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

Summary of Ph.D. thesis

Hristos Glavinas

Department of Medical Microbiology and Immunology
Faculty of Medicine
Albert Szent-Györgyi Medical and Pharmaceutical Center
University of Szeged
Szeged Hungary
2007

1 Introduction

1.1 Background

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. 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 gene coding for P-gp was cloned and based on the structure of the gene and sequence homologies it was proposed that it was an ABC transporter. These first observations showed that an ABC transporter is capable of transporting pharmaceuticals (chemotherapeutic drugs) thereby protecting cells from their toxic effect.

In 1994 it was accidentally discovered, that the product of the mouse Mdr1a gene 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 ivermectin 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. 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. Two transporters stand out as pivotal in this respect: ABCB1 (MDR1, Pgp) and ABCG2 (BCRP, MXR, ABCP). 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.

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

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.

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. 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 reconstituted from purified transporters and lipids offer excellent signal-to-noise ratio. However, preparation of these membranes is laborious, and these reagents are not available commercially.

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

Presently, the "gold standard" method for ABCB1 interaction studies – 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.

The ABCB1 ATPase assay is a fairly widely used screening tool in pharmaceutical industry. 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. 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 have been reported so far that aimed at dissecting the active and passive processes to test this theory.

In the recent couple of years the possible contribution of other transporters to the ADME properties of pharmaceuticals is 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 dugs 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 (e.g. ATPase assay) that can be used to detect the interaction of test compounds with ABCG2.

The first ABCG2 ATPase assays utilizing Sf9 membranes were reported on the R482G version of the transporter. 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. 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. It was proposed that the different glycosylation pattern and/or the different membrane composition of the Sf9 cells could be responsible for this phenomenon. Altogether, no ATPase assay suitable for the identification of transported substrates of wild type ABCG2 has been established so far.

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

All chemicals were purchased from commercial sources except from compounds of the validation set, which were molecules of the proprietary compound library of Altana Pharma, Konstanz, Germany.

3.2 Passive permeability

Passive permeability was determined using hexadecane artificial membrane permeability assay (HDM-PAMPA) or it was estimated by descriptors based on molecular structure.

3.3 Membrane preparation

Crude membrane preparations were prepared from cells (baculovirus infected Sf9 cells or selected human cell line) and membrane protein contents were determined using a modified Lowry procedure.

3.4 ABCG2 deglycosylation

Enzymatic deglycosylation was done using peptide-N-glycosidase F (PGNase F). Deglycosylation was followed by Western blotting.

3.5 Western blotting

ABCG2 expression and apparent molecular weight was detected by SDS-PAGE and subsequent western blotting using the specific anti-ABCG2 antibody BXP-21.

3.6 ATPase assay

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 with various concentrations of test drugs 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).

3.7 Vesicular transport assay

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 labeled reporter substrates and other compounds. 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.

3.8 Calcein assay

K562-MDR cells were collected and resuspended in HBSS at $1x10^6$ cells/ml. 100 μl of this suspension was added to each well of a 96-well plate. Test drugs and controls dissolved in DMSO were added. 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.

3.9 Hoechst assay

Selected human cells overexpressing ABCG2 were collected and resuspended in HBSS at $1x10^6$ cells/ml. 100 μl of this suspension was added to each well of a 96-well

plate. Test drugs and controls dissolved in DMSO were added. 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.

3.10 Caco-2 assay

Caco-2 cells were seeded on semi-permeable filter inserts at $2x10^5$ cells/cm^2. The cells on the inserts were then cultured for 21 to 25 days prior to use. Culture medium was removed and filters were washed with HBSS. Test compounds were added in HBSS and 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.

3.11 Sample analysis

Labeled compounds were measured by liquid scintillation. All other compounds were quantified using HPLC.

4 Results and discussion

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 the ABCB1 inhibitor GF120918. We found that the outcome of the different assays was dependent on passive permeability:

- The ATPase assay and the Calcein assay correlated perfectly for high permeability compounds.
- High permeability substrates of ABCB1 did not show up as transported substrates in the Caco-2 monolayer assay.
- For low permeability compounds there was a significant shift in apparent affinities in the Calcein assay when compared to the ATPase assay.
- Most, but not all low permeability substrates of ABCB1 showed up as transported substrates in the Caco-2 monolayer assay.

4.2 ABCG2 studies

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

We found the following similarities and differences between membrane prepared from a selected human cell line expressing ABCG2 (MXR-M) and from baculovirus transfected insect cells (MXR-Sf9):

- The apparent molecular weight of ABCG2 in the insect cell membranes was lower. This was due the different glycosilation pattern of the two cell types.
- The biochemical characteristics of the vanadate sensitive ATPase activity and methotrexate transport were similar in the two preparations. Inhibition of methotrexate transport showed identical substrate/inhibitor specificity.
- ABCG2 substrates sulfasalazine, topotecan and prazosin 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. On the contrary, ABCG2 in human cell membranes could be stimulated by all three compounds.
- 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 showed increasing ATPase activity above 1 μM

- Using PGNase F to deglycosylate ABCG2 in the mammalian membrane we found that it is unlikely that the different glycosylation is responsible for the differenced observed in the two assay systems.
- Later we showed that it is the different cholesterol content of the membrane preparations that causes this difference.

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

- The inhibition of ABCG2 ATPase activity by Ko143 and Ko134 could be reversed by using increased concentrations of sulfasalazine. Increasing concentrations of Ko143/Ko134 resulted in a shift in the apparent affinity of EC_{50} for the activating effect of sulfasalazine, suggesting that these interactions are competitive.
- Increasing concentrations of Ko134 inhibited the baseline vanadate sensitive ATPase activity, however, there was no shift in the EC_{50} values of the stimulatory effect of prazosin or topotecan suggesting that these interactions are not competitive.
- We screened a library of 30 compounds at 100 μM using both membrane preparations with and without 100 nM Ko143. No interaction was detected with MXR-Sf9, yet using Ko143 allowed the detection of the 3 known ABCG2 substrates. 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.

4.2.3 *Validation of the assay*

- 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 showed competitive interaction with Ko134. In the vesicular transport assay estrone-3-sulfate was transported with a K_M of 7.8 μM and Ko134 inhibition was also competitive.
- We compared the results obtained by 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, sulfasalazin, 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.

5 Summary

We designed and preformed a detailed study on the effect of passive permeability on the outcome of different *in vitro* assays used in pharmaceutical industry to identify 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 (100-200nM) 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.

6 Acknowledgments

I would like to thank Péter Krajcsi for the tremendous amount of help and instructions I received from him. I would also like to thank all colleagues at Solvo Biotechnology for their contribution to this work and for creating the excellent work atmosphere at Solvo. I am also very grateful for Prof. Ernő Duda for the opportunity to work in his group and for giving me the first important scientific guidance and encouragement. I am also very thankful for all collegues of his research group whom I had the chance to work with.

Original publications directly related to the thesis:

Glavinas H, Kis E, Pal A, Kovacs R, Jani M, Vagi E, Molnar E, Banshagi S, Kele Z, Janaky T, Bathori G, von Richter O, Koomen GJ, Krajcsi P.
ABCG2 (BCRP/MXR) ATPase assay – a useful tool to detect drug – transporter interactions
Drug Metab Dispos. 2007 May 30;
IF: 4.015

Pal A, Mehn D, Molnar ED, Gedey S, Meszaros P, Nagy T, Glavinas H, Janaky T, von Richter O, Bathori G, Szente L, Krajcsi P.
Cholesterol potentiates ABCG2 activity in a heterologous expression system - improved in vitro model to study function of human ABCG2.
J Pharmacol Exp Ther. 2007 Jun;321(3):1085-94.
IF: 4.098

Fantin M, Quintieri L, Kusz E, Kis E, Glavinas H, Floreani M, Padrini R, Duda E, Vizler C.
Pentoxifylline and its major oxidative metabolites exhibit different pharmacological properties.
Eur J Pharmacol. 2006 Mar 27;535(1-3):301-9.
IF: 2.477

Other publications directly related to the thesis:

Oliver von Richter, Hristos Glavinas, Peter Krajcsi, Stephanie Liehner, Beate Siewert, Karl Zech
A novel screening strategy to identify ABCB1 substrates and inhibitors
Manuscript under revision

Glavinas H, Krajcsi P, Cserepes J, Sarkadi B.
The Role of ABC Transporters in Drug Resistance, Metabolism and Toxicity
Current Drug Delivery,2004 1(1) 27-42. Review

Other publications in the field of the thesis:

Williamson G, Aeberli I, Miguet L, Zhang Z, Sanchez MB, Crespy V, Barron D, Needs P, Kroon PA, Glavinas H, Krajcsi P, Grigorov M.
Interaction of positional isomers of quercetin glucuronides with the transporter (CMOAT, MRP2).
Drug Metab Dispos. 2007 May 3;

Lespine A, Dupuy J, Orlowski S, Nagy T, Glavinas H, Krajcsi P, Alvinerie M.
Interaction of ivermectin with multidrug resistance proteins (MRP1, 2 and 3).
Chem Biol Interact. 2006 Feb 25;159(3):169-79.

Schmitt U, Abou El-Ela A, Guo LJ, Glavinas H, Krajcsi P, Baron JM, Tillmann C, Hiemke C, Langguth P, Hartter S.
Cyclosporine A (CsA) affects the pharmacodynamics and pharmacokinetics of the atypical antipsychotic amisulpride probably via inhibition of P-glycoprotein (P-gp).
J Neural Transm. 2005 Oct 27.

Cserepes J, Szentpetery Z, Seres L, Ozvegy-Laczka C, Langmann T, Schmitz G, Glavinas H, Klein I, Homolya L, Varadi A, Sarkadi B, Elkind NB.
Functional expression and characterization of the human ABCG1 and ABCG4 proteins: indications for heterodimerization.
Biochem Biophys Res Commun. 2004 Jul 30;320(3):860-7.

Other publications:

Letoha T, Gaal S, Somlai C, Venkei Z, Glavinas H, Kusz E, Duda E, Czajlik A, Petak F, Penke B.
Investigation of penetratin peptides. Part 2. In vitro uptake of penetratin and two of its derivatives.
J Pept Sci. 2005 Dec;11(12):805-11.

Nagy T, Glavinas H, Szincsak N, Hunyadi J, Janossy T, Duda E, Vizler C, Juhasz I.
Tumor cells expressing membrane-bound tumor necrosis factor activate macrophages and have a compromised growth in immunosuppressed and immunodeficient mice.
Cancer Lett. 2003. 196(1):49-56.

Vizler C, Nagy T, Kusz E, Glavinas H, Duda E.
Flow cytometric cytotoxicity assay for measuring mammalian and avian NK cell activity.
Cytometry. 2002. 47(3):158-62.