Utilization of membrane vesicles to study the interaction of the ABCB1 and ABCG2 transporters and small molecules to predict the ADME properties of pharmaceuticals

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Table of Contents

Abbreviations

ABC transporters: ATP-Binding Cassette transporters

ADME: Absorption-Distribution-Metabolism-Excretion

AM: acetoxy methylester

BSA: bovine serum albumin

ATP: adenosine triphosphate

BCRP: ABCG2

Caco-2: Human colonic adenocarcinoma cells

CHO: Chinese Hamster Ovary cells

DDI: drug-drug interactions

DMEM: Dulbecco's modified Eagle medium

DMSO: dimethyl sulfoxide

F12: Ham's F12 medium

FDA: Food and Drug Administration

GF: GF120918, elacridar

HBSS: Hanks' balanced salt solution

HDM-PAPMA: hexadecane artificial membrane permeability assay

HIV: human immunodeficiency virus

HPLC: high performance liquid chromatography

LLC-PK1: porcine kidney epithelial cells

MDCK: Madin-Darby canine kidney cells

MDR: multidrug resistance

MDR1: ABCB1 (P-glycoprotein)

MXR: ABCG2

P-gp: ABCB1 (P-glycoprotein)

R123: Rhodamine 123

Sf9 cells: *Spodoptera frugiperda* ovarian cells

1 Introduction

1.1 The first observations of the field

It has been long known that when tumor cells are treated with a specific chemotherapeutic agent resistance against the cytotoxin often develops. Surprisingly, the acquired resistance is not restricted to the cytotoxic agent used, but it is also extended to other, structurally unrelated cytotoxins. This phenomenon is called multidrug resistance. 30 years ago, when researchers were looking for the mechanism underlying multidrug resistance they identified a 170 kDa transmembrane glycoprotein in colchicine selected CHO cells (Juliano and Ling, 1976). They found that cells expressing this protein on their surface exhibit altered drug permeability, and therefore named it P-glycoprotein (P-gp). Twelve years later the amplification and extensive transcription of a gene in multidrug resistant cell lines was described (Shen et al., 1986). The gene was named mdr1 for multidrug resistance, and based on the structure of the gene and sequence homologies it was proposed that it was an ABC transporter (Chen et al., 1986). The same year it was shown that the mdr1 gene codes for P-glycoprotein (Ueda et al., 1986). These first observations showed that an ABC transporter is capable of transporting pharmaceuticals (chemotherapeutic drugs) thereby protecting cells from their toxic effect. Advance on the field of P-gp and multidrug resistance research played a central role in ABC transporter research ever since.

Several years later it was accidentally discovered, that the product of the mouse Mdr1a gene (rodents have two Mdr1 genes designated Mdr1a and Mdr1b) plays a central role in protecting mice from the neurotoxic effect of the antiparasite ivermectin. Mdr1a (-/-) mice were 100-fold more sensitive to orally administered ivermectin, due to significantly higher brain-to-plasma ratios of the antiparasite when compared to wild type mice. It was proposed that ivermectin is a substrate of Mdr1a that is located in the apical membrane of brain endothelial capillary cells and limits the entrance of ivermectin into the central nervous system (Schinkel et al., 1994). This was the first report showing that an ABC transporter can protect whole compartments of the human body from the toxic effects of a xenobiotic.

The last 15-20 years extensive research in this area resulted in significant advance in our understanding of how different ABC transporters modulate the different pharmacokinetic parameters of xenobiotics. It turned out that multiple ABC transporters located at different pharmacological barriers are important determinants of the ADME (Absorption-Distribution-Metabolism-Excretion) properties of several drug molecules. This current work does not aim to give a full overview of the countless examples of such drug-ABC transporter interactions. Recent reviews covering the topic in detail are available (Sarkadi et al., 2006), (Glavinas et al., 2004). Two transporters stand out as pivotal in determining the ADME properties of drugs: ABCB1 (MDR1, P-gp) and ABCG2 (BCRP, MXR). The aim of this work was to set up and validate *in vitro* methods to detect the interaction of drug molecules with these two transporters. Therefore, this introduction is focused on the structure and function of ABC transporters with particular attention on the biochemical characteristics of ABCB1 and ABCG2. Also, the currently available *in vitro* methods are reviewed and the current position and acceptance of these assays in pharmaceutical industry is analyzed in detail.

1.2 General overview of ABC transporters

1.2.1 The structure and function of ABC transporters

ABC proteins form one of the largest protein families known. The family is characterized by a conserved structure of ABC binding domains (containing the "ATP Binding Cassette" motif) and transmembrane domains. In mammals, the functional ABC protein contains two ATP binding domains and two transmembrane domains. The four domains can be present in one polypeptide chain ("full transporters") or might be set up by the homo- or heterodimerization of two polypeptides containing one of each domains ("half transporters"). Each transmembrane domain forms 6 transmembrane alpha-helices spanning the membrane. The two transmembrane domains together probably form a pore-like structure creating a channel across the membrane. The transmembrane domains are thought to exist in an "open" and "closed" conformation regulated by ATP binding and hydrolysis. The change in conformation might be responsible for the translocation of compounds across the cell membrane, might open and close ion channels or serve as

regulators for complex, multi-protein receptors. Based on homology searches using the conserved ABC motif 49 human ABC proteins have been identified in the human genome. Based on similarity in the gene structure, order of the domains, and sequence homology these proteins are grouped into 7 families designated with letters A-G. The members of each family are designated by a number following the letter.

ABC transporters transport substrates across the cell membrane against their concentration gradient (active transport). The energy requirement for this process is derived from ATP hydrolysis. It is believed that ATP binding and hydrolysis generates a conformation change in the ATP binding domain. The close interaction between the ATP binding domains and the transmembrane domains creates the transmission of force to the transmembrane domains resulting in a conformation change in the transmembrane domains driving substrate translocation. Several members of the ABC protein family were shown to be active transporters (Sarkadi et al., 2006). This work focuses on two such ABC proteins: ABCB1 and ABCG2.

1.3 ABCB1 and ABCG2

1.3.1 ABCB1

ABCB1 was the first ABC transporter discovered. It is a full transporter; a glycosylated protein expressed in the apical membrane of cells. It is localized at several pharmacological barriers including the intestinal brush-border membranes, the canalicular membrane or hepatocytes, the apical membrane of the endothelial cells of the blood-brain barrier and the placental brush-border membrane. ABCB1 has high transport capacity and broad substrate recognition. Different, chemically unrelated molecules (e.g. anticancer drugs, HIV-protease inhibitors, antibiotics, antidepressants, antiepileptics, and analgesics) were shown to be transported substrates of ABCB1. The fluorescent molecules calcein AM, rhodamine 123 (R123) and Hoechst 33342 were also shown to be transported by ABCB1. The structural basis for the promiscuous behavior of the transporter is still under investigation. Based on the physicochemical properties of substrates identified it was proposed that the model ABCB1 substrate is amphipatic, with a molecular mass of 300–2,000 Da (Sarkadi et al., 2006).

1.3.2 ABCG2

In 1998 researchers were studying mitoxantrone selected MCF-7 cells (derived from a breast cancer patient) and identified a novel ABC transporter that conferred resistance against mitoxantrone, doxorubicin, and daunorubicin (Doyle et al., 1998). The protein was named BCRP (Breast Cancer Resistance Protein) or MXR (Mitoxantrone Resistance Protein). In the standard nomenclature it is designated ABCG2. Later, it turned out that the selected cell line used in these studies expressed a mutant form of ABCG2 carrying a glycine at position 482. The wild type protein (482R) was cloned from human placenta (Allikmets et al., 1998).

The ABCG2 transporter is a half transporter. It was shown that it functions as a homodimer (Ozvegy et al., 2001) and it is localized to the plasma membrane of cells (Rocchi et al., 2000). It is expressed on the intestinal brush-border membranes, the canalicular membrane or hepatocytes, the apical membrane of the endothelial cells of the blood-brain barrier and in the placental brush-border membrane (Litman et al., 2001). Interestingly, ABCG2 was also shown to be expressed in the mammary gland of mammals and its expression is strongly induced during lactation (Jonker et al., 2005). Similarly to ABCB1, ABCG2 has wide substrate specificity. Several chemotherapeutic drugs (e.g. mitoxantrone, methotrexate, topotecan) are actively pumped out of cells expressing ABCG2 thereby rendering these cells resistant to these cytotoxic agents. This suggests that ABCG2 might be responsible for multidrug resistance unrelated to the expression of ABCB1 (Litman et al., 2001). Estrone-3-sulfate and other sulfate conjugates were also reported to be ABCG2 substrates and it was proposed that one of the physiological functions of ABCG2 is to clear sulfate conjugates that are formed in the cytoplasm during metabolism (Adachi et al., 2005). Interestingly, using knock-out mice it was shown that Abcg2 is responsible for protection against the toxic effects of pheophorbide A, a phototoxic degradation product of chlorophyll (Jonker et al., 2002).

1.4 *In vitro* ABC transporter assay systems in drug research and development

The discovery of the importance of drug-ABC transporter interactions in the ADME properties of drugs generated a new need in pharmaceutical industry: scientists involved in drug development realized, that by examining the interactions of the transporters with

pharmacological and toxic agents, a prediction for the cellular and tissue distribution of these compounds can be achieved. As the transporter interactions appear to be crucial in the later phases of drug development, scientists in pharmaceutical industry require low-cost, high-throughput methods that allow them to screen huge compound libraries (up to hundreds of thousands of molecules). This way they can select compounds with the most advantageous transporter interaction profile in the early phases of drug discovery (early ADME prediction) to avoid transporter-related problems in the later phases of drug development. Although knock-out mice, as well as mutant mice and rat strains, deficient in functional expression of some ABC transporter proteins, are available, only *in vitro* techniques meet the high-throughput requirements of early ADME studies.

1.4.1 Screening methods

The functional *in vitro* test systems mostly rely on the detection of the translocation of a substrate or the detection of the hydrolysis of ATP. The interaction of the test drug with the transporter can be determined indirectly, as it modulates the transport of a reporter substrate. Alternatively, the interaction can be detected by directly measuring the translocation of the test drug. The "direct" assay is more suitable for substrate screening, while assays utilizing a reporter substrate are more relevant in interaction studies, and in screens to identify inhibitors.

The target protein can be included in the assays in two major forms: in whole cells expressing the transporter, or in purified membrane vesicles. For whole cell studies a plethora of selected or transfected cell lines are available. Two major drawbacks of using selected cell lines are usually noted: (i) transporter expression pattern changes with time, and (ii) the cell line overexpresses other transporters with overlapping substrate specificities. Clearly, transfected cells, if available, are the experimental system of choice, as they harbor a well-defined modification and a more stable expression.

The function of the transporters can also be studied in membrane vesicles prepared from cells or tissues that overexpress the transporter. This is a widely used technique that can be used in different high-throughput assay formats. Utilization of inside-out vesicles carry major advantages, such as (i) the test compounds and ATP have direct access to the cytoplasmic domains of the transporter, and (ii) since the direction of the transport is

occurring from outside to inside, the transport can be assayed by measuring the amount of substrate trapped inside the vesicle compartment. Membrane preparations, purified from cells expressing the transporter, or reconstituted from purified protein and lipid components have been described.

Although membrane vesicles can be made from practically any kind of cells that express the transporter (e.g. selected cell lines, transfected cell lines, tissues), the most frequently used membrane preparations utilize baculovirus-infected Sf9 insect cells (Sarkadi et al., 1992). The advantage of this expression system is the particularly high transporter protein expression level (around 5-10 % of the total membrane protein is the expressed transporter). In addition, the heterologously expressed transporter is the only mammalian protein in this system. This makes the insect membranes a powerful tool in membrane-based assays. Membrane preparations from other expression systems are usually not suitable for ATPase measurements, due to low signal-to-noise ratio, as a consequence of a low transporter expression. Some ABC-transporter overexpressing vesicular membrane preparations are by now commercially available. Membrane preparations reconstituted from purified transporters and lipids offer excellent signal-to-noise ratio (Lu et al., 2001). However, preparation of these membranes is laborious, and these reagents are not available commercially.

1.4.2 Whole cell based assays

Efflux transporter-expressing cells actively pump substrates out or the cell, which results in a lower rate of substrate accumulation, lower intracellular concentration at steady state, or a faster rate of substrate elimination from cells loaded with the substrate. If the substrate is cytotoxic, cells overexpressing the transporter are less sensitive to the toxic effect than the parental ones, and inhibitors or competing non-cytotoxic substrates restore sensitivity. These types of screens are often used in search of substrates and reversing agents of transporters involved in multidrug resistance (Breuninger et al., 1995).

Radioactive substrates are also used to measure the transport across the cell membrane, as the accumulation or extrusion of the radioactive signal is different for the transporter overexpressing cell lines compared to parental ones (Reid et al., 2003). As

most test compounds are not available in a radioactively labeled form, the "indirect" set-up is more frequently used.

Transported substrates can also be labeled by fluorescent dyes. Furthermore, fluorescent molecules can be substrates of different transporters. As most test drugs are non-fluorescent, this assay is also usually performed in an "indirect" set-up, using a fluorescent reporter substrate, such as Rhodamine 123 (R123), which is a substrate of the ABCB1 transporter (Feller et al., 1995). When cells are incubated with R123, the dye accumulates at a slower rate in cells overexpressing ABCB1 than in the parental ones, or, alternatively, ABCB1-expressing cells loaded with R123 extrude the dye faster than parental ones. Inhibitors or competing substrates of the ABCB1 transporter modulate the rate of R123 accumulation/extrusion. The most commonly used application is the measurement of the efflux of R123. Multiple drawbacks of this assay have been reported: (i) the dye displays poor cellular retention, and it interacts with different intracellular compartments result in a spectral and intensity shift (143), (ii) the initial concentration is cell-type dependent and (iii) the concentration of R123 changes during the measurement. It has also been reported that after the initial loading, cells have to be incubated for 1-2 (Lee et al., 1994), or even 3-10 hours in clinical samples (Chaudhary and Roninson, 1991). This long time-frame and the extensive washing of the cells prior to the plate reader analysis, makes this assay inapplicable for high throughput screening.

Calcein AM has been used as a more suitable alternative for R123 transport assay (Homolya et al., 1993), (figure 1.B). Calcein AM is also a substrate for ABCB1, but it is nonfluorescent. Intracellularly calcein AM is hydrolyzed by endogenous esterases, yielding a fluorescent product, calcein. Therefore, accumulation of the fluorescent dye can be detected without the need for washing steps, making this assay suitable for a high throughput screen format. The presence of ABCB1 in the cell membrane strongly reduces calcein accumulation, and inhibitors of ABCB1 produce an increased rate of accumulation, up to the level measured in the absence of the transporter. calcein has favorable spectral properties, such as bright fluorescence, that is insensitive to pH or to Ca^{2+} as well as Mg^{2+} concentrations, and does not show spectral changes upon accumulation in intracellular compartments or upon binding to intracellular structures. Both calcein AM and free calcein are also transported by ABCC1, thus the calcein assay

is applicable for the quantitation of both ABCB1 and ABCC1 activities (Hollo et al., 1996). A suitable kit (MDQ kit) already exists for the specific diagnostic detection of ABCB1 and ABCC1 activities in clinical tumor samples (Karaszi et al., 2001).

Similar dye transport-based assays utilizing Hoechst 33342 or pheophorbide A have been worked out to measure the activity of ABCG2 (Kim et al., 2002), (Robey et al., 2004). Hoechst 33342 is a non-fluorescent substrate of both ABCB1 and ABCG2, and the dye becomes fluorescent after entering the cell and binding to DNA, making a high-throughput application of the assay feasible. Pheophorbide A is an ABCG2 specific substrate which becomes fluorescent after it is chemically modified inside the cells and can be used in a similar fashion as Hoechst 33342.

In vivo, drugs have to cross pharmacological barriers in order to get absorbed (intestinal epithelial cells), distributed (blood-brain barrier endothelial cells) or excreted (hepatocytes, proximal tubule epithelial cells). This transcellular movement is modeled by the monolayer efflux assays, alternatively termed as vectorial transport assays (figure 1.D). There are several cell lines that can be grown on a permeable support in a way that the cells form a confluent monolayer with tight junctions. The cell lines used in these assays are polarized epithelial or endothelial cells, with tight junctions. Most commonly the human intestinal epithelial line Caco-2, and transfected versions of the canine kidney cell lines MDCKI, MDCKII, or the porcine kidney epithelial cells LLC-PK1 are used. Test drugs can be applied to either side of the cell layer and the rate of transport across the monolayer is measured from the apical to basolateral (A-B) or from the basolateral to apical (B-A) direction. If an active transport process modulates the movement of the test drug across the cell membranes, the compound is transported at different rates in the two directions. Modulators, specific for efflux transporters, erase the difference in transport rate, confirming the drug – transporter interaction. Recently, a variation of the monolayer efflux assay, referred as equilibrium transport assay, has been described (Gaillard et al., 2000). In this set-up, the compounds are simultaneously placed at an equal concentration to both the apical and the basolateral side. The redistribution of the compounds is monitored over up to two days.

Once again, transfected cell lines seem to be more suitable for the studies as the data can be corrected for the contribution of endogenous transporters (Adachi et al., 2001),

and these cells provide more stable expression of the ABC transporters. It is important to note that at least some of the endogenous ABC transporter promoters are stress-sensitive (Sukhai and Piquette-Miller, 2000). Therefore, it is likely that cell lines that express ABC transporters from endogenous promoters will be more susceptible to mechanical and environmental stress (changes in temperature, acidity, oxygen and fuel supply) that may occur during cell culturing.

1.4.3 Membrane based assays

Membranes prepared form ABC transporter overexpressing cells, or reconstituted from purified protein and lipids can be used to assay the interaction of test drugs with ABC transporters in three basic assay formats: the ATPase assay, the vesicular transport assay and the nucleotide trapping assay.

The ATPase assay is one of the most widely used assays to search for compounds that interact with different ABC transporters (Sarkadi et al., 1992). The assay (figure 1.A) requires relatively high levels of transporter protein expression, thus mostly membrane vesicles prepared from recombinant baculovirus infected insect cells (e.g. Sf9 cells) are used for this purpose. These membrane preparations show a vanadate-sensitive ABC transporter ATPase activity that is modulated by interacting compounds. The rate of ATP hydrolysis is determined by measuring the liberation of inorganic phosphate. In the presence of transported substrates the ATPase activity of the transporter increases (activation protocol). Inhibitors that act in a non-competitive fashion or some slowly transported compounds inhibit the ATPase activity of the stimulated transporter (inhibition protocol). The main advantage of the ATPase assay is in its simplicity and reproducibility.

A more direct measurement of substrate translocation is possible by quantitation of the intravesicularly trapped substrates in the vesicular transport assay (figure 1.C). Successful vesicular transport studies have been reported using membranes from different sources (insect cells, transformed and selected cell lines, artificial membrane vesicle - see (Bodo et al., 2003), (Lu et al., 2001)). A suitable membrane preparation contains adequate amount of inside-out orientation vesicles (the rest is usually open fragments), and substrates are actively transported into the vesicles. Rapid filtration, using glass fiber

filters or nitrocellulose membranes, is used to separate the vesicles from the incubation solution, and the test compound, trapped inside the vesicles, is retained on the filter. The quantity of the transported unlabelled molecules can be determined by high resolution, high sensitivity analytical methods. Alternatively, the compounds are radiolabeled or a fluorescent tag is attached, and the radioactivity or fluorescence retained on the filter is quantified.

This assay can also be performed in an "indirect" set-up, where interacting test drugs modulate the transport rate of a labeled reporter compound. The "direct" assay clearly has an advantage, however, some "fast diffuser" compounds (e.g. verapamil) redistribute so fast that the translocation assays yield conflicting data (Litman et al., 2001). A high throughput format of the vesicular transport assay has recently been published (Tabas and Dantzig, 2002).

1.4.4 Screening strategies

It is generally accepted that none of the *in vitro* assay systems available is suitable for straightforward predictions for the *in vivo* situation. Some methods (e.g. ATPase or vesicular transport) provide simple, low-cost and high-throughput solutions, but do not model the actual physical setup of the pharmacological barriers of the human body. Other methods (e.g. monolayer assays) are expensive and time consuming, yet, their design is more relevant to the *in vivo* situation. Therefore, a successful attempt to identify and characterize the interaction of compounds with ABC transporters should include a combination of different *in vitro* assays performed in an optimized sequence. This sequence is termed 'screening strategy' in pharmaceutical industry. The optimal screening strategy depends on the overall aims of the study. For example, if we would like to identify transporter-interacting drugs in a large compounds library, indirect, fluorescence-based cellular assays and the ATPase assay can be used the screen the molecules. This gives a combination of direct and indirect assays; however, even these large-scale screening methods should be used in a complementary fashion, as false positive or false negative results could be obtained if only one of these methods is used. Hits can then be further characterized by more expensive, lower-throughput assays. Other

strategies for other situations can also be worked out using different combinations of *in vitro* transporter assays.

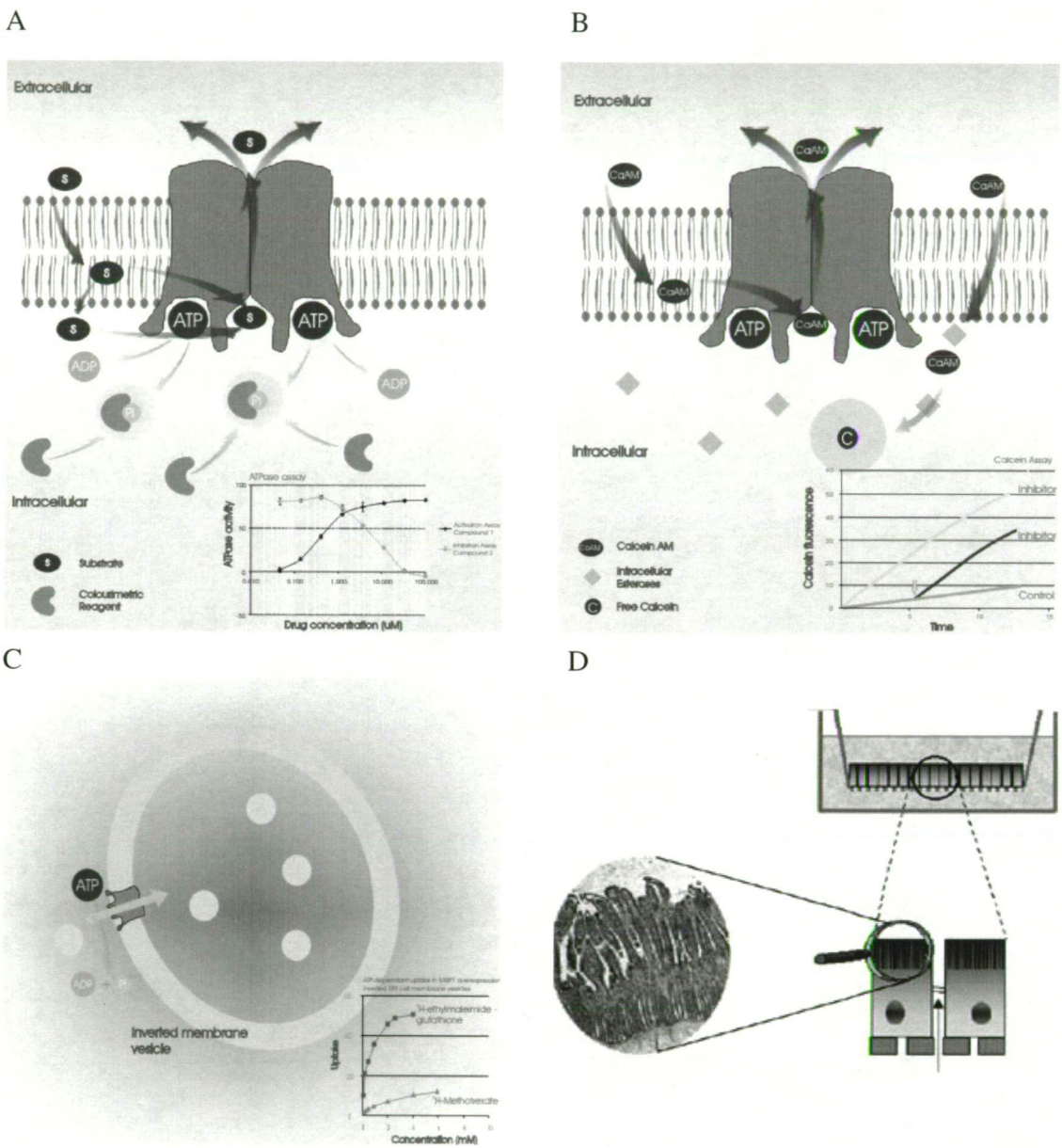


Figure 1. The principle and typical output of the ATPase assay (A), the Calcein assay (B), the direct vesicular transport assay (C). The physical setup of the monolayer efflux assay (D).

1.5 The current position and acceptance of *in vitro* transporter assays for ABCB1 and ABCG2 in the pharmaceutical industry

It is generally accepted that ABCB1 is an important determinant of the ADME properties of several pharmaceuticals. The numerous examples regarding mainly oral absorption and brain-to-plasma distribution of drugs made ABCB1 interaction screening a must in pharmaceutical industry. Presently, the "gold standard" method for this purpose – supported by current FDA regulations – is the Caco-2 monolayer assay. FDA suggests two types of assays: (1) A-B and B-A transport of the test compound, and (2) inhibition of the A-B/B-A transport of digoxin to detect ABCB1 mediated drug-drug interactions. Recent publications clearly pointed out, that this approach yields conflicting results (Polli et al., 2001), (Rautio et al., 2006). On one hand, several compounds that were shown to be substrates of ABCB1 by other methods (ATPase assay, cytotoxicity tests) do not appear to be transported substrates in the Caco-2 monolayer assay. On the other hand, some compounds that do not interact with ABCB1 in other assays show up as transported substrates in this assay. Caco-2 cells express a wide range of different transporters and it was suggested that reason for the discrepancies might be related to other active transport processes. Inhibitors are often used to overcome the problem of multiple transporters involved in the process. GF120918 is widely used in ABCB1 research as a specific inhibitor, although this inhibitor was also shown to inhibit ABCG2 (Volk et al., 2002). An important advance on the field was the discovery of a high affinity, specific inhibitor of ABCB1 (LY335979, zosuquidar, (Slate et al., 1995)). The widespread use of this compound as an inhibitor might help in dissolving some of the contradictions caused by multiple transporters expressed in Caco-2 cells. Other researchers suggested using transfected cell lines (e.g. MDCKII-MDR1) for the monolayer studies. Yet, it was also shown that the parental MDCKII cells express a functional canine transporter with very similar biochemical characteristics to human ABCB1 (Taub et al., 2005).

The Calcein assay and cytotoxicity tests are also widely used cell based assays. These methods are routinely used to screen for compounds that inhibit ABCB1. These compounds might cause ABCB1 mediated drug-drug interactions, but they can also be advantageous in overcoming ABCB1 mediated multidrug resistance (reversing agents).

Recent studies reported data suggesting that drugs that modulate the A-B/B-A transport of digoxin in Caco-2 cells also inhibit the calcein AM extrusion in the Calcein assay (Rautio et al., 2006) suggesting that the Calcein assay might be a suitable tool for the detection of compounds that cause ABCB1 mediated drug-drug interactions. This modification of the screening strategy would result in lower cost and higher throughput.

The ABCB1 ATPase assay is a fairly widely used screening tool in pharmaceutical industry (e.g. Fantin et al., 2006). It is generally accepted that compounds that are transported at high turnover rates are readily detected in this assay. Yet, the acceptance of this assay as a general screening tool is hindered by two observations. (1) It is well known that several compounds that were shown to be transported by ABCB1 do not stimulate the vanadate sensitive ATPase activity of membrane preparations containing ABCB1. It has been hypothesized that these compounds are transported by ABCB1 with a low turnover rate that do not yield detectable amount of inorganic phosphate in the ATPase assay. (2) Several compounds that are not transported in the Caco-2 assay stimulate the ABCB1 ATPase activity. It was proposed, that the passive permeability of the test compounds might be responsible for the different outcomes in the different assays (Rautio et al., 2006). Theoretically, monolayer studies measure the sum of passive permeability and active transport. In case of highly permeable substrates the rate of the passive process might be significantly higher than the active transport, thereby making the transporter interactions undetectable in the monolayer assay. No studies reported so far aimed at dissecting the active and passive processes to test this theory.

ABCB1 transports mainly hydrophobic substrates that easily cross the cell membrane; therefore, direct vesicular transport measurements for these substrates cannot be performed. There are some low permeability substrates (e.g. N-methyl-quinidine) for which successful vesicular transport experiments have been reported (Hooiveld et al., 2002), however, these assays are not used in pharmaceutical industry. It is perceivable, that successful vesicular transport systems utilizing drugs that are known to interact with ABCB1 *in vivo* (e.g. digoxin) would be more popular than the currently available vesicular transport assays.

In the recent couple of years the possible contribution of other transporters to the ADME properties of pharmaceuticals have been gaining wider acceptance. ABCG2 is

similar to ABCB1 in several respects: both proteins are expressed in several (and similar) pharmacological barriers, both transport a wide variety (and partially overlapping) substrates and both transporters are involved in the multidrug resistance phenomenon. Currently there are no FDA regulations regarding tests for the interaction of drugs with ABCG2, but the growing interest in the field indicates that ABCG2 might become an important transporter for pharmaceutical industry in the near future. Therefore, it is important to establish and validate high throughput functional assays that can be used to detect the interaction of test compounds with ABCG2.

It has been shown that Caco-2 cells express functional ABCG2 on their apical membrane (Xia et al., 2005). While this might be the reason for the conflicting data for ABCB1 studies, it makes Caco-2 cells suitable for ABCG2 studies. Also, MDCKII cells transfected with mouse Abcg2 and human ABCG2 have been established and described in detail (Breedveld et al., 2004), (Pavek et al., 2005). The development of these assays has been greatly facilitated by the availability of a high affinity, specific inhibitor of ABCG2: Ko143 (Allen et al., 2002). Using these cell lines and specific inhibitors might allow the establishment of valuable tools, yet, similarly to ABCB1 monolayer studies passive permeability of the test compounds could be a limiting factor when using these assays.

Fluorescent dye efflux assays (Hoechst 33342, pheophorbide A) are available to study the interaction of test compounds with ABCG2. These assays are mostly used to develop reversing agents against ABCG2 mediated multidrug resistance (Robey et al., 2004). This approach might also become interesting for pharmaceutical industry for identifying compounds causing ABCG2 mediated drug-drug interactions.

The first ABCG2 ATPase assays utilizing Sf9 membranes were reported on the R482G version of the transporter (Ozvegy et al., 2001). This membrane preparation had very high baseline vanadate sensitive ATPase activity, which could be stimulated by a number of known ABCG2 substrates. Later studies showed that a single amino acid change resulted in significant changes in the substrate specificity of this protein; highlighting the importance of amino acid 482 in substrate binding and/or transport activity of ABCG2 (Ozvegy et al., 2002), (Ozvegy-Laczka et al., 2005). Sf9 membranes containing the wild type (482R) version of the transporter also exhibit high baseline

vanadate sensitive ATPase activity, which cannot be further stimulated by known ABCG2 substrates (Ozvegy et al., 2002), (Ozvegy-Laczka et al., 2004). It was proposed that the different glycosylation pattern and/or the different membrane composition of the Sf9 cells could be responsible for this phenomenon (Ozvegy et al., 2001). Altogether, no ATPase assay suitable for the identification of transported substrates of wild type ABCG2 has been established so far.

The substrate specificity of ABCG2 partially overlaps with that of ABCB1 (Litman et al., 2000), yet, ABCG2 transports a number low permeability compounds like methotrexate and sulfate conjugates (Suzuki et al., 2003); (Imai et al., 2003). Vesicular transport assays utilizing ^{3}H-methotrexate and ^{3}H-estrone-3-sulfate have been established and are used in pharmaceutical industry to identify ABCG2 interacting compounds.

2 Aims of the work

1. For ABCB1 the major goal was to compare different membrane based and cell based *in vitro* assays to show that current "gold standard" methods used in pharmaceutical industry (Caco-2 and MDCKII-MDR1 monolayer studies) can be replaced by the ABCB1 ATPase assay. We were interested in the reason for the discrepancies that have been described. We hypothesized that passive permeability has a significant impact on the outcome of the different cell-based *in vitro* assay systems and might be an important input parameter of a successful screening strategy. Our overall aim was to propose a general, high-throughput, cost-effective screening strategy suitable for the detection of compounds that interact with ABCB1.
2. As no successful ABCG2 ATPase assay was reported suitable for the detection of transported substrates of the wild type (482R) ABCG2 transporter our primary goal was to set up such an ATPase assay. All reported ATPase assays were based on membranes prepared from the Sf9/baculovirus system, so we decided to change the expression system and prepared membranes from a selected human cell line overexpressing ABCG2. According to our hypothesis, the different glycosylation pattern and/or membrane composition might make these membrane preparations suitable for the detection of substrates of ABCG2 in the ATPase assay.
3. Several high affinity inhibitors (e.g. Ko143) of ABCG2 have been reported. We hypothesized that suppressing the baseline vanadate sensitive ATPase activity of the ABCG2 membrane preparations by inhibitors might result in better signal-to-background ratios and a more robust assay.

3 Materials and methods

3.1 Chemicals

Ritonavir and fluvastatin were obtained from Toronto Research Chemicals (North York, ON, Canada), topotecan was purchased from LKT Laboratories (St. Paul, MN, USA), doxorubicin was purchased from Alexis (Grünberg, Germany). Pantoprazole, pumafentrine, and tolafentrine were from ALTANA Pharma, Konstanz, Germany. GF120918 was synthesized at ALTANA Pharma, Konstanz, Germany. Ko143 and Ko134 was a kind gift of Dr. Gerrit-Jan Koomen, University of Amsterdam (Amsterdam, The Netherlands). [^{3}H]cimetidine and [^{3}H]methotrexate were acquired from Amersham Biosciences (Freiburg, Germany) or Moravek Biochemicals (Brea, CA, USA). calcein AM, [^{14}C]caffeine, [^{14}C]erythromycin and [^{3}H]estrone-3-sulfate were purchased from Perkin Elmer/NEN (Boston, MA, USA). [^{3}H]D-Mannitol was obtained from MP Biomedicals (Cambridge, UK), and [^{3}H]mitoxantrone from Biotrend (Cologne, Germany). BXP-21 antibody was purchased from Abcam (Cambridge, UK). All other chemicals were purchased from Sigma.

3.1.1 Training set

A set of 40 drugs was selected based on the compound's passive permeability (Wohnsland and Faller, 2001) and their interaction with ABCB1. This set contained the following compounds: acyclovir, alprenolol, amiloride, antipyrine, atenolol, ceftriaxon, chloramphenicol, chloroquine, cimetidine, cyclosporine A, desipramine, digoxin, doxorubicin, erythromycin, fluvastatin, furosemid, guanabenz, imipramine, lansoprazole, levadopa, methotrexate, metoprolol, midazolam, mitoxantrone, naproxen, omeprazole, pantoprazole, prazosin, propranolol, pumafentrine, quinidine, ranitidine, ritonavir, sulfasalazine, sulpirid, terbutaline, testosterone, tolafentrine, topotecan and verapamil.

3.1.2 Validation set

Out of 1296 small molecules originating from different chemical series for which ABCB1-ATPase and passive permeability using HDM-PAMPA had been determined,

two subsets of compounds were selected. Subsets 1 and 2 contained compounds with molecular weights greater than 300 Da (to exclude paracellular transport) and mass balance between 0.8 and 1.2. All compounds stimulated basal ABCB1-ATPase activity. The compounds were discriminated according to their passive permeability with high permeable compounds (HDM-PAMPA P_{app} >50 * 10^{-6} cm/sec) grouped in Subset 1 and low permeable compounds (HDM-PAMPA P_{app} <5 * 10^{-6} cm/sec) in Subset 2.

3.2 Passive permeability

3.2.1 HDM-PAMPA assay

Passive permeability was determined using hexadecane artificial membrane permeability assay (HDM-PAMPA) as described (Wohnsland and Faller, 2001). Assay plates were stirred on an orbital shaker at 100 rpm. Passive permeability was recorded after 5 or 96 h for compounds depending on high or low flux through the hexadecane layer. The sandwich was disassembled and the solutions in the acceptor and donor compartments were transferred to disposable UV-transparent plates (UV-Star®, Greiner-BioOne, Frickenhausen, Germany). UV absorption was measured with a SAFIRE microplate spectrophotometer (Tecan, Männedorf, Switzerland) scanning each compound from 240 to 450 nm, recording each compound at individual wavelengths yielding maximal UV absorption. Mass balance conditions were verified in order to assess retention of test compounds to the hexadecane layer or unspecific binding to assay plates.

3.2.2 Estimation of passive permeability from molecular structure

In case no experimental data was available for passive permeability (Ko143 and Ko134) we used the following equation (Guangli and Yiyu, 2006) to estimate P_{app}:

$\log P_{app}$ = -0.18HBD + 0.095CPSA10 + 0.0026CPSA20 – 0.0051 TPSA - 4.42

where *HDB* is the number of hydrogen-bond donors, *CPSA10, CPSA20* and *TPSA* are descriptors of the polar surface area of the molecule.

3.3 Membrane preparation

Transporter expressing Sf9 cells were obtained using recombinant baculoviruses. Recombinant baculoviruses encoding the wild type human ABCB1, ABCG2, inactive ABCG2-K86M mutant (carrying a mutation at a crucial position of the catalytic center of ATP binding and cleavage) or β-galactosidase were kindly provided by Prof. Sarkadi. The selected human cell line expressing wild type ABCG2 and the parental line were also kindly provided by Prof. Sarkadi. Human membrane vesicle preparations containing wild type ABCG2 (MXR-M) and control human membrane preparations (M-CTRL), as well as insect cell membranes containing the human ABCB1 (MDR1-Sf9) or ABCG2 (MXR-Sf9) transporter and control insect membranes (beta-gal-Sf9-CTRL, MXR-K86M-Sf9-CTRL) were prepared as described earlier (Sarkadi et al., 1992). Membrane protein contents were determined using a modified Lowry procedure (Bensadoun and Weinstein, 1976).

3.4 ABCG2 deglycosylation

Enzymatic deglycosylation was performed using peptide-N-glycosidase F (PGNase F, Sigma-Aldrich). 1 µl of the enzyme (500 U/ml) was added to 50 µl of membrane suspension (5 mg/ml), mixed and incubated at 37 °C for 10 min. Deglycosylation was detected by Western blotting.

3.5 Western blotting

ABCG2 expression and apparent molecular weight was detected by SDS-PAGE and subsequent western blotting using specific anti-ABCG2 antibody BXP-21, HRP-conjugated anti-mouse secondary antibody (Sigma) and enhanced chemiluminescence (ECL, Amersham Biosciences) as described earlier (Ozvegy et al., 2002).

3.6 ATPase assay

ATPase activity was measured as described earlier (Sarkadi et al., 1992). Briefly, membrane vesicles were incubated in 10 mM $MgCl_2$, 40 mM MOPS-Tris (pH 7.0), 50 mM KCl, 5 mM dithiothreitol, 0.1 mM EGTA, 4 mM sodium azide, 1 mM ouabain, 5 mM ATP, and various concentrations of test drugs with or without 1.2 mM sodium

orthovanadate at 37 °C. ATPase activities were determined as the difference of inorganic phosphate liberation measured in the presence or absence of 1.2 mM sodium orthovanadate (vanadate sensitive ATPase activity). Results are presented as vanadate sensitive ATPase activites, or as relative activities (%), where 100% is the baseline vanadate sensitive ATPase activity of the membrane suspension.

3.7 Vesicular transport assay

Vesicular transport studies were performed as described (Bodo et al., 2003). Briefly, membrane fraction containing inside-out membrane vesicles were incubated in the presence or absence of 4 mM ATP in a buffer containing 7.5 mM $MgCl_2$, 40 mM MOPS-Tris (pH 7.0), 70 mM KCl at 37 °C in the presence of the indicated substrate and other compounds for the indicated times. The transport was stopped by addition of 1 ml of cold wash buffer (40 mM MOPS-Tris (pH 7.0), 70 mM KCl) to the membrane suspensions and then rapidly filtered through class F glass fiber filters (pore size, 0.7 μm). Filters were washed with 2x5 ml of ice cold wash buffer. When labeled compounds were used as substrates radioactivity retained on the filter was measured by liquid scintillation counting. For unlabeled compounds quantification was performed by HPLC. ATP-dependent transport was calculated by subtracting the values obtained in the presence of AMP from those in the presence of ATP.

3.8 Calcein assay

K562-MDR cells, overexpressing ABCB1, were obtained from Dr. Sarkadi (National Medical Center, Institute of Hematology and Immunology, Budapest, Hungary). The cells were cultured as previously described (Homolya et al., 1996). After centrifugation at 500 g, culture medium was removed and cells were resuspended in HBSS, pH 7.4, at $1x10^6$ cells/ml. 100 μl of this suspension was added to each well of a 96well plate (Corning, Corning NY, USA). Test drugs and controls dissolved in DMSO were added at a volume of 2 μl and preincubated for 15 min at 37 °C. Calcein-AM dissolved in HBSS containing 50 μg/ml BSA was added to a final concentration of 250 nM to each well and fluorescence (excitation 485 nm, emission 538 nm) was recorded every 30 sec for 8 min. The slope of the fluorescence build-up for test drugs were plotted as *relative inhibition*

(%) based on fluorescence in the presence of 60 μM verapamil (positive control, 100%) and DMSO (blank control, 0%). The measurements were performed on a Fluoroskan Ascent microplate fluorometer (Thermo Electron Cooperation, Dreieich, Germany).

3.9 Hoechst assay

Selected human cells, overexpressing ABCG2, were obtained from Dr. Sarkadi (National Medical Center, Institute of Hematology and Immunology, Budapest, Hungary). The cells were cultured in DMEM:F12 1:1 mixture. After trypsinization and centrifugation at 500 g, culture medium was removed and cells were resuspended in HBSS, pH 7.4, at $1x10^6$ cells/ml. 100 μl of this suspension was added to each well of a 96well plate (Corning, Corning NY, USA). Test drugs and controls dissolved in DMSO were added at a volume of 2 μl and preincubated for 15 min at 37 °C. Hoechst 33342 dissolved in HBSS containing was added to a final concentration of 50 μM to each well and fluorescence (excitation 355 nm, emission 460 nm) was recorded every 30 sec for 15 min. The slope of the fluorescence build-up for test drugs were plotted as *relative inhibition (%)*, based on fluorescence detected in the presence of 1 μM Ko143 (positive control, 100%) and DMSO (blank control, 0%). The measurements were performed on a Fluoroskan Ascent microplate fluorometer (Thermo Electron Cooperation, Dreieich, Germany).

3.10 Caco-2 assay

Caco-2 cells (batch #2463691) were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA) at Passage 17. The cells were grown in culture medium consisting of DMEM (Biochrom, Berlin, Germany), supplemented with glutamine, 4.5 g/L glucose, heat-inactivated fetal calf serum (20% v/v), 100 U/ml penicillin, and 100 μg/ml streptomycin as described earlier (Yee, 1997).

For transport studies, cells from passage 55 to 70 were used by seeding them on semi-permeable filter inserts (24 wells inserts, 0.4 μm pore size, Greiner-BioOne, Frickenhausen, Germany) at $2x10^5$ cells per cm^2. The cells on the inserts were then cultured for 21 to 25 days prior to use. For transport experiments, the culture medium was removed from the filter inserts, which were then washed with HBSS and placed to a

new 24-well plate (Greiner BioOne, Frickenhausen, Germany) that had been pretreated by incubating 24 h with culture medium. The transport buffer was HBSS buffer containing 20 mM HEPES and 20 mM glucose monohydrate. The pH of both the apical and basolateral compartments was 7.4. The transport was started by addition of 350 µl of 10 µM or 100 µM compound dissolved in transport buffer to the apical compartment. Subsequently, a 50 µl sample was taken from the donor compartment at t = 0 min to determine the initial concentration of substrate (C_0) and stored at < -18 °C until further analysis. Then, 750 µl transport buffer was added to the 24-well plates, i.e. the basolateral compartment. The plates, containing the filter inserts, were then incubated in a humidified incubator at 37 °C for 120 min being stirred on an orbital shaker at 300 rpm. Samples from both compartments were collected and stored at < -18 °C. Markers for paracellular and transcellular flux, i.e. FITC-dextran and propranolol, were assayed in parallel in each set of experiments.

ABCB1 function, i.e. the effect of ABCB1 on the permeability of test compounds in the absorptive (apical to basolateral) direction, was assessed by calculating the ratio of apparent permeability coefficients in the presence and absence of the ABCB1 inhibitor GF120918, added to the apical and basolateral compartment at 1 µM. The ratio of digoxin permeabilities was determined for each batch of Caco-2 monolayers in the presence and absence of 1 µM GF120918 to ensure comparable ABCB1 expression. Only Caco-2 monolayers exhibiting digoxin permeability ratios > 2.5 were used for further experiments. Mass balance conditions were verified by additional sampling in the donor compartment in order to assess retention of test compounds to the cell monolayer or unspecific binding to assay plates. All experiments were carried out in triplicate.

3.11 Sample analysis

All compounds were determined using an Agilent HPLC System 1100 (Agilent, Waldbronn, Germany) equipped with a binary pump. Compounds in Set 1 were eluted using a Synergy Fusion 4.0 µm particle size, 75x4.6 mm reversed phase column (Phenomenex, Darmstadt, Germany) in combination with a RP18 security guard 10x3.0 mm precolumn as stationary phase. The mobile phases were: (A) 10 mM ammonium acetate buffer, pH 3.0 or 6.0 and (B) acetonitrile. Gradient runs were

programmed as follows: at a flow rate of 1.5 ml/min 5% B for 1 min, increase to 90% B in 6 min, 90% B for 1 min then re-equilibration with 5% B for 2 min, until the next sample was injected. Compounds in Set 2 were eluted using a Synergy Fusion, Mercury MS 2.0 µm particle size, 20x4.0 mm reversed phase column (Phenomenex, Darmstadt, Germany) as stationary phase. The mobile phases were: (A) 10 mM potassium dihydrogenphosphate, pH 3.0 or 10 mM ammonium acetate buffer pH 6.0 and (B) acetonitrile. Gradient runs were programmed as follows: at a flow rate of 2.0 ml/min 5% B, increase to 90% B in 3 min, 90% B, decreased to 5% B in 0.5 min followed by re-equilibration with 5% B for 1 min until the next sample was injected.

[^{14}C] and [^{3}H]-labeled compounds were determined by liquid scintillation counting on a Wallac Pharmacia S1414 scintillation counter (Perkin Elmer, Rodgau, Germany).

3.12 Data analysis

3.12.1 Apparent permeability (P_{app})

The apparent permeability coefficients (P_{app}) were calculated as follows:

$$P_{app} = -1 \frac{V_D \times V_R}{(V_D + V_R) \times A \times \varepsilon_a \times t} \times \ln\left(1 - \frac{C_R(t)}{C_{Eq}}\right)$$

where V_D is the volume of the donor compartment, V_R is the volume of the acceptor compartment, A is the accessible filter area, t is the incubation time, $C_R(t)$ is the concentration in the receiver compartment at time point t, and C_{Eq} is the concentration corresponding to a full equilibrium of the compound in the donor and acceptor compartment (Wohnsland and Faller, 2001). The P_{app} was corrected for the apparent porosity (ε_a) according to (Nielsen and Avdeef, 2004) using ε_a -HDM-PAMPA= 0.495 and ε_a – Caco-2= 1.0.

A retention factor (R_M) for all permeability experiments was calculated to assess mass balance conditions as follows:

$$R_M = 1 - \frac{C_D(t) + C_R(t)}{C_0}$$

where $C_D(t)$ and $C_R(t)$ are the concentrations in the donor and receiver compartment, respectively at time point t.

3.12.2 ABCB1-ATPase kinetics

V_{max} and K_M values for formation of inorganic phosphate from ABCB1-ATPase experiments were estimated by analysis of untransformed reaction velocity data via nonlinear regression. Compounds that stimulated ABCB1-ATPase activity without inhibition at higher concentrations were fitted to a modified form of the Michaelis-Menten equation:

$$V_S = V_0 \frac{(V_{max} - V_0)S}{(S + K_M)}$$

where V_S is the ATPase activity as a function of the substrate concentration S, V_0 is the basal activity (the activity in the absence of modulator).

Drugs that stimulated ATPase activity at low concentrations but inhibited activity at higher concentrations were fitted using the following equation.

$$V_S = \frac{K_1 K_2 V_0 + K_2 V_1 S + V_2 S^2}{K_1 K_2 + K_2 S + S^2}$$

where V_S is the ATPase activity as a function of the substrate concentration S, V_0 is the basal activity (the activity in the absence of modulator). V_1 is the maximal enzyme activity if only activation occurred and K_1 is the substrate concentration that gives the half maximal increment in this ATPase activity. V_2 is the activity at infinite concentration of the modulator, and K_2 is the substrate concentration that gives half-maximal reduction of ATPase activity from the value V_1. This equation is based on conventional enzyme kinetics algebra for a model that has an activity V_1 with one substrate molecule (S) bound, and a, lower, activity 1 of magnitude V_2 with two substrates (S) molecules bound (Litman et al., 1997). The data were analyzed by use of GraphPad Prism software (version 4.0 GraphPad, San Diego, USA).

3.12.3 ABCB1 inhibition

The inhibitory potency of compounds subjected to the Calcein assay was expressed as IC_{50} (concentration leading to half maximal inhibition). The IC_{50}–values were calculated using the four-parameter equation with a variable slope.

$$Inhibition = \frac{Bottom + (Top - Bottom)}{1 + 10^{((\log IC_{50} - S) \times h)}}$$

where *Bottom* is the minimal and *Top* is the maximal inhibition observed and *h* refers to the Hill constant.

3.12.4 Statistical analysis

Assays were run in duplicates or triplicates and unbiased ("n-1") standard deviation was calculated and plotted. Cutoff values in screening were defined as:

Cutoff = Baseline + 3Me,

where *Me* is the median of the unbiased ("n-1") standard deviations of the duplicate or triplicate measurements of the dataset.

4 Results

4.1 ABCB1 studies

In order to measure passive permeability and transporter interactions independently from each other we chose two different assays. We used HDM-PAMPA to determine passive permeability, while we used the ATPase assay utilizing inside-out membrane vesicles and open membrane fragments to measure transporter interactions. We then assayed the compounds with the Calcein assay and the Caco-2 monolayer efflux assay using GF120918 as inhibitor of ABCB1. In these two methods we expected that both passive permeability and transporter interactions can modulate the outcome of the assay. Table 1 shows the assay systems used, assay conditions and determined parameters.

Assay	Test compound concentration(s)	Determined parameters
HDM-PAMPA	100 µM	HDM-PAMPA P_{app}
ATPase assay	8 concentration points (3-fold dilution) starting at 100 or 300 µM	Stimulation (Y/N), K_M or K1 (ATPase K)
Calcein assay	8 concentration points (3-fold dilution) starting at 100 or 300 µM	IC_{50} (Calcein IC_{50})
Caco-2 (A-B) assay in the absence of GF120918	10 µM or 100 µM	P_{app} Caco-2 (A-B) -GF
Caco-2 (A-B) assay in the presence of 1 µM GF120918	10 µM or 100 µM	P_{app} Caco-2 (A-B) +GF

Table 1. Assays used, test compound concentrations and determined parameters.

We calculated the ratio of Calcein IC_{50} and ATPase K (Calcein IC_{50}/ATPase K) and the ratio of A-B permeability for Caco-2 cells in the presence and absence of GF120918 (Caco-2 P_{app} +GF/-GF). Using the determined and calculated values we tried to set up correlations to see if the other parameters are dependent on passive permeability (HDM-PAMPA P_{app}). Figure 2.A shows the correlation between HDM-PAMPA P_{app}, ATPase (Y/N) and Caco-2 P_{app} +GF/-GF for the training set. Compounds that did not stimulate the vanadate sensitive ATPase activity in the ATPase assay (open circles) did not show GF120918 sensitive A-B permeability in the Caco-2 assay (cutoff 1.5) regardless of HDM-PAMPA P_{app} values. Compounds that stimulated the vandate sensitive ATPase

activity in the ATPase assay (closed circles) with high permeability (HDM-PAMPA P_{app} > $50*10^{-6}$ cm/s) exhibited no GF120918 dependent A-B permeability in the Caco-2 assay, while compounds with low permeability (HDM-PAMPA P_{app} < $5*10^{-6}$ cm/s) exhibited GF120918 sensitive A-B permeability. Some compounds with medium permeability ($5*10^{-6}$ cm/s < HDM-PAMPA P_{app} < $50*10^{-6}$ cm/s) showed GF120918 dependent A-B permeability in the Caco-2 assay, while some did not. Figure 2.B shows the correlation between P_{app} PAMPA and Calcein IC_{50}/ATPase K for compounds of the training set that exhibited stimulation in the ATPase assay. The ratio for the kinetic constants fell in the 0.3 – 3.9 range with average ratio of 1.43 and showed no correlation with passive permeability.

We realized that the training set only contained four compounds that stimulated the vanadate sensitive ATPase activity in the ATPase assay, and showed low permeability (<$5*10^{-6}$ cm/s) in the HDM-PAMPA assay. Therefore, we screened 1296 molecules using the ATPase assay and the HDM-PAMPA assay. We chose 23 compounds that showed stimulation in the ATPase assay: 11 compounds with low permeability (HDM-PAMPA P_{app} < $5*10^{-6}$ cm/s) and 12 with very high permeability (HDM-PAMPA P_{app} > $50*10^{-6}$ cm/s) (validation set). We next measured and calculated the same parameters as we did for the training set. Figure 2.C shows the correlation between HDM-PAMPA P_{app} and Caco-2 P_{app} +GF/-GF for the validation set. High permeability compounds did not exhibit GF120918 sensitive A-B permeability in the Caco-2 assay, while most low permeability compounds did (10 out of 11, cutoff 1.5). Figure 2.D shows the correlation between P_{app} PAMPA and Calcein IC_{50}/ATPase K for the validation set. For the high permeability subset of compounds the ratio of the kinetic constants fell in the 0.27 – 2.6 range with average ratio of 1.47, while for the low permeability subset it fell in the 1.32 – 22.1 range with average ratio of 8.1.

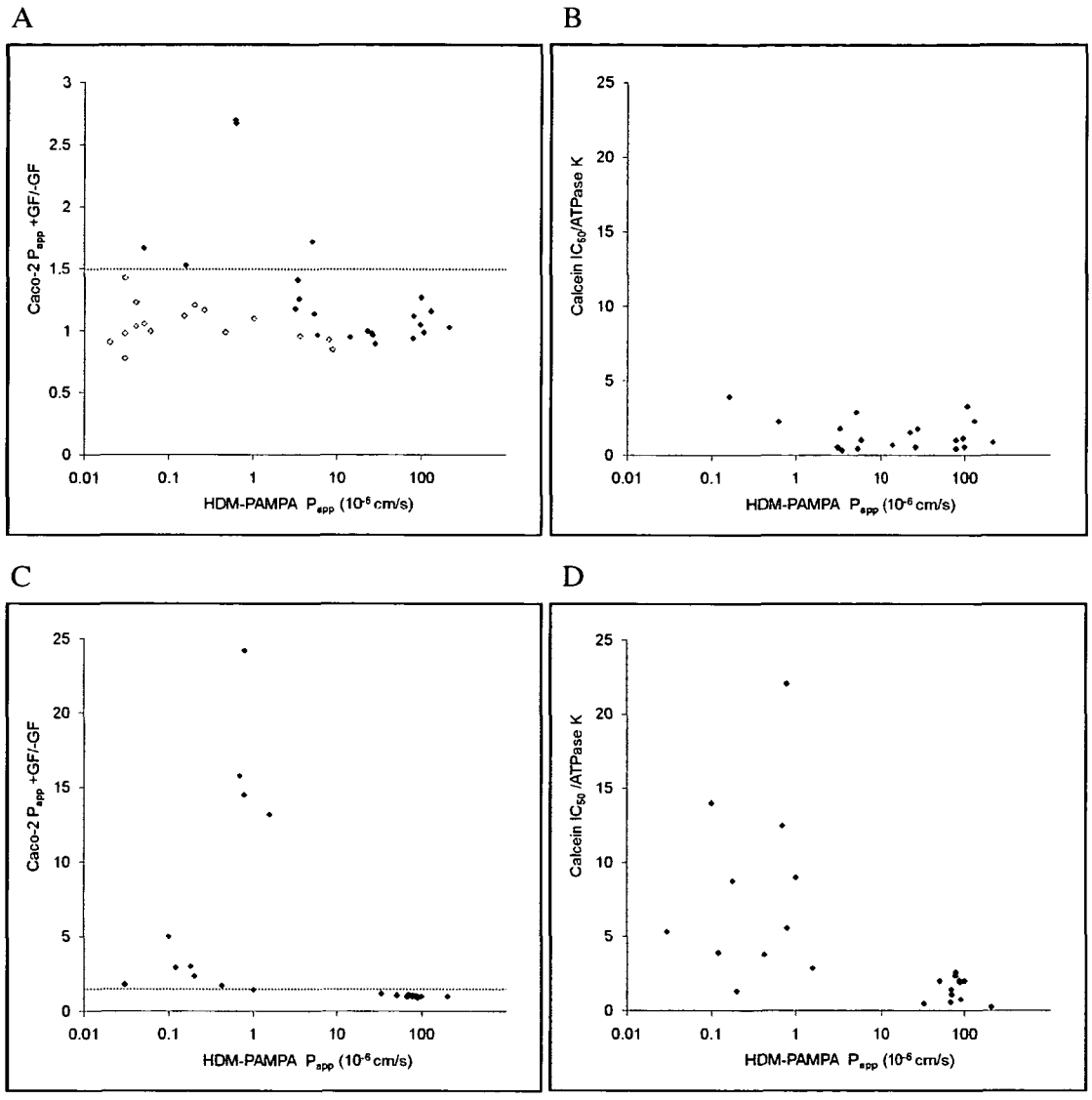


Figure 2. (A) Correlation between HDM-PAMPA P_{app}, ATPase (Y/N) and Caco-2 P_{app} +GF/-GF. Filled and open circles: compounds that stimulated and compounds that did not stimulate the vanadate sensitive ATPase activity in the ATPase assay, respectively. Cutoff (1.5) is also shown. (B) Correlation between HDM-PAMPA P_{app} and Calcein IC_{50}/ATPase K for compounds that stimulated the vanadate sensitive ATPase activity in the ATPase assay. (C) Correlation between HDM-PAMPA P_{app} and Caco-2 P_{app} +GF/-GF for the validation set. Cutoff (1.5) is also shown. (D) Correlation between HDM-PAMPA P_{app} and Calcein IC_{50}/ATPase K for the validation set. See text for further details.

4.2 ABCG2 studies

4.2.1 Comparison of ABCG2 activity in human (MXR-M) and in Sf9 (MXR-Sf9) membranes

To compare the ABCG2 content and glycosylation level of the membrane preparations we used western blotting using BXP-21, an ABCG2 specific antibody (figure 3.). Sf9 preparations containing the wild type or the defective (K86M) transporter (MXR-Sf9, lane 1 MXR-K86M-Sf9-CTRL, lane 2.) displayed a strong band with apparent molecular weight of 55-60 kDa. No band was detected in membrane preparations prepared from Sf9 cells infected with the baculovirus containing β-galactosidase gene (beta-gal-Sf9-CTRL, lane 3.). Membrane preparations from the selected human cell line exhibited a strong band with an apparent molecular weight of 70 kDa (MXR-M, lane 4.) that was absent in membrane preparations obtained from the parental line (M-CTRL, lane 5.).

To compare the MXR-M and the MXR-Sf9 membrane preparations we first determined the vanadate sensitive ATPase activity of the membrane preparations at different ATP concentrations. (figures 4.A-B). Both membrane preparations showed similar K_M-values for ATP (2.0 and 2.2 mM for MXR-Sf9 and MXR-M membranes, respectively). ATP dependent [^{3}H]-methotrexate vesicular transport measurements (figures 4.C-D) revealed that [^{3}H]-methotrexate transport had similar K_M-values (3.9 mM and 3.6 mM for MXR-Sf9 and MXR-M membranes, respectively). The transport of ^{3}H-methotrexate could not be observed in Sf9 membranes containing the K86M defective mutant version of ABCG2, nor in membranes prepared from the unselected, ABCG2 non-overexpressing human cell lines (data not shown).

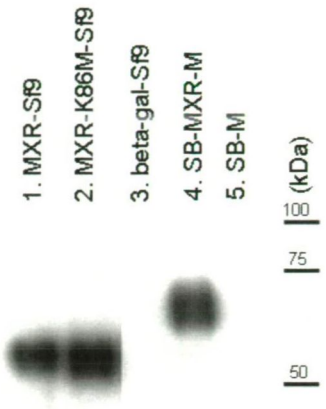


Figure 3. ABCG2 levels in the different membrane preparations as detected by the monoclonal antibody BXP-21 in western blot. 10 µg of total membrane protein/sample were separated by 10% SDS-PAGE and blotted on PVDF membranes. The presence of ABCG2 was detected by the monoclonal antibody BXP-21 and anti-mouse-HRP secondary antibody visualized with ECL detection.

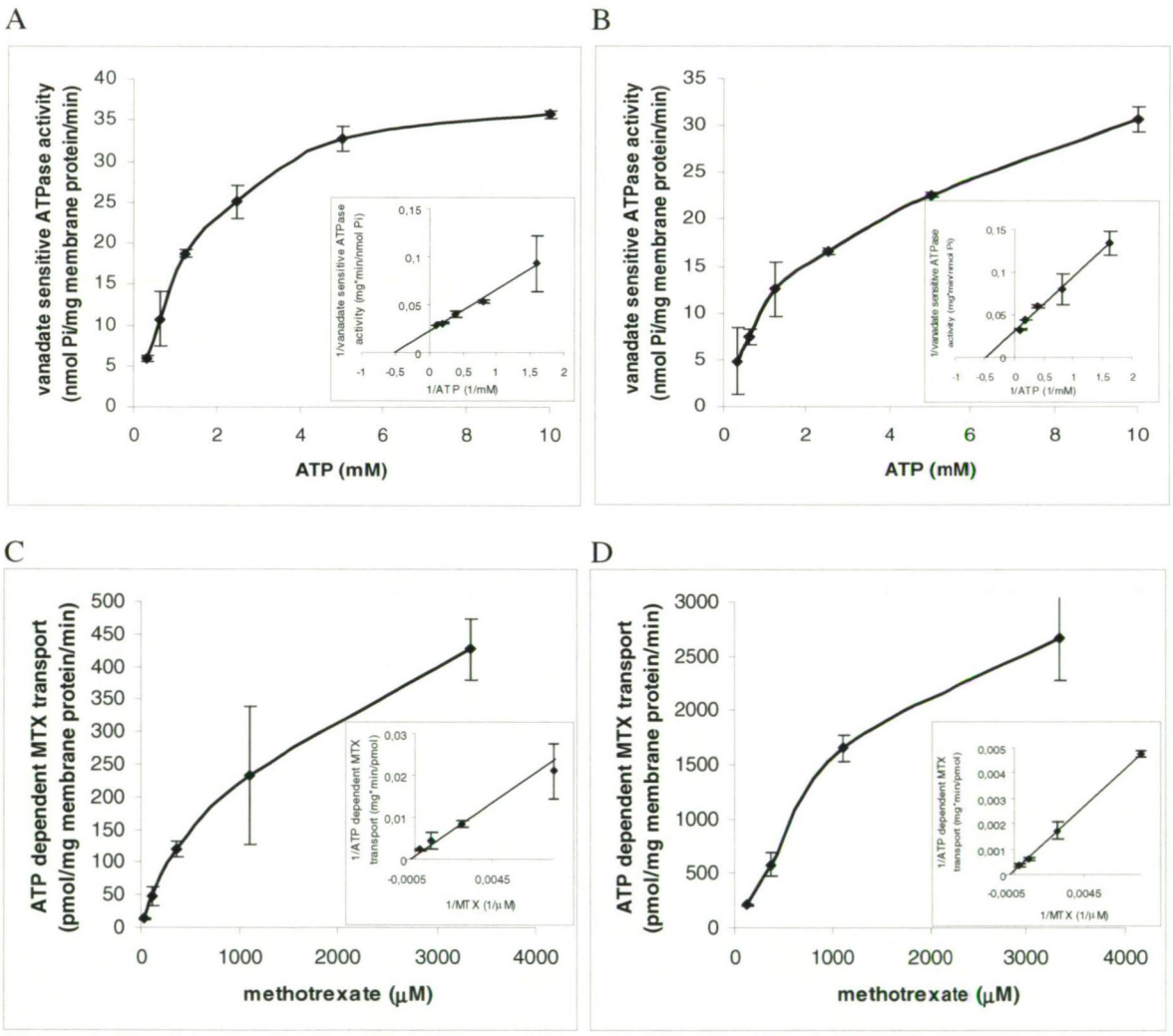


Figure 4. Vanadate sensitive baseline ATPase activity of MXR-Sf9 (A) and MXR-M (B) preparations in the presence of different ATP concentrations. Membranes containing 20 µg of total protein were incubated at 37 °C for 40 min. Insert: Lineweaver-Burk plot. ATP dependent [^{3}H]-methotrexate transport for MXR-Sf9 (C) and MXR-M (D) membrane vesicles at different methotrexate concentrations. Incubation time was the first linear phase of transport: 12 minutes and 4 minutes for MXR-Sf9 membrane vesicles and MXR-M vesicles, respectively. Insert: Lineweaver-Burk plot.

4.2.1 *ABCG2 ATPase activity in MXR-M and in MXR-Sf9 membranes – effect of substrates and inhibitors*

ABCG2 substrates sulfasalazine, topotecan and prazosin (Litman et al., 2000) (van der Heijden et al., 2004) showed only a slight modulation of the baseline vanadate sensitive ATPase activity of MXR-Sf9 membranes. Sulfasalazine appeared to be a weak stimulator, while topotecan and prazosin were weak inhibitors of the baseline activity (figure 5.A). The ABCG2 in human cell membranes could be stimulated by all three compounds (figure 5.B). All four inhibitors tested (Ko143, Ko134, Hoechst 33342 and GF120918) inhibited the baseline vanadate sensitive ATPase activity of the MXR-Sf9 preparation. Ko143, Ko134 and Hoechst 33342 inhibited the baseline vanadate sensitive ATPase activity of the MXR-M preparation. In contrast, GF120918 showed inhibition at lower concentrations, while it exhibited increasing ATPase activity above 1 μM (figures 5.C-D). The MXR-Sf9 membrane preparation shows ~10 nmol Pi/mg/min vanadate sensitive ATPase activity in the presence of 100 nM Ko143 or 200 nM Ko134. Sf9 membranes expressing the defective (MXR-K86M-Sf9-CTRL) version of ABCG2 show similar baseline vanadate sensitive ATPase activity, which was not modulated by any of the substrates tested (figure 5.E). The MXR-M membrane preparation shows no vanadate sensitive ATPase activity in the presence of 100 nM Ko143 or 200 nM Ko134. Control membranes prepared from the unselected, ABCG2 non-overexpressing human cell line also did not have any vanadate sensitive ATPase activity and could not be stimulated by any of the substrates tested (figure 5.F).

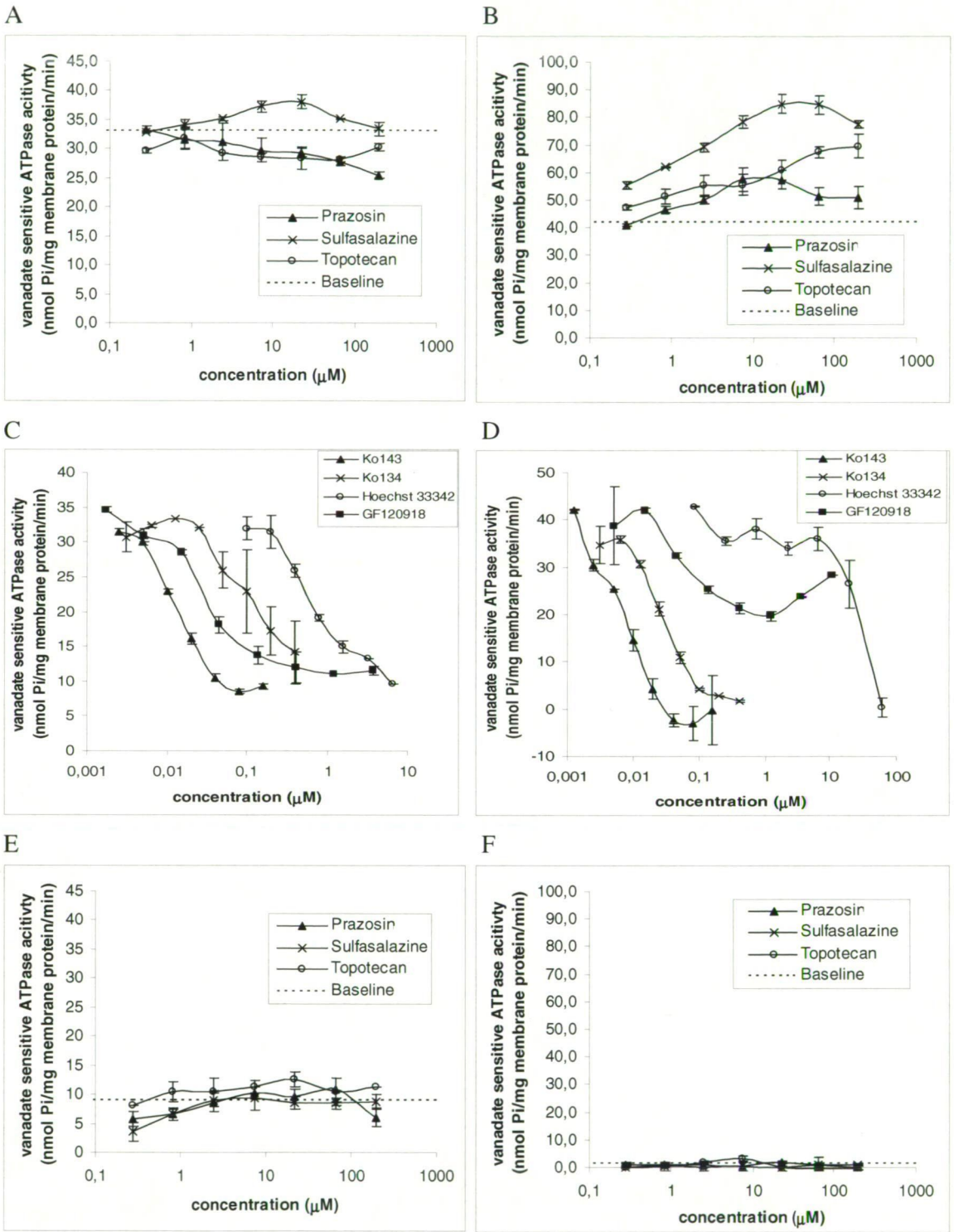


Figure 5. Vanadate sensitive ATPase activity of MXR-Sf9 (A, C) and MXR-M (B, D), defMXR-K86M-Sf9 (E) and M-CTRL (F) preparations in the presence of ABCG2 substrates and inhibitors at different concentrations. Membranes containing 20 μg of total protein were incubated at 37 °C for 40 min in the presence of different concentrations of test compounds.

4.2.2 *Deglycosylation of ABCG2*

We were interested if the different glycosylation of proteins in insect cells and mammalian cells is responsible for the difference observed in the ATPase assay. To test this hypothesis we enzymatically deglycosylated (PGNase F treatment) ABCG2 in the mammalian membrane. We found that the apparent molecular weight of ABCG2 in PGNase F treated mammalian membrane preparation was reduced to the apparent molecular weight of ABCG2 in the Sf9 membrane preparation as detected by western blot (Figure 6.A). We compared the vanadate sensitive ATPase activity of the treated and untreated human membrane preparations in the presence of different concentrations of sulfasalazine and found significant stimulation for both membranes (Figure 6.B).

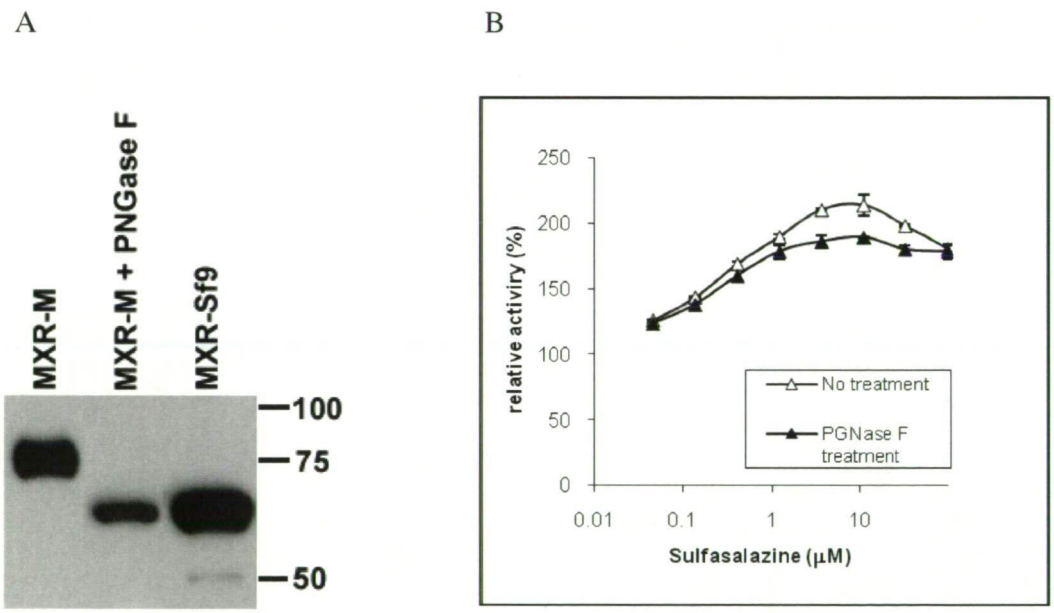


Figure 6. (A) PGNase treatment of MXR-M membrane preparation as detected by the monoclonal antibody BXP-21 in western blot. 10 µg of total membrane protein/sample were separated by 10% SDS-PAGE and blotted on PVDF membranes. The presence of ABCG2 was detected by the monoclonal antibody BXP-21 and anti-mouse-HRP secondary antibody visualized with ECL detection. (B) Vanadate sensitive ATPase activity in the presence of sulfasalazine for PGNase treated and untreated MXR-M membranes. Membranes containing 20 µg of total protein were incubated at 37 °C for 40 min in the presence of different concentrations of sulfasalazine.

4.2.3 Drug-stimulated ABCG2 ATPase activity in a low background system

We have studied whether by suppressing the basal ATPase activity employing an ABCG2 inhibitor we can acquire a more sensitive system that allows the detection of substrate stimulated ATPase. Using different concentrations of sulfasalazine as activator we tested all four inhibitors at different concentrations on both membrane preparations. The inhibition of ABCG2 ATPase activity by Ko143 (figures 7.A-B) and Ko134 (figures 7.C-D) could be reversed by using increased concentrations of sulfasalazine. The apparent EC_{50} for the activating effect of sulfasalazine shifted to higher concentrations (for MXR-M membranes 1.5 μM and 9.6 μM in the presence of 0 and 80 nM Ko143, respectively) suggesting that these interactions are competitive. Due to this competitive interaction as the inhibitors were added at higher concentrations the activating effect of sulfasalazine became evident for the MXR-Sf9 membranes. The inhibition by GF120918 was similar to Ko143 and Ko134, except that increasing concentrations of GF120918 caused a smaller shift in the apparent EC_{50} values than in the case of Ko143/Ko134 (figures 7.G-H). Inhibition by Hoechst 33342 could not be reversed even by the highest concentration of sulfasalazine used (figures 7.E-F). Therefore, inhibition by Ko143/Ko134 and GF120918 appeared to be competitive, while inhibition by Hoechst 33342 did not.

The assay using ABCG2 in membranes derived from human cells, with or without inhibition with Ko134 or Ko143 showed the best results, so we ran the experiment using different concentrations of Ko134 and the other two reference activators. Increasing concentrations of Ko134 inhibited the baseline vanadate sensitive ATPase activity as earlier, however, when looking at the activating effect of these two compounds we did not see any shift in apparent EC_{50} values suggesting that the interaction of these two compounds with Ko134 is not competitive (figure 8.)

We screened a library of 30 compounds at 100 μM using both membrane preparations with and without 100 nM Ko143 (table 2.). No interaction was detected with MXR-Sf9, yet using Ko143 allowed the detection of the known ABCG2 substrates sulfasalazine, topotecan and prazosin. The MXR-M preparation also detected the 3 known substrates in the absence of inhibitors, while applying Ko143 increased the signal-to-background (fold

activation) values up-to more than 4-fold. No false positives were seen for these 30 compounds in any assay setup used.

	Assay system (cutoff value)			
	MXR-Sf9 (1.22)	**MXR-Sf9 + Ko143 (1.17)**	**MXR-M (1.23)**	**MXR-M + Ko143 (1.97)**
Acyclovir	0.9	1.0	1.1	1.1
Alprenolol-HCl	0.9	1.0	1.1	0.6
Amilorid	1.0	0.9	1.1	1.1
Antipyrin	0.9	0.9	1.0	1.0
Atenolol	1.0	1.0	1.0	1.2
Ceftriaxon	0.9	1.0	0.9	1.1
Chloramphenicol	0.4	1.1	0.9	1.7
Desipramin	1.0	0.9	0.6	0.2
Digoxin	0.2	1.0	0.3	0.8
Doxorubicin	0.8	1.1	1.1	0.1
Erythromycin	0.9	1.1	0.9	0.7
Guanabenz	0.5	1.0	1.0	0.2
Imipramine	0.9	0.9	0.6	0.0
Lansoprazol	0.2	1.0	0.4	0.9
Levadopa	0.9	1.0	1.0	0.4
Metoprolol	1.0	1.0	1.0	0.6
Naproxen	0.5	1.1	1.0	0.6
Omeprazol	0.5	1.1	0.8	0.5
Prazosin	0.7	1.4	1.5	5.0
Propanolol	0.8	1.0	1.0	1.0
Pumafentrin	0.6	0.8	0.6	0.1
Quinidin	0.4	0.9	0.8	0.3
Ranitidine	1.0	1.0	1.2	0.8
Ritonavir	0.1	0.9	0.0	0.1
Sulfasalazine	1.0	2.4	2.0	7.2
Sulpirid	0.9	1.0	1.0	0.6
Terbutalin	0.5	1.0	0.9	0.7
Testosteron	0.7	1.1	0.4	0.0
Topotecan	0.8	1.3	2.1	2.7
Verapamil	0.4	1.0	0.7	0.2

Table 2. Fold activation (signal-to-background) values measured for the different assays in the presence of the compounds listed (100 µM). Values greater than the respective cutoff values are shaded.

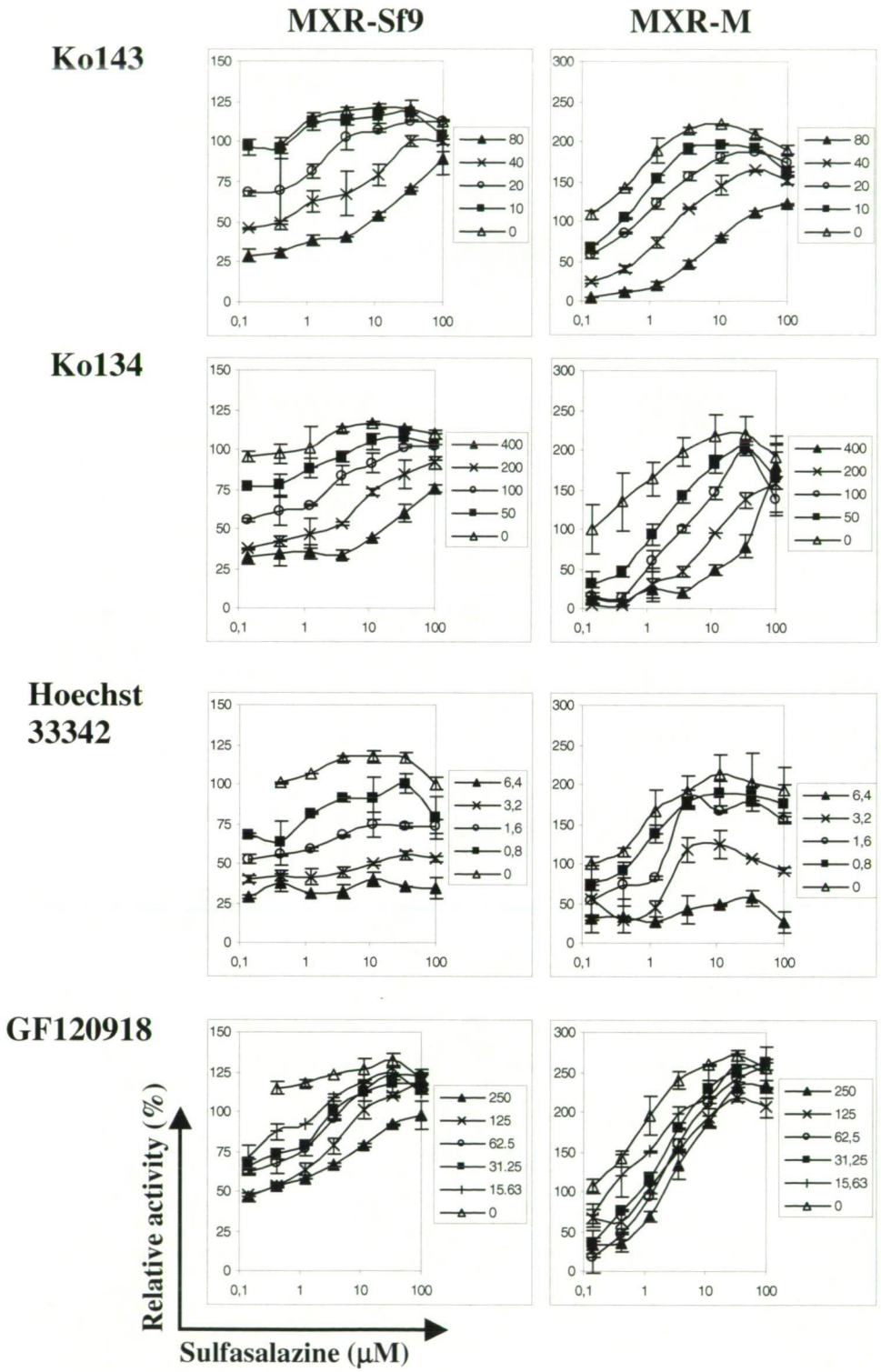


Figure 7. Vanadate sensitive ATPase activity plotted as relative activity (%) of MXR-Sf9 and MXR-M preparations in the presence of different concentrations of sulfasalazine (x-axis) and inhibitors (legend, A-B Ko143 (nM), C-D Ko134 (nM), E-F Hoechst 33342 (μM), G-H GF120918 (μM)).

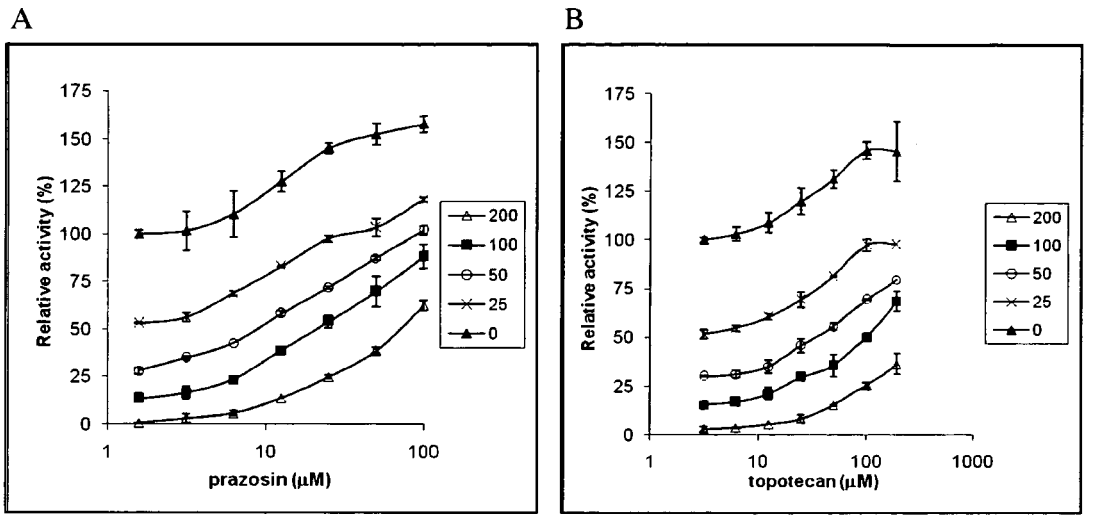
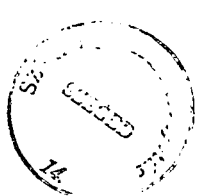


Figure 8. Vanadate sensitive ATPase activity plotted as relative activity (%) of the MXR-M membrane preparation in the presence of different concentrations of prazosin (A) or topotecan (B) and Ko134 (legend, nM). Membranes containing 20 μg of total protein were incubated at 37 °C for 40 min.

4.2.4 Estrone-3-sulfate ATPase and vesicular transport

To compare the kinetic parameters of ATPase and vesicular transport we measured the vanadate sensitive ATPase activity and the vesicular transport of the ABCG2 substrate estrone-3-sulfate at different estrone-3-sulfate and Ko134 concentrations. In the ATPase assay estrone-3-sulfate stimulated the baseline ATPase activity with a K_M value of 22 μM. It also showed a competitive interaction with Ko134 similar to that of sulfasalazine. In the vesicular transport assay estrone-3-sulfate was transported with a K_M of 7.8 μM and Ko134 inhibition was also competitive (figure 9.).



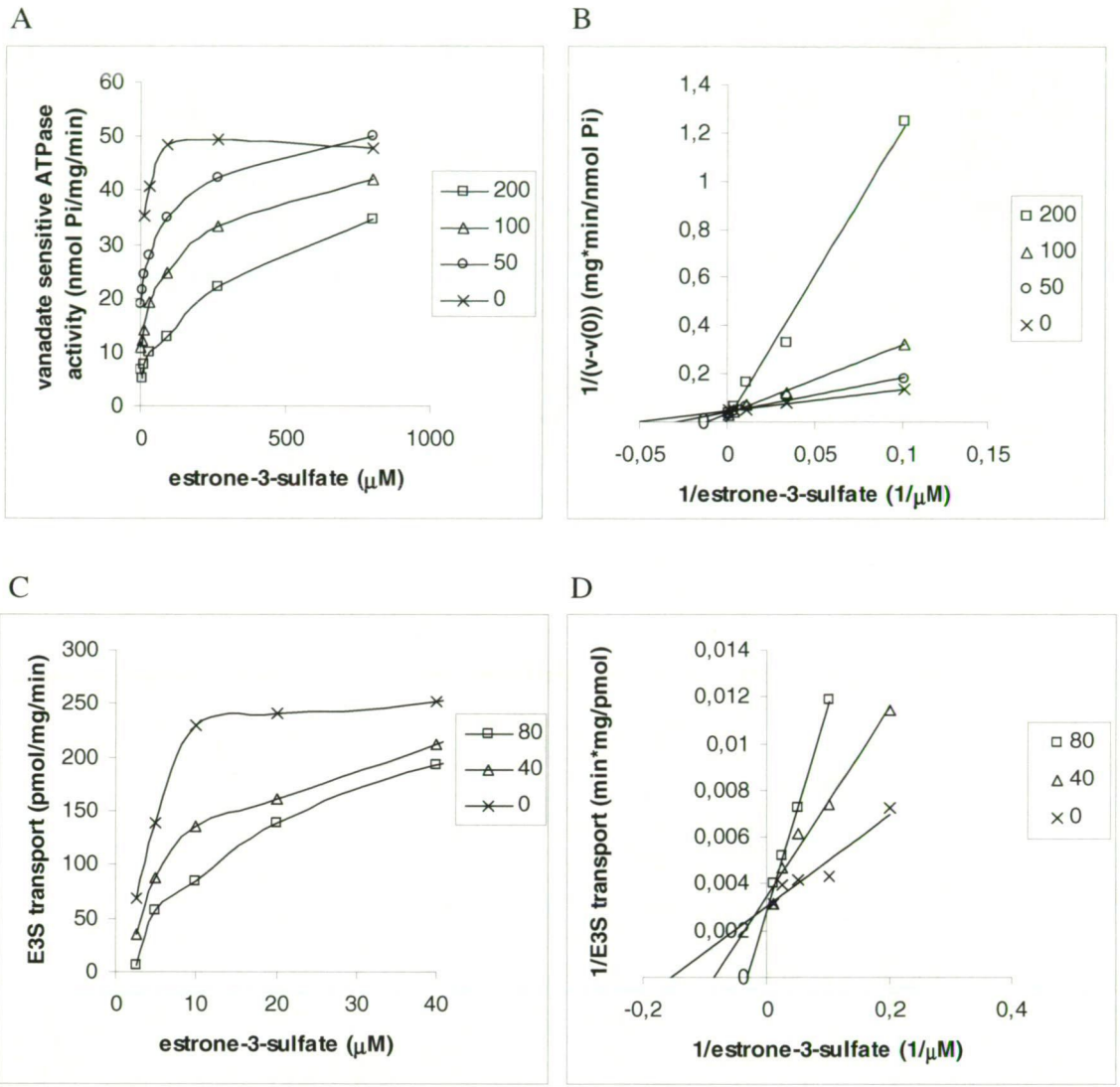


Figure 9. (A) Vanadate sensitive ATPase activity of MXR-M preparations in the presence of different concentrations of estrone-3-sulfate and Ko134 (nM, legend). Membranes containing 20 μg of total protein were incubated at 37 °C for 40 min. (B) Lineweaver-Burk plot of the estrone-3-sulfate stimulated vanadate sensitive ATPase activity (v-v(0)) of MXR-M preparations in the presence of different concentrations of Ko134 (nM, legend). v-v(0) was calculated by subtracting the vanadate sensitive ATPase activity in the presence of the respective Ko134 concentration from the vanadate sensitive ATPase activity determined in the presence of both estrone-3-sulfate Ko134. (C) ATP dependent [^{3}H]-estrone-3-sulfate transport for MXR-M membrane vesicles at different estrone-3-sulfate concentrations in the presence of different concentrations of Ko134 (nM, legend). Incubation time was 1 minute. (D) Lineweaver-Burk plot of figure C.

4.2.5 Comparison of membrane-based ABCG2 assays and the Hoechst assay

During our validation work we compared the results obtained using the different membrane based assays with a whole cell based ABCG2 assay (Hoechst assay). Some compounds that modulated the vanadate sensitive ATPase activity in the ATPase assay and/or were detected as transported substrates in the vesicular transport assay also inhibited Hoechst 33342 extrusion (prazosin, Ko143, Ko134), while others (topotecan, sulfasalazine, methotrexate, estron-3-sulfate) did not. We measured (HDM-PAMPA) or estimated the P_{app} value for these compounds and found that compounds that inhibited Hoechst 33342 extrusion were shown/estimated to have significantly higher apparent permeability than compounds that did not modulate Hoechst 33342 extrusion (table 3.).

Compound	Inhibition of Hoechst 33342 efflux (% of Ko143)	P_{app} (10^{-6} cm/s)
Prazosin	104 +/- 11.5	3.13
Ko143	100 +/- 3.7	60.25[#]
Ko134	98 +/- 4.8	75.85[#]
Topotecan	1.1 +/- 5.8	0.61
Methotrexate	4.5 +/- 3.9	0.02
Sulfasalazine	-4.3 +/- 7.3	0.03
Estrone-3-sulfate	1.7 +/- 6.3	0[*]

Table 3. Inhibition of Hoechst 33342 efflux measured in the Hoechst assay and passive permeability measured in the HDM-PAMPA assay or estimated from molecular structure ([#]) for compounds that showed interaction with ABCG2 in membrane based assays. [*]: permeability was below the level of detection in the HDM-PAMPA assay.

5 Discussion

5.1 ABCB1 studies

Using two independent sets of compounds with high diversity in molecular structure we determined the correlation between results of HDM-PAMPA permeability, the ATPase assay, the Calcein assay and the Caco-2 monolayer efflux assay (figure 2.). We found that highly permeable compounds that stimulate the vanadate sensitive ATPase activity in the ATPase assay do not show ABCB1 dependent transport in the monolayer efflux assay. Results of the training set are in accordance with data previously reported (Rautio et al., 2006), (Polli et al., 2001). This finding strongly suggests that for highly permeable molecules the rate of passive transport across the cell monolayer is significantly higher than the rate of active transport making the transporter interactions undetectable in the monolayer assay. These "false negatives" generated by the monolayer efflux assay can become crucial in drug development, as these compounds do interact with the transporter and might cause ABCB1 mediated drug-drug interactions in the later phases of drug development *in vivo*. This possibility is clearly demonstrated by examining the correlation between the ATPase assay and the Calcein assay (an *in vitro* DDI model). For highly permeable molecules the two assays correlate perfectly with no striking differences (less than 3-fold) in the apparent kinetic constants.

Despite of the limited number of low permeability compounds included in the study we can still conclude that the correlation of the different assays show a strikingly different pattern when compared with highly permeable ones. Most low permeability compounds exhibited GF120918 sensitive transport in the Caco-2 monolayer efflux assay suggesting that the rate of active transport is an important determinant of the movement of these molecules across cell membranes. Also, most of the low permeability ABCB1 interacting compounds exhibited significantly higher IC_{50} in the Calcein assay (up to 22-fold) than apparent affinity in the ATPase assay suggesting the intracellular concentration of these compounds can be significantly different from concentrations applied to the test buffer. However, there were some compounds where no ABCB1 mediated efflux or significant shift in the apparent binding constant in the Calcein assay was detected. One

possible explanation is that there are other active transport processes (uptake of efflux) that modulate the outcome of the cellular assays. Further studies on low permeability compounds are necessary to gain further insight into processes that are responsible for these differences.

Passive permeability (10^{-6} cm/s)
(HDM-PAMPA)

ATPase	LOW $P_{app} < 1$	MEDIUM $1 < P_{app} < 50$	HIGH $P_{app} > 50$
No interaction	No need for further ABCB1 testing	No need for further ABCB1 testing	No need for further ABCB1 testing
Interaction (activation or inhibition)	Possible ABCB1 mediated modulation of permeability – to confirm run Caco2 or MDCKII-MDR1 **Very rare!**	Possible ABCB1 mediated modulation of permeability – to confirm run Caco2 or MDCKII-MDR1 Possible P-gP mediated DDI – to confirm run the Calcein assay	Possible ABCB1 mediated DDI – to confirm run the Calcein assay **Most frequent!**

Figure 11. The proposed screening strategy to identify ABCB1 interacting compounds and to predict the likely *in vivo* consequence of the interaction based on the ATPase assay and HDM-PAMPA assay.

Based on these findings we set up a general screening strategy to describe the interaction of test compounds with ABCB1 (figure 11.). We suggest using the ATPase assay to identify compounds that stimulate ABCB1 ATPase activity. We also suggest running the assay in the presence of a known substrate of ABCB1 (e.g. verapamil). This assay system identifies inhibitors and slowly transported compounds as inhibitors of the verapamil stimulated vanadate sensitive ATPase activity (von Richter et al., manuscript under revision). For compounds that showed interaction in the ATPase assay (activation or inhibition) we suggest determining their passive permeability using the HDM-PAMPA assay. In case of low permeability compounds it is important to determine whether the compound exhibits ABCB1 dependent transport in the monolayer assay (Caco-2 or MDCKII-MDR1). This would indicate if the interaction with ABCB1 would likely result in limited absorption and/or limited BBB penetration *in vivo*. For high permeability compounds our recommendation is to run the Calcein assay. This assay would indicate if the compound is likely to cause ABCB1 mediated drug-drug interactions. It is important to note that the ATPase assay, the HDM-PAMPA assay and the Calcein assay are low

cost, high-throughput methods, while monolayer assays are expensive and time-consuming studies. Also, most ABCB1 interacting compounds show high passive permeability (e.g. we had to screen 1296 compounds to find 11 ABCB1 interacting compounds with low passive permeability). Therefore, this strategy is also the most cost-effective approach to screen for ABCB1 interactions and to predict the likely *in vivo* consequence of such interactions.

5.2 ABCG2 studies

We performed a comparative analysis of wild type ABCG2 in membrane preparations derived from baculovirus transfected Sf9 cells and selected human cells (MXR-Sf9 and MXR-M, respectively). Both transporter preparations had similar affinity for ATP and for [^{3}H]-methotrexate. The MXR-M preparations showed significantly higher v_{max} for [^{3}H]-methotrexate transport (figure 4.). One of the possible reasons for this difference is the difference in vesicular content of the two membrane preparations. Substrates and inhibitors of ABCG2 inhibited the ATP dependent [^{3}H]methotrexate transport in both membrane preparations, while non-interacting drugs did not modulate it (Glavinas et al., 2007). In contrast, in the ATPase assays the same set of compounds showed a strikingly different pattern. In case of MXR-Sf9 preparations the baseline vanadate sensitive ATPase activity could not be further stimulated by ABCG2 substrates, while stimulation was detected for all substrates tested for MXR-M preparations (figure 5.). The substrate specificity of the transporter in the MXR-M ATPase assay correlated well with the substrate specificity in the vesicular transport inhibition experiment, and is in good agreement with published data (Litman et al., 2000) (van der Heijden et al., 2004). We showed that it is unlikely that the different glycosylation pattern of insect and mammalian preparations is responsible for the different behavior of the Sf9 and mammalian membrane preparations (figure 6.). In a later study we also showed that the reason for this difference is the different membrane cholesterol content of the insect and mammalian membrane preparations (Pal et al., 2007).

When the basal ABCG2-dependent ATPase activity was suppressed by Ko143/Ko134, all known substrates of ABCG2 stimulated the ATPase activity in both types of ABCG2 preparations (MXR-Sf9 and MXR-M). This shows that the reason for

the unresponsiveness of the MXR-Sf9 preparation without inhibitors is not caused by altered substrate specificity of the transporter, but is due to the high baseline ATPase activity that cannot be further stimulated by ABCG2 substrates. By using ABCG2 inhibitors the MXR-Sf9 membrane preparation was suitable for substrate identification, and it increased the signal-to-background ratio of the MXR-M preparation (table 2.). Due to the competitive nature of the interaction for sulfasalazine and Ko143/Ko134 using higher inhibitor concentrations resulted in an increased apparent EC_{50} (figure 7.). Therefore, the concentration of the inhibitor has to be set as a compromise between reasonable shift in EC_{50} and adequately low baseline activities. For Ko143 this falls in the 50-100 nM range, while due to its somewhat lower affinity for ABCG2 it is in the 100-200 nM range for Ko134. For prazosin and topotecan increasing Ko143/Ko134 concentrations did not result in an increased apparent EC_{50} suggesting that these interactions are not competitive (figure 8.). This suggests that Ko143/Ko134 binds to the same binding site as the hypothesized endogenous substrate, sulfasalazine and estrone-3 sulfate, while prazosin and topotecan binds to a different one. Multiple substrate/inhibitor binding sites for ABCG2 have been suggested previously (Ozvegy-Laczka et al., 2005). Our results also indicate the presence of more than one substrate binding site for the ABCG2 transporter.

Estrone-3-sulfate was one of the best stimulator of the ATPase assay indicating high transport rate for this compound. The transport could also be detected in the vesicular transport assay, which allowed us to compare the biochemical characteristics of ATPase stimulation and vesicular transport. The K_M value for ATPase stimulation (22 μM) and K_M for vesicular transport (7.8 μM) were comparable. Stimulation and transport could be inhibited by Ko134 in a similar, competitive fashion (figure 9.). These results show that for estrone-3-sulfate kinetic parameters determined in the ATPase assay are reflective of the kinetic parameters of the actual transport. Further studies are necessary to see if the correlation of kinetic parameters holds true for different ABCG2 substrates.

We used the Hoechst assay to test compounds that showed interaction with ABCG2 in membrane-based assays and we found very poor correlation. This situation is similar to correlation studies conducted on membrane-based and cell-based ABCB1 assays. Therefore, we checked if the passive permeability of the test compounds can be

responsible for the poor correlation (table 3.). Our limited data (one cell-based assay, limited number of compounds, single concentration point) suggest that similarly to ABCB1 passive permeability of the compound tested is an important determinant in the outcome of cell-based ABCG2 assays. Fortunately, low permeability substrates of ABCG2 are not as uncommon as low permeability substrates of ABCB1. Therefore, it looks easier to design studies to set up correlations between membrane-based and cell-based ABCG2 assays with regard to passive permeability than it was for ABCB1.

6 Summary

We designed and preformed a detailed study on the effect of passive permeability on the outcome of different *in vitro* assays used to detect compounds that interact with ABCB1. We found that passive permeability is a crucial parameter in choosing the right *in vitro* tool to detect ABCB1 interacting compounds. We set up a screening strategy based on the passive permeability of test compounds as detected by HDM-PAMPA which looks to be a cost-effective approach to screen for ABCB1 interactions. The screening strategy was also designed to avoid "false negatives" that might lead to significant consequences in the later phases of drug development.

We successfully established an ABCG2 ATPase assay to identify substrates of the transporter. We showed that the human membrane preparation containing ABCG2 can distinguish between substrates and non-substrates. Suppressing the baseline vanadate sensitive ATPase activity by ABCG2 inhibitors Ko143 (50-100 nM) or Ko134 (100-200 nM) allowed us to increase the signal-to-background ratio of the assay. In essence, using the human membrane preparation containing ABCG2 with Ko134 or Ko143 is a reliable, sensitive and robust assay that could be the preferred choice in screening for substrates of ABCG2. Hits of this high-throughput, inexpensive method can be further characterized in lower throughput, and significantly more expensive direct transport experiments using vesicular transport or monolayer studies. This way, the improved ATPase assay can be a valuable tool in drug development as part of a screening strategy.

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