Investigation of interactions between ABC transporters and their lipid environment - Effect of membrane cholesterol content on the function of human ABCG2 (BCRP/MXR)

Summary of PhD Thesis

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Publications directly related to the thesis

I.
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: 3.956 (2006)

II.
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 Sep; 35(9):1533-42.
IF: 3.638 (2006)

III.
Bathori Gy, Mehn D, Pal A, Krajcsi P, Szente L, Fenyvesi E, Telbisz A, Sarkadi B, Varadi A, Kis E, Molnar E, Gedey S, Glavinas H, Nagy T, Nemeth A.
Test systems for transporter proteins
Patent, P0600408 (2006)

IV.
Pál Á, Kis E, Méhn D, Molnár É , Glavinas H, Nagy T, Mészáros P, Báthori Gy., Krajcsi P és Falkay Gy.
ABC transzporterek -különös tekintettel az ABCG2- és gyógyszerjelölt molekulák kölcsönhatásának predikciójára alkalmas in vitro módszerek
Acta. Pharma. Hung. (2007), in press

1. Introduction

1.1 ABC transporter proteins

The proteins of the ATP binding cassette (ABC) transporter superfamily form one of the largest family of transmembrane proteins. Most of them are transporter proteins that use the energy from ATP hydrolysis to pump substrates through intra- and extracellular membranes against concentration gradients. The transport in most cases is unidirectional; eukaryotes usually efflux substrates from the cytoplasm to the extracellular matrix. The transporter family includes many members (currently 49 human ABC proteins are known,) that were classified after phylogenetic analysis and domain structure organization into 7 subfamilies (from ABCA-ABCG). Deficiency or malfunction of ABC transporters can lead to diseases, like genetic diseases cystic fibrosis, Stargardt disease, adrenoleukodystrophy and Tangier disease

Within the ABC transporter superfamily several transporters act as multidrug resistance transporters. These are transporter proteins that are able to extrude a variety of xenobiotics, including drugs from the cell via active transport. In many cases increase of their activity can lead to resistance against drug therapy. Several members of the transporter family, e.g. ABCB1 (MDR1), ABCC1 (MRP1) and ABCG2 (BCRP /MXR) have been demonstrated to play a role in the development of drug resistance. The transporters significantly influence the kinetics of drug absorption even without any overexpression or amplification of their activity. On one hand, as several drugs are substrates of a multidrug transporter, it is of crucial importance during drug development for most indications to exclude potential drug candidate molecules that can be substrates of such transporters, already at an early phase: early ADME, an acronym of „Absorption, Distribution, Metabolism, Excretion". On the other hand, efflux transporter interactions of non-CNS drugs may be beneficial by inhibiting brain

penetration and thus reducing central side effects of drugs (e.g. second generation antihistamines).

1.2 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. 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 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 conformational change in the ATP binding domain. Most members of the ABC protein family were shown to be active transporters.

1.3 The ABCG2

The ABCG2 transporter is a half transporter. It was shown that it functions as a homodimer and it is localized to the plasma membrane of cells. ABCG2 (also referred to as BCRP, MXR) is one of the most important efflux transporters in endothelial and epithelial cells, modulating ADME properties of drugs and other xenobiotics. ABCG2 exhibits a

broad substrate specificity as it transports hydrophobic, anionic as well as cationic drugs.

1.4 The ABCB1

Multidrug resistance 1 (MDR1/P-glycoprotein/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 bush-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 rate and a broad substrate specificity.

1.5 The ABCB11

The liver plays an important role in the excretion of xenobiotics, including many kinds of drugs. It has been reported that several kinds of uptake and efflux transporter are expressed on both the sinusoidal and canalicular membrane in the liver to excrete drugs efficiently into the bile. It is generally accepted that the bile salt export pump (BSEP/ABCB11) mainly transports bile acids and plays an indispensable role in their biliary excretion. Blockade of ABCB11 may lead to drug-induced intrahepatic cholestasis that is one of the major causes of hepatotoxicity. This is often observed during the drug discovery and development process.

1.6 Interaction of cholesterol with ABC transporters

A few studies addressing the effect of cholesterol on ABC transporters have been published so far. ABCB1 is the only ABC transporter where the effect of cholesterol on transporter activity has been investigated in detail.

2. In vitro membrane based assays for studying transporter-drug interactions

Membrane transporters are commonly studied in cellular or membrane based assays. For membrane assays, the transporters of interest are overexpressed in cellular systems and following expression of the transporters, membrane vesicles are isolated from these cells. Insect cell based expression systems, like the insect cell - baculovirus system, are frequently applied for protein expression. Insect cells, such as Sf9 are most frequently derived from the ovary cells of the moth, *Spodoptera frugiperda.*

The activity of the transporter is usually measured i) either by the rate of ATP hydrolysis (ATPase assay) ii) or by the direct transport of labeled substrates (vesicular transport and monolayer efflux assay). In ATPase assays (i) the transport itself is not measured directly, but via stimulation of ATPase activity, as transported substrates enhance the ATPase activity of the transporter. These substrates are also referred as activators. Among transport assays (ii) the vesicular transport method is of particular importance in the study of transporters. In this assay inside-out vesicles from insect or mammalian cell membranes are utilized; and the substrate is transported into the vesicle, where it accumulates and can be detected.

3. Objectives

Drug-transporter interactions are commonly screened by high throughput systems using membranes from infected insect cell lines with very high expression level of certain ABC transporters. It is of pivotal importance that *in vitro* insect cell models closely mimic *in vitro* mammalian model systems. Our study was focused on membrane preparations made from human ABCG2 overexpressing human (ABCG2-M) and baculovirus-infected Sf9 insect cells (ABCG2-Sf9) with different ATPase activity profile of ABCG2.

ABCG2 is not fully glycosylated in insect cells and the membrane lipid composition (much lower cholesterol content) is really different from human/mammalian cell membranes. We were interested in finding the underlying causes of different ATPase activity of the human ABCG2 in the two model systems:

To explore the molecular basis of this difference, we investigated if the difference in glycosylation can cause substantially different ATPase activity in the Sf9 system.

Our next goal was to investigate the influence of cholesterol, using various cyclodextrins, on ABCG2-ATPase and transport activity.

Finally, we made specificity studies testing sensitivity of other ABC transporters, most important in ADME studies, overexpressed in Sf9 insect cells to the membrane cholesterol content.

4. Materials and methods

4.1 Chemicals and Biochemicals

All chemicals and biochemicals were purchased from commercial sources.

4.2 Membrane preparation

Human membrane vesicle preparations (ABCG2-M) as well as membrane vesicle preparations obtained from insect cells expressing ABCG2 (ABCG2-Sf9), ABCB1 (ABCB1-Sf9), ABCB11s (human ABCB11-Sf9, mouse Abcb11-Sf9 and rat Abcb11) were obtained from Solvo Biotechnology. The insect membrane vesicle preparations were produced using

recombinant baculoviruses encoding ABCG2, ABCB1 and ABCB11s.

4.3 Western blotting

ABCG2 expression and apparent molecular weight was detected by SDS-page and subsequent western blotting using anti-ABCG2 antibody, BXP21.

4.4 ATPase assay

In the experiments presented PREDEASY ATPase Kit (Solvo Biotechnology, Szeged, Hungary) was used for the determination of ATPase activity.

4.5 Vesicular transport assay

Inside-out membrane vesicles were incubated in the presence or absence of ATP. The incubation mix was then rapidly filtered through glass fiber filters. Filters were washed and radioactivity retained on the filter was measured by liquid scintillation counting. ATP-dependent transport was calculated by subtracting the values obtained in the absence of ATP from those in the presence of ATP.

4.6 Determination of cholesterol content

The cholesterol content of the membranes was determined using the cholesterol oxidase method. Reaction mix was mixed with cholesterol oxidase enzyme and membranes vesicles. After centrifugation the supernatant were analyzed by HPLC. The oxidised cholesterol was detected using UV detector.

4.7 Cholesterol loading and depletion

Cholesterol loading of ABCG2-Sf9, human ABCB11-Sf9, mouse Abcb11-Sf9, and rat Abcb11-Sf9 membranes were carried in the presence of cholesterol@RAMEB (Randomly methylated beta-cyclodextrin cholesterol complex) complex. In cholesterol depletion studies membranes were incubated with RAMEB (Randomly methylated beta-cyclodextrin).

The cholesterol loaded, transporter containing membrane preparations are a proprietary technology of SOLVO Biotechnology.

5. Results

5.1 Results for ABCG2 studies

Identical substrate specificity, different ATPase profile

We performed a comparative analysis of wild type ABCG2 in membrane preparations derived from baculovirus infected Sf9 cells and selected human cells (ABCG2-Sf9 and ABCG2-M, respectively). Both transporter preparations had similar affinity for ATP for methotrexate. Substrates and inhibitors of ABCG2 inhibited, while non-interacting drugs did not modulate the ATP dependent methotrexate transport in either membrane preparation.

In contrast, in the ATPase assay the same set of compounds showed a strikingly different pattern. In case of ABCG2-Sf9 preparations the baseline vanadate sensitive ATPase activity could not be further stimulated by ABCG2 substrates, while stimulation was detected for all substrates tested in ABCG2-M preparations.

Cholesterol loading potentiates drug induced ATPase stimulation of ABCG2 expressing ABCG2-Sf9 membranes

It has been known that lipid composition of insect and mammalian membranes significantly differs. One of the major differences is the cholesterol content, as Sf9 plasma membrane contains about 5-10-fold less cholesterol than human plasma membrane. As expected, the cholesterol contents of the ABCG2-Sf9 and ABCG2-M vesicles containing ABCG2 protein were markedly different, with ABCG2-Sf9 displaying an about 4¬5-fold lower cholesterol level. Cholesterol loading of both membranes using cholesterol@RAMEB treatment resulted in about a fifteen-fold increased cholesterol content in ABCG2-Sf9 vesicles and a three-fold increase in ABCG2-M vesicles. On the other hand, RAMEB treatment removed the cholesterol from both membranes very efficiently.

Differences in cholesterol content confer the difference in ATPase stimulation of ABCG2 expressing ABCG2-Sf9 and ABCG2-M membranes

Cholesterol loading significantly potentiated sulfasalazine induced activation of ABCG2-ATPase. In contrast, ABCG2 ATPase activity of ABCG2-M membranes was stimulated by sulfasalazine in a concentration dependent manner without requiring cholesterol treatment. Depletion of cholesterol of the ABCG2-M membranes impaired the ABCG2-ATPase response. All substrates, sulfasalazine, prazosin, topotecan stimulated the basal ATPase activity in a concentration dependent manner.

Enhanced ABCG2-mediated vesicular transport into cholesterol loaded ABCG2-Sf9 inside-out vesicles

To study if cholesterol loading affects transport kinetics of ABCG2 expressing ABCG2-Sf9 vesicles we monitored the transport of a known ABCG2 substrate, methotrexate, commonly used in VT studies. In order to evaluate the effect of cholesterol V_{max} and K_M has been calculated. Cholesterol loading dramatically increased maximal velocity of the

transport (2144 pmol/mg/min vs. 367 pmol/mg/min) without affecting K_M (1068 µM vs. 935 µM). We also studied prazosin transport. Indeed, little ATP-dependent transport (V_{max}: 85 pmol/mg/min) was seen in the ABCG2-Sf9 vesicles. On the contrary, a very significant transport was seen in the cholesterol loaded ABCG2-Sf9 vesicles with a maximal velocity of 702 pmol/mg/min.

Effects of cholesterol on ATPase and VT measurements correlate

To study the effect of membrane cholesterol levels on ABCG2-ATPase activity and vesicular transport, estrone-3-sulfate was used as a substrate. Cholesterol loading increased V_{max} of transport of estrone-3-sulfate by more than 20-fold (3408 pmol/mg/min vs 122.7 pmol/mg/min) with relatively little change in K_M (14.11 µM vs 27.58 µM). In the ABCG2-M membranes a similar effect of membrane cholesterol level was observed, as cholesterol depletion decreased V_{max} by about 5-fold (455.5 pmol/mg/min vs 99.02 pmol/mg/min) with little change in K_M (5.49 µM vs 7.89 µM) (Fig. 7B). The increased transport rate was paralleled in the ATPase assay, as low cholesterol membranes (ABCG2-Sf9 and cholesterol depleted ABCG2-M) displayed an impaired activation upon estrone-3-sulfate treatment. Moreover, the K_M and V_{max} values for estrone-3-sulfate induced ABCG2-ATPase activity showed an acceptable correlation between the cholesterol loaded ABCG2-Sf9 and ABCG2-M membranes.

5.2 Results for other ABC transporters

Effect of cholesterol on transporters most important in ADME studies

We selected ABCB1, ABCB11 and ABCC2 transporters, since they have the major impact to influence on ADME properties of drugs. To investigate the specificity effect of cholesterol on these transporters, we applied the most suitable

assay types to the transporters: ATPase assay with high permeability drug (verapamil) for ABCB1 and vesicular transport assay for ABCB11s and ABCC2 studies.

In case of human ABCB1, the increased cholesterol level resulted in only an elevated basal ATPase activity in Sf9 membrane vesicles.

Effect of cholesterol on transport activity of ABCB11

Cholesterol increases the taurocholate transport activity (V_{max}) of vesicles prepared from Sf9 cells containing either the human ABCB11, the mouse Abcb11 or the rat Abcb11 transporters.

In case of ABCC2, no significant effect was observed.

6. Summary

In our study for a first step, we investigated the underlying difference between the ABCG2 ATPase activity expressed in Sf9 and in human cell membrane. We established that different glycosylation level of ABCG2 in the Sf9 and the human membranes does not explain the different ATPase activity profile of the protein.
We found that the low-cholesterol content of the insect membranes profoundly attenuated the drug stimulated ATPase activity of the expressed ABCG2 protein. Along the same lines, cholesterol loading transformed the ATPase activity profile of Sf9 membranes to a profile similar to the native, cholesterol-rich mammalian membranes.

We investigated sensitivity of other ABC transporters (ABCB1, ABCC2 and ABCB11s) overexpressed in Sf9 insect cells to the membrane cholesterol content and we found positive effect (increased V_{max}) of higher membrane cholesterol content in case of ABCB11 vesicular transports.

However, the extent of improvement was not as significant as in case of ABCG2-Sf9 vesicles.

Cholesterol loaded ABCG2 insect cell membranes are suitable models to study drug transport by ABCG2 and drug-drug interactions in the ATPase assay and in the vesicular transport assay as well.
Based on our experiences, we think that membrane based assays utilizing the cholesterol-loaded insect cell membranes (*mammalian "mimic" model membranes)* are useful tools to develop new assays for studying drug – ABCG2 interactions.

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