ABC transporter blockers and reversal of drug resistance in microorganisms

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Abstract

One of the major causes of drug resistance and chemotherapeutic failure in both cancer and anti-infective therapies is the decrease of effective drug concentrations at their target place, due to the increased action of ATP-binding cassette (ABC) transporters. ABC transporters encompass membrane proteins that couple the energy derived from ATP hydrolysis to the translocation of solutes across biological membranes. Their functions include ancient and conserved mechanisms related to physiological mechanisms in prokaryotes and eukaryotes, emphasizing the biological relevance of ABC transporters. Since ABC transporter blockers can be used in combination with current drugs to increase their effective intracellular concentration, the possible impact of ABC transporter inhibitors is of great clinical interest. Special concern exists since scarce ABC transporter blockers that may be useful to increase the efficacy of current drugs have entered clinical trials and are available to be used in therapeutic regimes. Herein we review the progress made in recent years in the identification, design, availability and applicability of compounds that may work as ABC transporter blockers, as well as the roles of these compounds not only by directly blocking ABC transporters but also in the pharmacokinetics and pharmacodynamics of therapeutic drugs used against infectious diseases. These data may be helpful in the design of strategies to circumvent drug resistance in microorganisms including parasites in clinical circumstances.

Introduction

Chemotherapy has been one of the most potent tools of contemporary medicine, enabling successful treatment of many microbial infections and cancer diseases. However, the great achievements of chemotherapy have been endangered by the development of mutational or phenotypic resistance to the drug effect (Borowski et al., 2005).

Drug resistance is the capacity of disease-causing microorganisms or cancer cells to withstand exposure to drugs previously toxic to them. That is, the ability (or efficacy) of a compound to kill or to decrease the growth velocity or the virulence of a microorganism is dramatically reduced. Efficacy involves many functional steps, from signal transduction and generation of second messengers to regulation of gene expression and modulation of the biological response (Galandrin et al., 2007). Comprehensive understanding of cell metabolism, differentiation check points and intracellular cascades is therefore fundamental to assist in drug research, especially in the field of drug resistance. Fortunately, molecular and biochemical data regarding genome sequences of many bacteria and fungi and of many unicellular parasites are now available at http://www.sanger.ac.uk/Projects/Microbes.

Development of drug resistance is a relatively new concept; it originated in the twentieth century. Resistance to a particular compound was immediately associated with physiological changes that could be overcome if other drugs were used. Indeed, the use of different drugs addressing different targets could circumvent changes in their intracellular concentrations, due to changes in the equilibrium between influx vs. efflux; decreased efficacy due to structural changes in the drug-target; and increased levels of detoxification systems or activation of DNA repair systems (Fig. 1) (Cohen, 1992).

About 60 years ago a more complex scenario appeared for both cancer and infectious diseases, and the concept of ***m***ulti-***d***rug ***r***esistance (MDR) had to be incorporated into the lexicon.

It referred to the loss of a drug's efficacy associated with the selection of cells, or strains of bacteria, fungi or parasites, that were simultaneously resistant to more than one type of drug (Borowski et al., 2005).

MDR was immediately associated with the over-expression of a specific group of transporters (Juliano and Ling, 1976), the ***A***TP ***b***inding ***c***assette (ABC) transporters. Movement of molecules through ABC transporters is coupled to ATP hydrolysis (Holland and Blight, 1999; Saurin et al., 1999). More than 100 members of this family have been described (Pedersen, 2005; Sheps and Ling, 2006); however, the role of many of these conserved proteins still waits to be characterized. As described in chapter 5, ABC transporters in bacteria may export substrates, including drugs and antibiotics, or mediate the uptake of essential nutrients (Sheps and Ling, 2006). In fact, ABC transporters from prokaryote systems mediate the uptake of small solutes like histidine, maltose, peptides or ribose (Ehrmann et al., 1998; Holland and Blight, 1999). On the other hand, in fungi and parasites most ABC transporters function as exporters, mediating the translocation of substrates from the ATP-rich cytosol out of the cell through the plasma membrane or into intracellular organelles, where they collaborate to sequester the drug (Saurin et al., 1999; Sheps and Ling, 2006; Teodori et al., 2006). Most cases of drug resistance are due to the increased expression of ABC transporters that actively decrease the concentration of the drug in the cytosol. It is clear then that, at least theoretically, inhibition of ABC transporters could increase the drug sensitivity of a drug-resistant cell and therefore restore the "original" activity and efficacy of the given drug. Consequently, among other strategies the development of molecules that might inhibit the function of a given ABC transporter has been the subject of intensive research.

One of the main goals of this research has been to identify the minimal characteristics that a compound must possess in order to function as an ABC transporter blocker and hence therapeutically help to restore the original activity of a given drug. The present chapter

summarizes the progress made in recent years in the identification, design, feasibility and value of these so-called ABC transporter blockers. Since the main work has been done in the field of cancer, these data will also be mentioned and incorporated into the scenario of infectious diseases caused by microorganisms.

Drug-resistant microorganisms, physiological implications for infectivity and virulence

It has been suggested that continued therapy upon emergence of drug resistance may be justified as a way to maintain a less healthy microorganism and to reduce the load of microorganisms without drug-mediated cytostatic or cytotoxic effects. This proposal is based on the assumption that, when microorganisms acquire drug resistance, they often have a reduced ability to replicate and cause disease (Lenski, 1998; Geretti, 2005; Quinones-Mateu and Arts, 2006; Gagneux et al., 2006). However, the extent of impairment in microorganism replication varies widely in resistant strains; additionally, a broad variability in the capacity of microorganisms to replicate also exists in wild-type strains due to the natural genetic polymorphism. Moreover, the "cost" paid to achieve resistance, for example in bacteria, depends on the specific mutation that confers drug resistance and on the strain's genetic background. This "cost" can be reversed by compensatory mutations (McAdams et al., 2004; Geretti, 2005; Gagneux et al., 2006) and, therefore, the dynamic value of the regulatory adaptations involved is difficult to estimate (McAdams et al.., 2004). Specifically, each system within an organism makes a contribution that is linked to its overall fitness strategy to survive in a particular environment. We still do not know which implications these processes could have for drug resistance, but understanding the plasticity of microorganisms, as well as the pressures that modulate their metabolic and functional states,

could certainly help to establish general guidelines for clinical decisions in the case of patients suspected of harboring drug-resistant microorganisms.

Decrease in fitness (Natera et al., 2007) has also been a cause of debate regarding parasites (Williams and Day, 2001; Peyron et al., 2004; Hastings and Donelly, 2005). In general, it has been suggested that drug-resistant parasites have a lower reproductive efficiency and a compromised ability to compete with other isolates. However, although drug-resistant mutant parasites have been found to grow more slowly in the presence of drug pressure than its drug-sensitive progenitor, they grow faster when seeded in the absence of drug (Hastings and Watkins, 2005). This finding suggests that compensatory mutations allow the parasite to lead a successful life. Another concept that has been used to define fitness in parasites is virulence, a function that is essential for the survival and pathogenesis of the parasite (Uliana et al., 1999; Beverley, 2001; Williams and Day, 2001; Hastings and Watkins 2005) and changes upon drug resistance development. In fact, continued therapy upon emergence of drug resistance may be viewed as maintenance of a less healthy (virulent) variant and as means to reduce the parasitic load in the absence of actual drug-mediated inhibition. However, the evolution of new levels of virulence in response to medical interventions such as drugs is an issue not yet solved in parasite populations.

Due to the importance that decreased fitness may clinically have, a proposal has been made to create novel organisms with decreased fitness and a minimal probability of developing into resistant microorganisms (McAdams et al., 2004). These organisms should then compete with the original strains. Removal of the best-fit (resistant) strains could allow the less-fit strains to expand into the newly vacated niche space within a host but with less virulence. However, a less competent drug-resistant microorganism could eventually evolve into a competent isolate, i.e., one that is stable and that upon removal of drug pressure can be transmitted to new human recipients. These drawbacks therefore impose a word of caution and indicate that the

maintenance of less efficient resistant strains is still certainly not a tool to be used in the clinic in order to circumvent drug resistance.

Types of ABC transporters

As defined in chapters 1, 2 and 3, an ABC transporter is a transport ATPase. ABC means for "***A***TP ***b***inding ***c***assette", a phrase used by Hyde (1990) and Higgins (1992) to indicate the distinguishing attribute of this transport ATPase class (Pedersen, 2005). ABC transporters contain transmembrane domains, which anchor the protein to the membrane and form a pore through which the transport of substrates occurs. Additionally they have the so called "Walker A and B consensus motifs" (see chapter 1) twice in the same polypeptide chain, denoting the presence of two nucleotide binding sites; however, depending on the studied organisms, these two nucleotide binding sites can also be found in different polypeptides (McKeegan et al*.,* 2004; Pedersen, 2005). The nomenclature of ABC transporters is provided and frequently updated at the following URL: http://www.gene.ucl.ac.uk/nomenclature/genefamily/abc.html.

The best studied ABC transporter is the organic cation pump P-gp (mammalian P-glycoprotein) that is the product of the *ABCB1* gene (Leonard et al*.,* 2003; Higgins, 2007) and is associated with MDR. This is a membrane protein whose hydropathy profile contains an extensive transmembrane region similar to the one present in bacterial transporters belonging to a multifunctional import-export protein family. Both types of proteins (from bacteria and humans) are pore-forming plasma membrane molecules that contain C-terminal sequences homologous to the nucleotide-binding domains of ATP-binding proteins (Gerlach et al*.,* 1986).

This homology, as well as the close similarity of the P-gp with the hemolysin B transport protein, prompted the recognition of P-gp as a eukaryotic homologue of this multifunctional family of bacterial transporters, later known as the ABC super-family (Sheps and Ling, 2006).

Additionally, this data confirmed that ABC transporters, including P-gp, the multidrug resistance associated protein (MRP) (also known as ABCC1) (Cole and Deeley., 1998; McKeegan et al., 2004), and the breast cancer resistance proteins (BCRP, ABCG2; mitoxantrone resistance proteins, MXR) (Doyle et al., 1998; Maliepaard et al., 1999) are pumps, which transport an immense variety of substrates (Gerlach et al., 1986; Hyde et al., 1990).

ABC transporters belong to a superfamily that involves evolutionarily conserved molecules spanning from bacteria to humans (Saurin, 1999; Dassa and Boiuge, 2001; Lage, 2003) and perform a large variety of functions. It is therefore fundamental to determine the structure of many of these proteins, with and without substrates, for a comprehensive understanding of their interaction (McKeegan et al., 2004; Higgins 2007). However, the three-dimensional structures that have been resolved up to now provide only a coarse and static view of the molecule (McKeegan et al., 2004), especially since high-resolution structural data are available for only few of the ABC transporters (Holland and Blight, 1999; McKeegan et al., 2004; Higgins, 2007).

Due to limited writing space and since this chapter will cover only a specific area of the literature related to ABC transporters and their blockers, readers are referred to the following comprehensive reviews: Hyde et al., 1990; Higgins 1992; van Veen and Konings, 1997; Ambdukar et al., 1999; Holland and Blight, 1999; Dassa and Bouige, 2001; Paulsen et al., 2001; Paulsen, 2003; McKeegan et al., 2004; Jones and George, 2005; Dean et al., 2006; Shilling *et al*., 2006; Higgins, 2007; Ponte-Sucre, 2007). Readers are also referred to the chapters (1, 2 and 3) dedicated to the structure and function of ABC transporters including details on their evolution, distribution, biology and physiology.

Strategies to circumvent drug resistance

There are several ways to circumvent drug resistance: to apply higher concentrations of the drugs subjected to resistance, to search for new antibiotics and anti-parasitic drugs that are not ABC transporter substrates, or to block the extrusion of compounds by decreasing the activity of ABC transporters. This chapter deals especially with the third approach; however, the advantages and drawbacks of each strategy will be briefly discussed herein.

Higher concentrations of compounds may partly overcome resistance, but they may increase side effects, and the toxicity of the compound might exceed its benefits. On the other hand, since the substrate specificity of ABC transporters is so broad, the probability of finding and identifying compounds that are not substrates of ABC transporters is rather low (Fojo and Bates, 2003). The search for new antibiotics and anti-parasitic drugs that are not ABC transporter substrates has been going on since the drug efflux mediated by these proteins was identified as a mechanism of drug resistance. In fact, new ketolides, macrolides, and tetracyclines as well as fluoroquines are designed against both Gram-positive and Gram-negative bacteria (Lomovskaya and Watkins, 2001, Lomovskaya et al., 2001). For example, the new tetracyclines (glycylcyclines) do not generate drug resistance because they are not recognized by ABC transporters, and therefore are not transported out of the cell. But approaches to modify existing classes of drugs to enable them to avoid efflux pumps have been less successful for Gram-negative than for Gram-positive bacteria. One of the reasons for this is that drugs have to be amphiphilic in order to cross both membranes of the Gram-negative bacteria, a property that makes them good substrates for ABC transporters (Lomovoskaya and Watkins, 2001).

The most trustworthy avenue to overcoming drug resistance, then, is the development of agents that increase the pharmacodynamics of the existing drugs by inhibiting ABC transporters.

The aim of this latter approach is to interfere with either the expression or the function of the transporters. These goals could be attained using genetic approaches (to decrease the ABC transporter gene expression) or through more pharmacological approaches such as the design of compounds that modulate the activity and the function of the ABC transporter proteins (Borowski et al*.,* 2005).

Although ABC transporters have a strongly conserved primary sequence, few orthologous pairs of transporters are shared between phyla (Sheps et al., 2004). This characteristic, together with the broad substrate recognition of ABC transporters, suggests that redundancy in ABC transporters for the same substrate renders gene loses tolerable (Sheps et al., 2004) and therefore that the regulation of ABC transporter gene expression as a tool to revert drug resistance would not necessarily be reflected in a better therapy for the patient.

On the other hand, in the field of cancer research much attention has been paid to the control of MDR expression by interfering mRNA translation with antisense oligonucleotides (Borowski et al*.,* 2005). This methodology is dealt with in chapter 10 and will therefore not be discussed in the present chapter.

As already mentioned, pharmacological approaches to modulate the activity and the function of ABC transporters could result in a better therapy for the patient. During the remaining part of this chapter we will concentrate on the use of this strategy to circumvent drug resistance in infectious diseases. Initially we will discuss issues that are fundamental to understand how difficult it has been to use this approach and which advantages and drawbacks have resulted from the knowledge we have so far.

ABC transporters contribute to the pharmacokinetics and pharmacodynamics of drugs

ABC transporters are promiscuous

Despite the structural variations found among ABC transporters, most of these proteins recognize similar dyes, toxic ions, and clinically relevant antibiotics and anticancer drugs (van Veen, 2001). Regrettably, the broad spectrum of substrates that are accepted by ABC transporters and the poor specificity and low affinity that the substrates hold for the binding site, have impaired a successful development of effective blockers that might enter the clinical pipeline and be approved for therapeutic use (Teodori et al*.,* 2006).

In fact, questions that stimulate scientists regarding ABC transporters include: how can these proteins recognize the so many different substrates they should accommodate? Do they discriminate the substrates at separated recognition sites? Are they specific, are they promiscuous; is it necessary to inhibit all the transporters in a cell to obtain a therapeutic response? Or is it enough to inhibit a small percentage of them? (Teodori et al*.,* 2006). The information obtained up to now suggests that one large polymorphous recognition domain accommodates a great variety of substrates (Loo and Clarke, 2000; Loo and Clarke, 2001; Kaur, 2002; Pajeva and Wiese, 2002; Avendaño and Menendez, 2002, 2004; Yu et al., 2003; Murray et al., 2004; Pajeva et al*.,* 2004; Pleban et al*.,* 2005; Teodori et al*.,* 2005, 2006).

ABC transporters are ubiquitous

The wide distribution and multiple functions of ABC transporters complicate more than facilitate the strategies to overcome drug resistance. In fact, their ubiquity affects the absorption,

distribution and clearance of many families of drugs both at the cellular and at the systemic level. Moreover, compounds that inhibit ABC transporters can affect their own distribution.

ABC transporters are extensively localized in normal tissues. Their participation in many functions may control the tissue levels of exogenous compounds, medicaments and xenobiotics (van Veen and Konings, 1997). This means that although a key issue in designing ABC transporter blockers is the inhibition of drug efflux to enhance its effect, additional (side) effects related to the physiological functions of ABC transporters should be expected (Holland and Blight, 1999).

More challenging is the fact that any foodstuff or metabolite that modulates the activity of ABC transporters affects not only the movement of substrates but also the pharmacokinetics of any drug present. This all means that the physiological role of P-gp and sister proteins requires modulators that are more potent with proper selectivity and pharmacokinetics in order to avoid unwanted side effects (Teodori et al., 2006). The resolution of the structures of strictly related transporters allows the improvement of models that would give useful information on the drug recognition site of P-gp and MRP1. Consequently, medicinal chemists are changing their random screening to rational design of molecules, tailored to interact with high affinity with the recognition site of extruding pumps and lacking the unwanted properties (Teodori et al., 2006).

It is known that ABC transporters located in the gastrointestinal tract may limit the absorption of drugs and an area currently not explored regarding the availability of antibiotics and anti-parasitic drugs is the use of ABC transporter blockers to enhance the systemic levels of drugs that have been orally administered (Sun et al., 2004). The use of ABC transporter blockers may therefore increase the systemic availability of drugs whose transport back to the lumen of the gastrointestinal track depends on ABC transporters. The impact of this could be an immediate change in drug pharmacokinetics (Leonard et al., 2003; Higgins, 2007).

Plasma proteins mimic the binding of compounds to ABC transporters

The binding properties of ABC transporters associated with chemo-resistance are very similar to those of the plasma protein α1-acid glycoprotein (AGP). AGP is a component of plasma and is normally present at concentrations between 0.5 to 1.0 mg ml^{-1}. Together with albumin, AGP belongs to the most important proteins in plasma that determine the free drug concentrations available for the pharmacological effect. In general it is said that AGP and ABC transporters associated with drug resistance might act synergistically in protecting cells from xenobiotics. This is especially true since both proteins have a considerable overlap in the type of compounds they bind and the molecules that can induce their expression (Zsila and Iwao, 2007) [Box one]. This means that understanding the binding characteristics AGP could help in the prediction of drug interaction and disposition in chemo-sensitive and chemo-resistant scenarios. Moreover, an analysis of the behavior of AGP should be considered in situations where optimization of pharmacotherapy is desired, especially since its manipulation can pragmatically lead to changes in the systemic levels of drugs.

AGP belongs to the so-called group of "acute phase proteins" and its levels correlate with the severity of a disease, the stage of a disease, and the success of the therapy (Zsila and Iwao, 2007). In fact, at least in the case of antimalarial drugs, AGP has been identified as the source of failure in the reversal of chloroquine resistance in *Plasmodium falciparum* (Zsila et al., 2008). Furthermore, the development of a promising anti-HIV protease inhibitor was discontinued because its extensive binding to AGP markedly decreased its anti-HIV properties (Zsila and Iwao, 2007). Also, it has been demonstrated that the effectiveness of verapamil-type compounds is greatly reduced because they extensively bind to AGP rather than to ABC transporters (Gbotosho et al., 2006).

Biology of ABC transporter substrates and modulators

It has been recognized that substances that permeate the membrane slowly can be transported out of the cell by P-gp, whereas those that permeate rapidly act as P-gp inhibitors (Sheps and Ling, 2006). This means, that at least theoretically, P-gp inhibitors can be cycled repeatedly through the cell membrane and in this way prevent the export of the more slowly cycled accompanying drug, increasing its concentration in the cell; in this case, the modulator acts as a competitive inhibitor (Varma et al., 2003). Therefore, the speed at which P-gp and ABC transporters in general move substrates across membranes is crucial, and the analysis of resistance of a cell to a given compound must thus consider the membrane and the transporter as a functionally integrated unit (Sheps and Ling, 2006). For example, this is fundamental in the case of hydrophobic compounds and their interaction with P-gp, (Etian et al., 1996). Furthermore, changes in membrane dynamics might allow drugs to enter the cell more rapidly than P-gp can export them, thereby reducing resistance (Kamau et al., 2005). Taken together, this means that the critical parameter determining whether a cell resists a drug is not the extracellular concentration of drug, but rather the intracellular concentration set by the equilibrium between drug inflow through the membrane and drug export by the cell's competent transporters (Sheps and Ling, 2006).

Classification of ABC transporter blockers

The importance of transporter blockers to circumvent drug resistance was originally recognized by Tsuruo (1983). His group was the first one to describe that the calcium channel inhibitor verapamil and the phenotiazine derivative trifluoperazin potentiate the activity of vincristine (Tsuruo, 1983). Since then, the discovery and/or design of transporter blockers has become a clinically relevant endeavor.

P-gp modulation occurs by diverse means for different classes of compounds. P-gp inhibitors may interact directly with the binding site of the ABC transporter through one or more of the P-gp binding sites, thereby blocking (competitively or non-competitively) the transport of substrates. Interestingly, the data suggest that P-gp has multiple, interacting drug-binding sites, but can also possess one single, large, flexible pocket (Higgins, 2007). Alternatively, an inhibitor may prevent ATP binding, ATP hydrolysis, or the coupling of ATP hydrolysis to the translocation of substrates (Ambudkar et al., 1999).

Presently ABC transporter blockers are classified in three generations. First generation inhibitors are pharmaceuticals already in use for other treatments but also able to block P-gp, such as calcium channel inhibitors like verapamil, immunosupressants like cyclosporin A, anti-arrhythmics and neuroleptics like quinidine, reserpine, and yohimbine, and antiestrogens like tamoxifen and toremifene (Varma et al., 2003). The clinical efficacy of these compounds is limited by their toxicity (Khrishna and Mayer, 2000; McKeegan et al., 2004). For example, verapamil produces cardiac cytotoxicity at the concentration needed to inhibit drug resistance. This fundamental drawback has stimulated the development of modulators lacking the toxic side effects of first generation compounds.

Second generation P-gp modulators like R-verapamil, GF120918, MS-209, PSC-833, VX-710, or VX-853 were derived from first generation P-gp modulators with the specific purpose of decreasing drug resistance. For example, the compound PSC-833 (valspodar) was developed in an effort to find a non-immune-suppressant analogue of the natural chemosensitizer cyclosporine A (Boesch et al., 1991). This compound is 10-fold more active than cyclosporine in blocking the ABC transporters associated to drug resistance, and has already undergone clinical trials (Fracasso, 2001). Unfortunately, although second generation modulators are much less toxic than first generation modulators, they can still produce extreme side effects (Krishna and Mayer,

2001; Fracasso, 2001; McKeegan et al., 2004), especially due to an inhibition of multiple cell ABC transporters and to drug-drug interactions (Balayssac et al., 2005; Varma et al., 2003).

Third generation modulators, like biricodar, laniquidar, zosuquidar, LY335979, OC144093, R101933 and XR9576, are highly selective inhibitors of P-gp still under development (Dantzig et al., 2003). Clinical trials with third generation modulators specifically developed for MDR reversal are in progress. The results however are not encouraging and it may be that the perfect reverser does not exist (Nobili et al., 2006.

Structure-activity relationship in ABC transporter blockers

An ABC transporter blocker should selectively block the ABC transporters associated with drug resistance without affecting its own pharmacokinetics or the pharmacokinetics of the targeted compound (Seelig, 1998). [Box two]

In the 1990s, a minimum of structural features needed for compounds to be substrates of ABC transporters was proposed. These features included a basic nitrogen atom and two planar aromatic domains. Furthermore, at that time it was stressed that the aromatic domains should adopt a well-defined conformation, although at that same time it was demonstrated that compounds lacking these domains could also interact with ABC transporters. Until now, being hydrophobic and amphiphilic, as well as possessing a basic amine have emerged as the main structural characteristic of compounds that are substrates of ABC transporters (Seelig, 1998). Following these guidelines, numerous compounds have been either synthesized or isolated from plants, and tested as ABC transporter blockers especially against P-gp and MRP-1 (See table 1) (Avendaño and Menendez, 2004).

The literature involving the structure-activity relationship (SAR) of ABC transporters in general and especially the proteins involved in drug resistance, is large and complex and is

extensively discussed in chapter 3. Herein we will briefly summarize of the results that are fundamental for our discussion.

Although the various approaches focus mainly on the development of ABC transporter blockers or modulators, in many cases addressing the substrates of these transporters cannot be avoided (Stouch and Gudmundsson, 2002). Furthermore, it is often difficult to differentiate between substrates, modulators and inhibitors. In fact, as is described in chapter 3, information is still not complete regarding the structural design of drug and modulator binding sites, the way in which the energy released from the ABC domains is transferred to the transport module, and the details of the transport mechanism (Pedersen, 2005; Sheps and Ling, 2006; Higgins, 2007). This information is essential for designing drugs to modulate the activity of ABC transporters (van der Heide and Poolman, 2002; Shilling et al*.,* 2006).

On the other hand, as already mentioned ABC transporters translocate many compounds and it is not easy to find compounds that do not behave as substrates. Therefore, the analysis and definition of the available compounds is normally biased towards the characteristics substrates should have. Furthermore, many of the compounds are pesticides, and many are structurally related. These characteristics prevent the determination of a truly general SAR especially if methods employing structural features or fragments as descriptors are used (Stouch and Gudmundsson, 2002).

An additional issue is the variability in the methods, cell lines, assays and record systems employed to collect the data. In many cases, the data cannot be compared, making the task to develop a realistic and valid SAR for ABC transporters even more difficult. In fact, several factors, including the assays employed, the binding sites, the cell lines, the inducer and the mechanism used to stimulate the expression of the ABC transporters, the membrane partition of

the compounds, and the permeation rates of them could be so varied that the data obtained cannot be definitively compared (Stouch and Gudmundsson, 2002).

On top of this, in the conditional case that all factors considered are equivalent, an intrinsic dilemma arises: as described in chapter 3, a minimum of two binding sites have been described, further complicating the situation (Wiese and Pajeva, 2001). Additional problems are the cytotoxicity of the compounds or the alteration of membrane properties promoted by ABC transporter blockers, or reentry of the compounds into the cell, thus producing an unspecific component in the function of ABC transporters (Stouch and Gudmundsson, 2002).

Troubles usually include the fact that binding or affinities have been used as terms of functionality instead of using substrate efflux or ATP degradation. Binding does not necessarily confer activity and therefore the use of this concept may induce an additional complexity and should be avoided. Additionally, the use of descriptors such as *log P* (logarithm of the ratio of the concentrations of the un-ionized solute in the solvents), van der Waals forces or additional molecular properties has not been consistent, and imposes additional complexities that complicate the real meaning of the SAR studies. (Stouch and Gudmundsson, 2002).

As a result of this complicated panorama many studies have abandoned the quantitative analysis of the SAR and have simply classified the compounds as substrates and inhibitors. Although with this approach the effect of weakly active molecules is lost, at least a sensitive and sensible result is obtained. There is consensus in that the following properties should be fulfilled by the pharmacophore: (i) the molecule should have a *log P* value of at least 2.92 or higher to allow a hydrophobic van der Waals interaction with the ABC transporter (which is lipophilic); (ii) the molecule should have a long chain axis higher than 18 carbon atoms, to cover more than one unit of protein to increase the strength of the binding; (iii) the molecule should promote a good nucleophillic interaction of the molecule with the ABC transporter (that is, the energy

should favor a better nucleophillic attack); and (iv) in order to form a cation at physiological pH the molecule should have at least one tertiary amine, so that the ionic/hydrogen bond is strengthened (Wang et al., 2002), [Box three].

Of the many SAR studies found in the literature for ABC transporters (Ecker and Chiba (1995; Seelig, 1998; Krishna and Mayer, 2001; Geney et al., 2002; Stouch and Gudmundsson, 2002; Wang et al., 2002, Dantzig et al., 2003, McKeegan et al., 2004; Tsakovska and Pajeva, 2006., the one by Seelig (1998) is especially elegant. Although already somewhat old, the description made by this author is very sophisticated, relates the compounds to their activity and classifies them as pharmacophores, i.e., substrates, no substrates, and inducers.

In her description of the features that should be fulfilled by compounds, Seelig (1998) describes two recognition elements based on electron donor characteristics: one (named Type I), is constituted by two electron donor groups separated 2.5 $\pm$ 0.3 Å; a second one (named type II), is constituted by two electron donor groups separated 4.6 $\pm$ 0.6 Å; in some cases a third electron donating group may be located between the outer other two. Seelig (1998) analyzed 100 compounds from the literature and could identify the following characteristics: compounds classified as inducers contained one type II recognition element and many type I recognition elements, and their capacity to form hydrogen bonding correlated with their capacity to induce the expression of ABC transporters. Unfortunately, inhibitors were hard to find and only 7 could be analyzed; additionally, although somewhat similar to the substrates, they did not present the recognition elements.

In the model of Seelig (1998), partitioning into the membrane is the rate limiting step in the interaction between ABC transporters and compounds; additionally, dissociation of the compound from the transporter is related to the strength of the hydrogen bonds. However, Seelig

did not take in consideration molecular weight and hydrophobicity; a compound can bind and still have structural modifications that render it inactive, originating an inhibitor or blocker.
The good correlation between the number of recognition elements and the strength of the binding suggests that ABC transporters may have more than one binding site, all of them similar or equivalent. Additionally, the structural variety of substrates means that different drugs can occupy different receptor sites in different binding modes, the most active compounds being those that occupy simultaneously the highest number of receptor sites (Avendaño and Menendez, 2004).

A further attempt to make SAR analysis for ABC transporters was done by Stouch and Gudmundsson (2002). They performed a study where descriptors such as molecular weight, shape, lipophilicity, complexity, and electronic conformation were analyzed to evaluate most of the compounds used by Seelig (1998). Unfortunately their analysis could only modestly differentiate between substrates and blockers; the best descriptors were molecular shape and electrostatic charge across the surface. In an additional attempt to make a SAR analysis for ABC transporters, flavonoids with 3-OH group have been described to play a role as antioxidants, while flavonoids with 5-OH and/or 7-OH groups show a higher cytotoxicity and flavonoids with 3'-OMe and/or 5'-OMe groups are good as P-gp inhibitors. Of note, flavonoids with 6-OMe groups are not good P-gp inhibitors (Jeong et al*.,* 2007). It is believed that these SAR results can be taken into account for the development of flavonoids with high therapeutic index (Jeong et al*.,* 2007).

From the previous discussion it is clear that additional data are urgently needed in order to have a better knowledge of the SAR of ABC transporters. However, although many problems need to be solved, the information obtained to date has been helpful for understanding the architecture of the binding sites for drug and modulator and the link between energy generation

and drug translocation functions, as well as for the rational design of drugs to block the activity of ABC transporters.

Examples of the real use of ABC transporter blockers

As we already mentioned, the design and isolation of specific blockers for ABC transporters is one of the most promising approaches to overcome the limited success of antimicrobial and anti-parasitic chemotherapy. For example, quinolone derivatives and other alkoxyquinoline derivatives as well as semi-synthetic derivatives of tetracyclines inhibit the efflux of antibiotics via ABC transporters and restore, at least partially, the susceptibility of bacteria to different antibiotics (Nelson and Levy, 1999; Mallea et al*.,* 2003; McKeegan et al*.,* 2004). However, the success of this approach has so far been limited. Further, the activity of classical ketolides and macrolides against Gram-positive macrolide-resistant bacteria is enhanced by the presence of an ABC transporter blocker, but this is not the case for newly designed macrolides or fluoroquinolines (Lomovskaya and Watkins, 2001). The reader is referred to chapter 4 for a deeper discussion on this theme.

The analysis of compounds isolated from natural sources is still in its infancy and the number of groups searching for these compounds is very small. Plants should have evolved compounds to evade (or promote) mechanisms of accumulation of xenobiotics in different ecological scenarios and this information should be very useful in the design of ABC transporter blockers (Stavri et al*.,* 2007).

Natural inhibitors include flavonoids extracted from plants. They have a very low toxicity and increase the cellular accumulation of substances like the fluorescent substrate rhodamine 123 in KB-C2 cells overexpressing P-gp, due to a selective inhibition of ABC transporters (Kitagawa, 2006). These flavonoids are many times more active than verapamil, their inhibitory activity was

found to correlate with their hydrophobicity as measured through their capacity to dissolve in octanol, and are much safer than previously designed chemosensitizers (Choi et al., 2002). Of note, flavonoids do not bind to the transmembrane domains but rather to the nucleotide binding domain of ABC transporters, although in a site different from that occupied by ATP (Dayan et al., 1997).

Although chapters 6 and 7 discuss which compounds are inhibitors of ABC transporters in *Leishmania* and *Plasmodium* we want to mention very briefly the pharmacological value of dihydriethanoanthracene derivatives (Pradines et al., 2002) and dihydro-β–agarofuran sesquiterpenes isolated from the roots of *Mayetna magallanica* and *M. chubutensis* (Kennedy et al., 2001; Pradines et al., 2002) against parasitic microorganisms. Dihydriethanoanthracene derivatives restore the therapeutic efficacy of chloroquine against resistant parasites when simultaneously administered. On the other hand, dihydro-β–agarofuran sesquiterpenes revert the resistant phenotype and modulate intracellular drug accumulation in *Leishmania* parasites, probably affecting ABC transporters. These results suggest that the administration of an additional compound available at relatively low cost may be an effective strategy to enhance the activity of the common antiparasitic drugs.

It is also worth to mention that Hayeshi et al. (2006) described that out of 21 classical anti-parasitic drugs most of them flavonoids, 14 inhibit ABC transporters expressed in human intestinal epithelial CaCo-2 cells. Of these 21, only quinine, which also classified as the most active of all 21 compounds, is simultaneously an inhibitor and a substrate of the ABC transporters expressed in these cells; the rest of the anti-parasitic drugs act only as inhibitors. Finally, ABC transporter blockers have been used efficiently *in vivo* to increase the potency of classical drugs and reduce the size of lesions in BALB/c mice infected with drug-resistant *Leishmania* (Serrano-Martin et al., 2006). Interestingly, the drug-resistant parasites used in this

assay were selected and characterized using previously described protocols (Ponte-Sucre et al., 1997; Silva et al., 2004; Uzcátegui et al., 2005; Machuca et al., 2006). The herein described results suggest that, at least in experimental settings, the simultaneous administration of ABC transporter blockers increases the efficiency of the primarily administered drug.

Recently designed compounds include inhibitors having the 17-azapentacyclo[6,6,5,$0^{2,7}$,$0^{9,14}$,$0^{15,19}$]-nonadeca-2,4,6,9(14),10,12-esene-16,18 dione scaffold. These compounds inhibited the translocation of fluorescent rhodamine 123 through ABC transporters expressed in L5178 mouse T-cell lymphoma cells infected with the pHq MDR1/A retrovirus (Bisi et al., 2006). Importantly, compounds with the dione scaffold inhibit drug translocation 5-fold more effectively than verapamil.

Finally, improving the bioavailability of orally administered drugs is a fundamental challenge that if resolved would mean the real introduction of ABC transporter modulators in the combination therapy with the concomitant result of a decreased development of drug resistance (Edafiogho et al., 2007). In an attempt to reach this goal, Edafiogho et al. (2007) proposed to develop enaminone derivatives that retain their physicochemical properties pertinent to P-gp recognition. This proposal is based on the fact that enaminones are weak substrates of P-gp, and therefore could act as potent inhibitors of the pump. Interestingly, the lack of specificity for action on P-gp is considered to be an inherent disadvantage for most if not all ABC transporters modulators. Pharmacokinetic complications could occur from the interaction of the ABC transporter inhibitors with cytochromes, involved in drug metabolism. Fortunately, in the case of the enaminones like LY335979 or OC144-093, an increased specificity and consequently a decreased occurrence of pharmacokinetic alterations has been demonstrated.

Many P-gp inhibitors have also been tested against MRP-1 or BCRP but unfortunately, most of them had no inhibitory effect (Allen et al., 2002; Boumendjel et al., 2005). This was somehow

predictable, as P-gp recognizes hydrophobic compounds while MRP-1 recognizes hydrophilic substrates (Boumendjel et al., 2005). However, this result was difficult to understand since BCRP transports structurally and functionally diverse organic substrates, including hydrophobic compounds, weak bases, organic anions, and glucuronide-, sulfate-, glutamylate- and glutathione-conjugates (van Herwaarden and Schinkel, 2006). Additionally, P-gp inhibitors should prevent drug extrusion from cells by ABC transporters and thus increase the cytotoxic action of drugs transported by ABC transporters, but since they act on multiple ABC transporters, they could also induce an enhanced level of toxicity at different non-target sites (Sheps and Ling, 2006).

Conclusions and future trends

The increasing development of drug resistance is one of the major reasons why cancer chemotherapy fails, and is predicted to constitute one of the main impediments to successful chemotherapy against microorganisms in the near future. Although the design of new drugs not transported by ABC transporters is in progress, the isolation or design of blockers to ABC transporters constitutes one of the most promising approaches to overcoming the lack of success in the aforementioned chemotherapeutic approaches. Several ABC transporter blockers have been successfully tried against bacteria and parasites; however, ABC transporter inhibitors are not yet in standard use for the treatment of bacterial or parasitic infections or for cancer diseases. Reasons for this are numerous and include the low substrate specificity and the multiple functions subserved by ABC transporters.

The central paradigm of SAR is that compounds with similar structures act at the same site and with the same mechanism. Unfortunately, as already discussed, inhibition and/or binding at P-gp, MRP-1, or ABC transporters in general can occur via different mechanisms as well as at a number of different sites, and a successful design of ABC transporter substrates, modulators, and

blockers has thus been hindered by an incomplete understanding of the mechanisms and biology of these transporters.

In this regard, various organizations are devoted to encourage the development of anti-infective drugs in general and ABC transporter modulators in particular. In this regard, two Web sites, http://www.TDRtargets.org and http://www.dndi.org/, focus on many diseases that have not been high on the pharmaceutical industry's priority list because most of them largely afflict people from developing countries.

Finally, as already mentioned by Higgins (2007), probably the time has come not to fight against drug resistance but to find alternatives to avoid and avert it. In this regard, a promising avenue is the use of ABC transporter blockers to actively prevent the emergence of drug resistance. For example, it would be interesting to explore whether the use of ABC transporter blockers prevents the selection of resistant strains with increased expression of ABC transporters like MDR or MRP. Prevention of chemo-resistance through the combination of ABC transporter blockers with the selected drug has, however, the potential drawback that resistance to the inhibitor itself could appear. Hence, the risk/benefit profile of the joint use of such combinations should be evaluated on a case-by-case basis.

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Figure legend

Figure 1: Main resistance mechanisms to drugs observed in cellular systems

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