP associated with drug resistance (84)</td>
<td></td>
</tr>
<tr>
<td>ABCC1</td>
<td>3435C &gt; T</td>
<td>30 early-onset and 77 late-onset Parkinson’s patients</td>
<td>Age at onset of Parkinson’s disease</td>
<td>CT and TT genotypes associated with increased risk (85)</td>
<td></td>
</tr>
<tr>
<td>ABCC1</td>
<td>-129T &gt; C 2677G &gt; T/A 3435C &gt; T</td>
<td>25 early-onset and 70 late-onset Parkinson’s patients</td>
<td>Incidence and onset of Parkinson’s disease</td>
<td>3435TT genotype highest in early-onset patients (86)</td>
<td></td>
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<tr>
<td>ABCC1</td>
<td>multiple</td>
<td>206 Parkinson’s patients</td>
<td>Incidence of Parkinson’s disease</td>
<td>1236C, 2677G, and 3435C independently associated with risk of late-onset Parkinson’s disease (87)</td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Polymorphism</td>
<td>Population</td>
<td>Outcome Marker</td>
<td>Finding</td>
<td>References</td>
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<tr>
<td>ABCB1</td>
<td>3435C &gt; T</td>
<td>249 ulcerative colitis, 179 Crohn’s disease patients</td>
<td>Risk of disease</td>
<td>rs3789243 genotype associated with susceptibility to ulcerative colitis but not Crohn’s disease</td>
<td>(90)</td>
</tr>
<tr>
<td></td>
<td>tSNPs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCB1</td>
<td>3435C &gt; T</td>
<td>149 ulcerative colitis, 126 Crohn’s disease patients</td>
<td>Risk of disease</td>
<td>3435TT genotype associated with ulcerative colitis, no association with Crohn’s disease</td>
<td>(91)</td>
</tr>
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</tr>
<tr>
<td>ABCB1</td>
<td>3435C &gt; T</td>
<td>300 ulcerative colitis patients</td>
<td>Risk of disease</td>
<td>3435T allele associated with ulcerative colitis</td>
<td>(92)</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>ABCB1</td>
<td>-129T &gt; C, 1236C &gt; T, 2677G &gt; T/A, 3435C &gt; T others</td>
<td>144 ulcerative colitis, 163 Crohn’s disease patients</td>
<td>Risk of disease and refractory disease</td>
<td>1236T/2677T/3435T haplotype associated with higher risk for refractory Crohn’s disease and ulcerative colitis</td>
<td>(93)</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>ABCB1</td>
<td>2677G &gt; T, 3435C &gt; T</td>
<td>828 Crohn’s disease and 580 ulcerative colitis patients</td>
<td>Risk and severity of disease, steroid use</td>
<td>2677TT genotype associated with severe ulcerative colitis and steroid use</td>
<td>(94)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ABCB1</td>
<td>2677G &gt; T, 3435C &gt; T</td>
<td>211 Crohn’s disease, 97 ulcerative colitis patients</td>
<td>Risk of disease</td>
<td>3435T increased risk of ileo-colonic Crohn’s disease</td>
<td>(95)</td>
</tr>
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<tr>
<td>ABCB1</td>
<td>1236C &gt; T, 3435C &gt; T tSNPs</td>
<td>533 Crohn’s disease, 224 ulcerative colitis</td>
<td>Risk of disease</td>
<td>No statistical association</td>
<td>(96)</td>
</tr>
<tr>
<td>Gene</td>
<td>Finding</td>
<td>Population</td>
<td>Outcome</td>
<td>References</td>
<td></td>
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</tr>
<tr>
<td>ABCB1</td>
<td>2677G &gt; T/A 3435C &gt; T</td>
<td>265 Crohn's disease, 149 ulcerative colitis</td>
<td>Risk and severity of disease, steroid use</td>
<td>No statistical association (97)</td>
<td></td>
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<tr>
<td>ABCB1</td>
<td>34G &gt; A 421C &gt; A</td>
<td>200 Crohn's disease, 186 ulcerative colitis</td>
<td>Risk of disease</td>
<td>No statistical association (98)</td>
<td></td>
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<tr>
<td>ABCB1</td>
<td>1236C &gt; T 2677G &gt; T/A</td>
<td>405 AML patients</td>
<td>Response to chemotherapy</td>
<td>3435CC and CGC/GGC haplotype associated with decreased survival and increased risk of relapse (105)</td>
<td></td>
</tr>
<tr>
<td>ABCB1</td>
<td>2677G &gt; T/A 3435C &gt; T</td>
<td>101 AML patients</td>
<td>Response to chemotherapy</td>
<td>2677G and 3435C associated with higher remission rates and 3-year event-free survival (106)</td>
<td></td>
</tr>
<tr>
<td>ABCB1</td>
<td>3435C &gt; T</td>
<td>200 AML patients</td>
<td>Response to chemotherapy</td>
<td>No statistical association (107)</td>
<td></td>
</tr>
<tr>
<td>ABCB1</td>
<td>1236C &gt; T 2677G &gt; T/A</td>
<td>150 AML patients</td>
<td>Response to chemotherapy</td>
<td>No statistical association (108)</td>
<td></td>
</tr>
<tr>
<td>ABCB1</td>
<td>5UTR SNP</td>
<td>110 AML patients</td>
<td>Response to chemotherapy</td>
<td>Associated with increased relapse rate and poor overall survival (109)</td>
<td></td>
</tr>
<tr>
<td>ABCB1</td>
<td>3435C &gt; T</td>
<td>44 ALL patients</td>
<td>Response to chemotherapy</td>
<td>No statistical association (110)</td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Polymorphism</td>
<td>Population</td>
<td>Outcome Marker</td>
<td>Finding</td>
<td>References</td>
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</tr>
<tr>
<td>ABCB1</td>
<td>3435C &gt; T</td>
<td>53 ALL patients</td>
<td>Response to chemotherapy</td>
<td>No statistical association</td>
<td>(111)</td>
</tr>
<tr>
<td>ABCB1</td>
<td>-129T &gt; C</td>
<td>45 ALL patients</td>
<td>Response to chemotherapy</td>
<td>No statistical association</td>
<td>(112)</td>
</tr>
<tr>
<td>ABCB1</td>
<td>1236C &gt; T</td>
<td>113 patients</td>
<td>Response to chemotherapy</td>
<td>3435CC decreased event-free and overall survival</td>
<td>(114)</td>
</tr>
<tr>
<td>ABCB1</td>
<td>2677G &gt; T/A</td>
<td>914 ovarian cancer patients</td>
<td>Response to chemotherapy</td>
<td>No statistical association</td>
<td>(115, 116)</td>
</tr>
<tr>
<td>ABCC1</td>
<td>4002A &gt; G</td>
<td>62 nonsmall-cell lung cancer patients</td>
<td>Response to chemotherapy</td>
<td>No statistical association</td>
<td>(117)</td>
</tr>
<tr>
<td>ABCB1</td>
<td>IVS18 -30C &gt; G</td>
<td>93 breast cancer patients</td>
<td>Response to chemotherapy</td>
<td>No statistical association</td>
<td>(118)</td>
</tr>
<tr>
<td>ABCG2</td>
<td>421C &gt; A</td>
<td>68 breast cancer patients</td>
<td>Response to chemotherapy</td>
<td>3435CC associated with clinical complete response</td>
<td>(119)</td>
</tr>
<tr>
<td>ABCB1</td>
<td>3435C &gt; T</td>
<td>192 breast cancer patients</td>
<td>Response to chemotherapy</td>
<td>3435CC together with MTHFR and GSTP1 SNPs associated with early relapse</td>
<td>(120)</td>
</tr>
<tr>
<td>Gene</td>
<td>Marker</td>
<td>Finding</td>
<td>Outcome</td>
<td>References</td>
<td></td>
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</tr>
<tr>
<td><strong>ABCB1</strong></td>
<td>3435C &gt; T</td>
<td>41 breast cancer patients</td>
<td>Response to chemotherapy</td>
<td>3435TT associated with complete pathological response (121)</td>
<td></td>
</tr>
<tr>
<td><strong>ABCB1</strong></td>
<td>2677G &gt; T 3435C &gt; T</td>
<td>69 nonsmall-cell lung cancer patients</td>
<td>Response to chemotherapy</td>
<td>3435CC, 2677GG, and GC haplotype associated with better response (122)</td>
<td></td>
</tr>
<tr>
<td><strong>ABCB1</strong></td>
<td>2677G &gt; T 3435C &gt; T</td>
<td>54 small cell lung cancer patients</td>
<td>Response to chemotherapy</td>
<td>3435CC and GC haplotype associated with better response (123)</td>
<td></td>
</tr>
<tr>
<td><strong>ABCB1</strong></td>
<td>3435C &gt; T</td>
<td>123 HIV patients</td>
<td>CD4 cell increase</td>
<td>TT &gt; CT &gt; CC (124)</td>
<td></td>
</tr>
<tr>
<td><strong>ABCB1</strong></td>
<td>3435C &gt; T</td>
<td>340 HIV patients</td>
<td>Virological response</td>
<td>TT genotype associated with increased virological response (125)</td>
<td></td>
</tr>
<tr>
<td><strong>ABCB1</strong></td>
<td>3435C &gt; T</td>
<td>461 HIV patients</td>
<td>Time to virological or immunologic failure</td>
<td>No statistical association (126)</td>
<td></td>
</tr>
<tr>
<td><strong>ABCB1</strong></td>
<td>3435C &gt; T</td>
<td>149 HIV patients</td>
<td>CD4 cell increase</td>
<td>No statistical association (127)</td>
<td></td>
</tr>
<tr>
<td><strong>ABCB1</strong></td>
<td>2677G &gt; T/A 3435C &gt; T</td>
<td>155 HIV patients</td>
<td>Virological failure</td>
<td>Associated with an interaction between ABCB1 2677G &gt; T and CYP2B6 516G &gt; T (128)</td>
<td></td>
</tr>
<tr>
<td><strong>ABCB1</strong></td>
<td>2677G &gt; T/A <strong>ABCC4</strong> 5724G &gt; A 4131T &gt; G</td>
<td>35 HIV patients</td>
<td>Viral response</td>
<td>2677TT &gt; GT &gt; GG (132)</td>
<td></td>
</tr>
</tbody>
</table>
in fact, one of these intronic SNPs was significantly associated with multidrug resistance in epileptic patients (84).

Germline polymorphisms in ABC transporters have also been associated with phenotypes of altered drug or toxin exposure in other complex clinical conditions, such as Parkinson’s disease. Several studies have reported that the \( ABCB1 \) 3435TT genotype is associated with early-onset Parkinson’s disease, although these did not reach statistical significance (85, 86). Another group found that the 3435TT genotype was associated with late-onset (but not early-onset) Parkinson’s disease (87). Because haplotypes containing the 3435T allele have been linked to decreased \( ABCB1 \) expression and function \textit{in vitro} (88), it was hypothesized that 3435T carriers would have decreased P-glycoprotein at the blood–brain barrier and thereby increased neuronal exposure to potentially toxic xenobiotics, predisposing them to Parkinson’s disease. While there is some evidence to support this hypothesis, research in this area is preliminary and remains inconclusive at this time.

A similar hypothesis has been proposed for inflammatory bowel disease (IBD); decreased intestinal \( ABCB1 \) expression may correlate with a weaker physiological defense against intestinal bacteria or toxins, leading to IBD. In support of this hypothesis, it has been shown that \textit{mdr1a} knockout mice develop intestinal inflammation similar to IBD (89). Polymorphisms in \( ABCB1 \) have been associated with susceptibility to IBD; an intronic SNP (rs3789243) and haplotypes including this SNP were highly correlated with the incidence of IBD (90). The 3435TT genotype has also been correlated with ulcerative colitis, a type of IBD (90–94), and with a specific subset of Crohn’s disease (95). However, other studies have not found an association between \( ABCB1 \) genotype and incidence or response to treatment of IBD (96–98). These conflicting reports may be due to the complex pathophysiology of IBD, and the difficulty in phenotyping and subphenotyping patients with different forms of this disease.

ABC transporters were initially implicated as a mechanism for drug resistance because of their ability to efflux cancer chemotherapeutic agents out of tumor cells, thereby preventing their cytotoxicity (1). The effects of ABC transporters are compounded by their overexpression in tumor cells. Numerous studies have shown that overexpression in tumors is due to gene amplification at the chromosomal level (99). The role of ABC polymorphisms in multidrug resistance to cancer chemotherapeutics has also been explored. This has been most extensively studied for hematological malignancies, where expression of ABC transporters has long been recognized as a marker of drug response. For example, increased expression and/or function of MDR1 (P-glycoprotein), MRP3, and MXR have all been associated with remission and/or survival in acute myeloid leukemia (AML) patients (100–103). Interestingly, expression of P-glycoprotein, multidrug resistance protein (MRP1) and MXR is highest in immature, self-renewable, and quiescent leukemic stem cells, consistent with a role for these transporters in multidrug resistance (104). It is less clear whether germline polymorphisms in these transporters influence transporter
expression and drug response. The first and largest pharmacogenetic study in AML examined three common \textit{ABCB1} coding region polymorphisms in 405 Caucasian AML patients (105). The \textit{ABCB1} 3435CC genotype was associated with an increased probability of relapse and decreased overall survival. A similar association with relapse and overall survival was observed in patients homozygous for the CGC/CGC haplotype comprising the 1236C > T, 2677G > T/A, and 3435C > T polymorphisms. These findings could not be directly related to an increased efflux of drugs from the blast cells, since the CGC/CGC haplotype was also associated with the lowest levels of MDR1 mRNA in blasts. The authors suggest that the poor response in these patients was related to pharmacokinetic changes resulting from decreased P-glycoprotein function, but this remains to be shown. In contrast, a similar study in Asian patients \((n = 101)\) found a higher probability of complete remission and 3-year event-free survival in patients with the 3435CC and 2677GG genotypes, consistent with decreased P-glycoprotein function in blast cells from patients with the 3435CC genotype (106). Additional studies show no association between the \textit{ABCB1} 1236C > T, 2677G > T/A, or 3435C > T polymorphisms and response to AML treatment, \textit{ABCB1} mRNA expression, or P-glycoprotein function in blasts (107, 108). In addition to these widely studied \textit{ABCB1} SNPs, a SNP in the 3'-untranslated region (UTR) has been associated with an increased relapse rate and worse overall survival in AML patients (109).

The possibility of polymorphisms in \textit{ABC} genes influencing drug resistance has also been explored in acute lymphoblastic leukemia (ALL) and in solid tumors, and the data are similarly discordant. In general, the \textit{ABCB1} 3435C > T SNP has no effect on treatment outcome, \textit{ABCB1} expression, or P-glycoprotein function in ALL (110–112), although a few positive associations have been weakly suggested (113, 114). In ovarian cancer, the largest study included 914 patients, and there were no associations between common SNPs in \textit{ABCB1}, \textit{ABCC1}, \textit{ABCC2}, or \textit{ABCG2} and response to treatment with carboplatin plus taxanes (115, 116). Most studies in breast and lung cancer have been conducted in very small populations, and any associations must be considered tentative (117–123). Interestingly, a combination of SNPs in three genes (\textit{ABCB1}, \textit{MTHFR}, and \textit{GSTP1}) is the best predictor of postoperative early relapse in breast cancer patients treated with 5-fluorouracil, epirubicin, and cyclophosphamide (120). This highlights the complexity of drug response pathways and the need to consider polymorphisms in multiple genes.

\textit{ABC} transporters expressed on the cell surface of lymphocytes have also been implicated in limiting the intracellular concentration of antiretroviral drugs used to treat HIV and can therefore play a role in drug resistance to antiretroviral therapy. Fellay et al. reported an association between the \textit{ABCB1} 3435TT genotype and increased immunologic response following protease inhibitor-based therapy, indicating that patients with this genotype may accumulate more of a drug inside target lymphocytes, resulting in increased efficacy (124). However, this has not been replicated by others (125–127). The \textit{ABCB1} 3435TT and 2677TT genotypes have also been correlated with a decreased
likelihood of virological failure and a decreased emergence of efavirenz-resistant virus, with no effect on efavirenz plasma levels (125). The results with efavirenz are surprising, given the lack of data supporting a role for P-glycoprotein in the transport of efavirenz. Consistent with the findings of Haas and coworkers (125), the ABCB1 3435CC genotype shows a trend toward earlier virological failure (126). The importance of considering drug response pathways is again illustrated by analyses suggesting that virological failure with efavirenz is best predicted by a two-locus interaction between ABCB1 2677G > T and CYP2B6 516G > T (128).

MRP4 might also be of importance in antiretroviral therapy, since its substrates include nucleoside and nucleotide analogs (129–131). A SNP in the 3′-UTR of ABCC4 was associated with intralymphocytic concentrations of the anti-HIV drug lamivudine (132). The field of antiretroviral pharmacogenetics is still in its infancy and none of the findings to date have been appropriately replicated. However, the importance of optimal antiviral therapy in AIDS patients worldwide makes this an area ripe for further investigation.

15.5. FUTURE PERSPECTIVES

The controversy surrounding the role of ABC polymorphisms in response to antiepileptic drugs, antiretrovirals, and cancer chemotherapeutic agents illustrates some of the major hurdles facing pharmacogenetics. The collection of large populations with detailed drug response phenotype data for use in exploratory and replicative pharmacogenetic studies is particularly limiting. A majority of the studies discussed above have been considerably underpowered to detect clinically meaningful associations, and very few positive findings have been appropriately replicated. There are also significant challenges in defining drug response phenotypes. By necessity, pharmacogenetic studies require the collection of drug response data across multiple sites. It is therefore necessary to develop standard definitions of drug response and toxicity to insure that the proper data are routinely collected and that data sets are available for replication. As with all studies of human genetics, pharmacogenetic associations should be able to withstand the rigor of thorough replication studies before they are considered valid.

As the field of pharmacogenomics progresses, new reports describing intriguing pharmacogenetic associations with ABC transporters continue to appear. For example, a recent publication demonstrated that the ABCG2 421C > A SNP is associated with the pharmacokinetics of the anticancer drug gefitinib (133). Another important focus in ABC transporter pharmacogenomics should be to move beyond single SNP/single gene association studies and to consider detailed drug response pathways. This approach will be aided by the discovery of novel genes implicated in drug response through the application of whole genome SNP scans and further development of animal models for human ABC transporters. It is also critical that the mechanistic basis for
ABC transporter associations is described. While most studies of ABC transporter pharmacogenetics must still be considered preliminary in nature, there is much excitement about the potential for applying such knowledge for the optimization of drug therapy.

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434 GENETIC POLYMORPHISMS IN ABC TRANSPORTERS


The discovery of the major superfamily of proteins called ATP-binding cassette (ABC) proteins originally comes from the study of drug resistance in cancer cells. This can be considered as a paradigm of how a concrete therapeutic problem, when it is approached by highly motivated basic scientists, can lead to fundamental discoveries for cellular and molecular biology. Great hopes were put in the possibility of circumventing clinical resistance of human cancers, but this remains a dream that has not yet been realized in the current practice. Whereas cancer chemotherapy has made considerable progress in the past decade with the discovery, development, and now current availability of targeted therapies, a leading example of which is imatinib, the multidrug resistance phenomenon has hampered the real benefit of chemotherapy regimens for decades.

Multidrug resistance is a public health problem encountered in the treatment of cancer, as well as in the treatment of infectious diseases induced by pathogenic microorganisms such as *Plasmodium falciparum* or trypanosomes. One of the mechanisms by which human cells and bacteria can acquire multidrug resistance involves the expression of membrane proteins that induce an active extrusion of drugs from the inside of the target cells. These efflux pumps have an important physiological role in detoxification, are related to natural defense mechanisms, and influence the bioavailability and disposition of various xenobiotics. In cancer cells, overexpression of these molecules contributes to multidrug resistance by decreasing the intracellular or tissue level of active cytostatics.
In the last 20 years, an important amount of work has been dedicated to determining the structures and physiological functions of ABC proteins, as well as to experimenting with new chemosensitizers that may be able to reverse drug resistance. Major limitations to chemotherapy regimens in which ABC protein modulators were included arose from the toxic effect of these agents on normal cells. For example, the primitive stem cells, which are known to express P-glycoprotein (P-gp) or breast cancer resistance protein (BCRP), can be unwanted targets, thereby inducing myelosuppression. On the other hand, a strategy using gene transfer targeting proteins implicated in multidrug resistance can induce myeloprotection. Multidrug resistance is thus still a major hurdle in efficiently treating cancer and infectious diseases, especially malaria. Even if clinical trials with the third generation of modulators are still ongoing, the preliminary results are not encouraging. This is probably because the perfect reverser does not exist, and each individual patient case would need a specific schedule of chemotherapy according to its expression pattern of ABC transporters.

Chemical modulation is the most widely used approach for overcoming drug resistance because it is the easiest to administer compared to antibodies or antisense strategies. But it should be borne in mind, as emphasized in several chapters of this book, that the clinical trials of the modulators have not always been conducted with the best design for allowing positive end points. We still think that reversal of clinical multidrug resistance could significantly improve the therapeutic response in a large number of cancer patients, but the lack of 3D structures of ABC proteins makes any rational design relayed on “structure-based drug design” very difficult, although some groups have enthusiastically undertaken such approaches.

Another way of circumventing multidrug resistance would be to develop anticancer agents that are not substrates of the multidrug transporters. Epothilones, for example, are a new class of microtubule targeting drugs. They are known to induce microtubule polymerization in the same way as the taxanes; they have a structure quite similar to that of macrolides; and they are not substrates of P-gp. Preclinical experiments have demonstrated a wide range of effects of epothilones on various cancers, but only a few derivatives are currently under clinical trial. A new class of taxanes that is less affected by an overexpression of ABC proteins is also under evaluation (MAC-321 and DJ-927). Other drugs inhibiting tubulin polymerization are currently under study, such as a series of quinazoline derivatives, which have the advantage of not being substrates of P-gp. Some anthracyclines also appear to concentrate in tumor cells independently from P-gp. Although they may still be substrates of the transporter, idarubicin or theprubicin are highly lipophilic, and their rapid uptake cannot be compensated by the slower process of P-gp-mediated extrusion. There are currently numerous other promising agents under development, but their activity against ABC transporters needs to be evaluated.

At the present time, despite the invaluable progress in basic cellular and molecular biology originating from the study of multidrug resistance, no
routine diagnostic or therapeutic approaches to human cancer have ensued. Individualization of prescriptions has appeared within a few years as a leading concept in cancer chemotherapy. It will be the challenge of a new generation of scientists to apply this concept to the transport mechanisms involved in drug resistance and to provide clinicians with adapted tests and personalized prescriptions aimed at successfully combating this disease.
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