Summary of Ph.D. thesis

In vitro methods to test drug – ABC transporter interaction –optimization and validation studies

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Introduction

The ATP binding cassette (ABC) transporter superfamily is one of the largest family in field of the transmembrane proteins. Members of this superfamily cover a broad spectrum of physiological functions including detoxification, defense against xenobiotics and oxidative stress, absorption, distribution and excretion processes as well as lipid metabolism.

The multidrug resistance phenotype in tumors can be associated with the overexpression of certain ABC transporters, termed MDR proteins. The P-glycoprotein -ABCB1- mediated multidrug resistance was discovered first and probably still is the most widely observed mechanism in clinical multidrug resistance. Two other ABC transporters have been demonstrated to participate in the multidrug resistance of tumors: the multidrug resistance associated protein 1 (ABCC1), and the breast cancer resistance protein (ABCG2).

Rheumatoid arthritis (RA) is a chronic disease characterized by inflammation of the lining or synovium of the joints. There are three general classes of drugs commonly used in the treatment of RA: non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and disease modifying anti-rheumatic drugs (DMARDs). One of the drawbacks is that while NSAIDs and corticosteroids have a short onset of action, DMARDs can take several weeks or months to display clinical effect. Moreover, due to the long-term application of the drugs many RA patients demonstrate loss of efficacy over time. DMARDs resistance may be a multifactorial event including enhanced drug efflux *via* ABC transporters, impaired drug uptake and drug activation, enhanced drug detoxification etc.

ABC transporters likely play a role in drug resistance to DMARDs. Previous studies revealed that ABCB1 expression was higher in the peripheral blood lymphocytes of prednisolone and DMARD-treated patients than in controls. Llorente and colleagues observed a difference in the expression of ABCB1 among patients who did not respond to therapy and those patients who did. Others found ABCC1 overexpression in the chloroquine resistant human CEM T cells. When human T lymphocytes were selected in the presence of sulfasalazine, the resistant population overexpressed ABCG2 and displayed lower sensitivity (5-fold) to leflunomide too. However, as the authors did not show that the phenomenon can be modelled in a transfected system they did not explicitly identify ABCG2 as the only factor responsible for resistance to sulfasalazine and leflunomide.

Leflunomide is a DMARD with an isoxazole ring that inhibits proliferation of mitogen-stimulated T- and B-lymphocytes by a non-cytotoxic. It inhibits the dihydroorotate

dehydrogenase, a key enzime in the pathway for *de novo* synthesis of UMP. Leflunomide is a pro-drug that undergoes rapid nonenzymatic conversion to its active form, A771726. Leflunomide is used either alone or in combination with methotrexate in the therapy of RA.

During the past few years it became clear that the plasma membrane not only consist of a uniform phospholipid bilayer but is rather laterally segregated into different plasma membrane domains that consist of a variety of different lipids and proteins. These small (10-200 nm) cholesterol-enriched microdomains are called lipid-raft or membrane-raft. The importance of the membrane lipid composition for the funtcion of membrane proteins has been demonstrated for a number a relevant proteins involved in signal transduction and membrane trafficking. In addition several reports found ABC transporters (ABCB1, ABCC1, ABCC2, ABCG2) localized in these microdomains. No study had been published on microdomain localization of the ABCB11 transporter, altough this transporter is expressed on the cholesterol-rich apical membrane of the hepatocyte.

Bile acids are oxidation products of cholesterol, synthesized by the hepatocytes, and transported as bile salts by the ABCB11 to the bile. In humans there is no compensatory mechanism for the loss of this transporter, as mutations of ABCB11 result in a genetic disease, called progressive familial intrahepatic cholestasis type 2 (PFIC2) and inhibition of ABCB11 by drug molecules leads to clinical cholestasis. Therefore there is an increasing need for reliable, validated screening tools suited to test drug candidates for ABCB11 interaction potential, during the process of drug development.

ABCB11 protein is highly identical with its rodent (rat and mouse) orthologs. Gene knockout or transgenic mouse models are important *in vivo* systems for studying the role and regulation of the Bsep gene. *In vitro* expression of the mouse, rat and human ABCB11 protein makes the analysis of the functional interspecies differences possible and forecast or validate the *in vivo* results through membrane based high throughput assays, like the vesicular transport assay and the ATPase assay.

Aims of the work

1./ The major goal was to test drugs from that application fields where MDR phenomenon appears (autoimmune disease, cancer disease, inflammation disease). Using relevant ABC transporter expressing test systems- ATPase assay, vesicular assay and whole cell based assays- we wanted to show and characterize earlier not described interactions.

2./ We hypotesized that cholesterol modulates rat, mouse and human ABCB11 activity which are localized in the cholesterol-rich canalicular membrane of the liver cells. As native Sf9 membranes contain much less cholesterol than mammalian cells, we have developed a cholesterol-enriched Sf9 membranes that are better mimics of the mmammalian membranes. We have also examined affinity differences between the mouse, rat and human ABCB11 mediated bile salts (taurocholate, glycocholate, taurochenodeoxycholate, glycochenodeoxycholate) transports using compounds which cause clinical cholestasis, namely cyclosporin A, troglitazone and glibenclamide.

3./ Cholesterol enrichment of mBsep-Sf9 (expressing mouse Abcb11) vesicles resulted in a membrane product that is suitable for high-throughput screening for bile salt export pump interactors using the ATPase assay. Our goal was to develop and optimize this nonradioactive methods.

Materials and methods

Cell lines and membrane preparations

HEK293 cells (obtained from the American Type Culture Collection), and the ABCG2 overexpressing clone (HEK293-BCRP) were maintained in MIX MEM medium (Hank's F12 : DMEM , 1:1) supplemented with 10%(v/v) heat-inactivated fetal-calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and L-Glutamin (2 mM) in a humidified atmosphere of 5% CO2 in air at 37°C. PLB985, PLB985-BCRP (overexpressing ABCG2), K562-MDR (overexpressing ABCB1), HL60-MRP (overexpressing ABCC1) were cultured in Advanced RPMI 1640 medium supplemented with 10%(v/v) heat-inactivated fetal-calf serum, penicillin

(100 units/ml), streptomycin (100 μg/ml) and L-Glutamin (2 mM) in a humidified atmosphere of 5% CO_2 in air at 37°C.

Membrane vesicle preparations obtained from insect cells expressing ABCG2, ABCB1, ABCC1, mouse, rat and human ABCB11 were obtained from Solvo Biotechnology.

Western blot

ABCG2 and ABCB11s expression and apparent molecular weight was detected by SDS-page and subsequent western blotting using anti-ABCG2 (BXP21) and anti-ABCB11 antibody.

Cholesterol loading and determination of cholesterol content

Cholesterol loading of the membranes were carried in the presence of 1 mM cholesterol@RAMEB complex at at 37°C for 30 min.

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

ATPase assay

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

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.

Hoechst assay and Calcein assay

Accumulation of Hoechst 33342 and Calcein dye was measured in a fluorescence spectrophotometer at 350 nm (excitation)/460 nm (emission) and 485 nm/538 nm. For maximum inhibition of the ABCG2 protein 300 nM Ko134 was applied in each experiment. In the case of Calcein assay 60 μM verapamil was used. The fluorescence intensities were recorded in a kinetic mode. Using the slope of the curve determined without inhibitors (R_{base}), the slope of the curve

in the presence of inhibitor (R_{max}), and the slope of the curve determined for any drug at the given drug concentration (R_{drug}), the *% inhibition* of Hoechst 33342/Calcein extrusion can be represented with the following formula:

$$Inhibition(\%) = \frac{R_{drog} - R_{base}}{R_{\max} - R_{base}} * 100$$

IC_{50} values were derived from these curves.

Cytotoxicity assay

Cytotoxicity assays were performed by seeding HEK293 and HEK293-BCRP cells at a density of 4000 cells per well in 96-well plates containing the culture medium. After 24 hours, drugs were prediluted in medium and added to the cells at different concentrations. The cells were further incubated with the drug in a humidified tissue-culture chamber (37°C, 5% CO_2) for 96 hours. Surviving cells were detected by the MTS method. IC_{50} values were calculated from dose-response curves (i.e. cell survival vs. drug concentration) obtained in triplicate experiments.

Data Analysis

Vesicular transport assay, ATPase assay and Hoechst/Calcein assay were run in duplicates, the cytotoxicity assays were run in triplicates. Data are presented as mean ± S.D.

The potencies of drugs to alter ATPase activity were obtained from plots of the rate of ATP hydrolysis as a function of logarithm of drug concentration by nonlinear regression of the general, sigmoid dose-response equation:

$$\nu = V_{\min} + \frac{V_{\max} - V_{\min}}{1 + 10^{(\log EC_{50} + [A]) x n_H}}$$

where v = response (nmolPi/mg protein/min), V_{min} = minimal response, V_{max} = maximal response, EC_{50} = ligand concentration producing 50% of maximal response (efficacy), A = the actual test drug concentration, and n_H = Hill slope, the parameter characterizing the degree of cooperativity.

In case of Vesicular transport assay, K_I values were determined using Cheng-Prusoff equation:

$$K_I = \frac{IC_{50}}{1 + \frac{[S]}{K_M}}$$

where K_I = the affinity of the inhibitor, IC_{50} = the concentration of the inhibitor where inhibits 50% of the transport, S = the concentration of the substrate, K_M = the affinity of the substrate.
In the cytotoxicity assay each concentration was tested in triplicate. IC_{50} values were calculated from dose-response curves obtained from four separate experiments.
For curve fitting, V_{max}, K_M, K_I and IC_{50} calculations PRISM 3.0 software was used. For statistical analyses Wilcoxon-probe and ANOVA were used.

Results and discussion

Detection and characterization of substrate-transporter interaction using HT *in vitro* assays

Different membrane and whole cell based assays were performed to investigate and characterize the interaction between the transporter, ABCG2 and the analyzed compounds, leflunomide and its metabolite, A771726. We have found that both compounds interacted with ABCG2 and are transported substrates of the transporter based on several facts: both compounds activated the ABCG2 transporter in the ATPase assay; both proved to be competitive inhibitors of methotrexate transport by ABCG2 and the resistance to their cytotoxic effects could be reversed in the presence of a ABCG2-specific inhibitor, Ko134.
ABCG2 was shown to be important in the defense mechanism of immune-competent cells, such as stem cells or monocyte-derived dendritic cells. ABCG2 could play an important role in inflammatory processes, because it was shown that it is expressed on endothelial cells and macrophages in the synovial sublining of RA patients. It was suggested that the expression of this ABC transporter is rather inflammation dependent than drug-induced. However, previous studies indicated that ABCG2 overexpressing population is selected upon treatment of cells with escalating dose of various drugs and the breast cancer resistance protein is important in the absorption and elimination of DMARDs, such as sulfasalazine and methotrexate.
Methotrexate, is a known ABCG2 substrate, which is commonly used in vesicular transport studies. This inhibition type membrane based assay is suitable to study the effect of the test drug on the accumulation of a transported substrate. By using a relevant drug molecule as a reporter substrate, potentially important drug-drug interactions can be detected in a simple *in vitro* system. Both leflunomide and A771726 inhibited the accumulation of methotrexate into inside-out BCRP-HAM-Sf9 vesicles in a dose-dependent manner, but with different affinities. Leflunomide inhibited methotrexate transport with higher K_I (1.86 μM) than A771726 (0.093

μM). Furthermore, the Dixon plots revelead that the mechanism of action between methotrexate and leflunomide or A771726 is competitive inhibition.

This difference in kinetic parameters is also observed in the ATPase assay. The modulation of baseline ATPase activity may indicate the substrate nature of a compound. Leflunomide and A771726 stimulated the vanadate sensitive ATPase activity of the ABCG2 transporter in the ATPase assay with 3.93 μM and 0.78 μM EC_{50} values, respectively.

Hoechst 33342 is a membrane permeable dye which is an excellent substrate of ABCG2. This cellular assay is suitable to follow the interaction between the ABCG2 transporter and test drugs. Both substrates and inhibitors increase the cellular accumulation of the dye through ABCG2 interaction. Leflunomide and its metabolite inhibited the Hoechst efflux with nearly same IC_{50} values; 4.53 μM and 2.87 μM.

We performed 96-hour cytotoxicity assays using empty vector transfected HEK293 cells and the ABCG2 transporter expressing clones. HEK293-BCRP cells showed 20.6 and 7.5-fold resistance compared to the mock HEK293 in the case of leflunomide and A771726. This increased resistance is attributable to the ABCG2 transporter because it could be reversed by a known specific ABCG2 inhibitor, Ko134. This assay set up is considered as a surrogate transport assay, as it refers directly to the involvement of a specific transporter and it's role in resistance to the drug tested.

Leflunomide is a commonly used new DMARD applied in monotherapy or in combination therapy with non steroidal anti-inflammatory drugs. Moreover, it is applicable to treat active rheumatoid arthritis in combination with other disease modifying antirheumatic drugs, like methotrexate, sulfasalazine, infliximab, adalimumab, etanercept and anakinra.

Leflunomide is used in combination therapy with methotrexate in case resistance is developed for the latter mentioned DMARD. Several studies confirmed that administration of leflunomide together with methotrexate can lead to improved patient condition. This may be at least in part due to the competitive inhibition between methotrexate and leflunomide or A771726 leading to increased local concentrations of one both drugs. Our findings can be the basis of new therapeutic approaches, such as the local administration of ABCG2 reversing agents in combination with DMARDs.

Effect of membrane cholesterol level on mouse, rat and human ABCB11 activity

We have shown that cholesterol treatment has an effect on ABCB11 activity, namely, increasing V_{max} values, without producing a significant shift in K_M values. Cholesterol loading had the most

pronounced effect on rat Abcb11 increasing intrinsic clearance values for taurocholate (TC), glycocholate (GC) and taurochenodeoxycholate (TCDC) 3-5-fold. In a study published simultaneously with our study raft localization of ABCB11 was shown and a study published right after our paper confirmed the crucial role of cholesterol in ABCB11 activity.

We tested 3 inhibitors with 4 substrates on ABCB11 transporters from 3 species (human, rat, mouse) in this novel high-throughput screening system. Our data show different patterns for cyclosporin A and troglitazone as well as glibenclamide. IC_{50} values for cyclosporin A varied greatly for all substrates for ABCB11 from the same species as well as more expectedly between species for the same substrate. For troglitazone and glibenclamide there was little variation within IC_{50} values for the different substrates within a species. In contrast, there were significant variations between species for the same substrate. Both glibenclamide and troglitazone displayed the lowest inhibitory potency towards the mouse protein. Interestingly though, for glibenclamide the IC_{50} values of the rat transporter are closer to the relevant data on the human transporter. While for troglitazone the IC_{50} values for the rat protein are closer to the data on the mouse protein.

Our results correlate reasonably well with published data. For human ABCB11-mediated TC transport inhibition the difference between our data and published results is about twofold for both glibenclamide and cyclosporin A. For rat Abcb11 mediated TC transport inhibition the differences between our data and published values differ 3-6-fold. However, the rankings (cyclosporin A<troglitazone~glibenclamide) in all studies are the same.

To ensure that the IC_{50} values obtained are valid we tested a small set of compounds for their abilities to inhibite ABCB11-mediated TC transport both control and cholesterol-loaded Sf9 vesicles containing human ABCB11. All compounds inhibited TC transport near identical IC_{50} values for the respective compounds in the membrane vesicles with different cholesterol content. The only outlier was cyclosporin A, where a difference close to 10-fold could be observed. Not only was this compound showing the greatest inter-species and bile salt variability, but also this was the one apparently sensitive for the cholesterol level of the membrane.

In general, we observed greater variations in species-specificity for inhibitors than for substrates.

Optimalization of the mouse Bsep-HAM ATPase assay

In this study the mouse Abcb11 protein also proved to be sensitive for the cholesterol content of its membrane environment. Transport rate of the reporter substrate (TCDC) and the amount of phosphate generated in the presence of TCDC both increased in cholesterol loaded Sf9 membranes. The elevated cholesterol level leads to an approximately two-fold increase of V_{max}

in the vesicular transport assay. In correlation with increased transport V_{max}, an increased maximal stimulation of ATPase activity was observed. However, in contrast to the results observed in the case of ABCG2, treatment of the Sf9 cells with cholesterol@RAMEB complex decreased the basal vanadate sensitive ATPase activity of the mouse Abcb11 protein. The combined effect of increased maximum value and decreased background resulted in a significantly better signal/ background ratio and a wider dynamic range.

The ATPase activity of the mBsep-HAM-Sf9 vesicles is time, temperature and ATP dependent. After the assay parameter set has been optimized, the results obtained from the mBsep-HAM-Sf9 ATPase assay were compared with those from the mBsep-HAM-Sf9 vesicles in the vesicular transport assay.Interestingly, slightly lower IC_{50} values were obtained by the ATPase assay than by vesicular transport.

ABCB11 transporter plays a key role in canalicular bile salt transport and in regulating the bile salt concentration in the hepatocytes as well as in the systemic circulation. Identification of ABCB11 inhibitors, that reduce bile salt secretion thus causing cholestasis, is crucial in the early phase of drug development. Membrane based rodent transporter studies are promising tools for prediction of human interaction. The current transport assays all require fairly cumbersome, nongeneral analytical methods like LC/MS or the application of radiolabeled compounds. In contrast mBsep-HAM-Sf9 ATPase is a simple, high-throughput, sensitive, nonradioactive assay designed to detect drug-ABCB11 interaction. It can complement the human ABCB11 mediated TC transport inhibition studies and the *in vivo* rodent studies.

List of publications

Publications directly related to the thesis:

Kis E, Rajnai Z, Ioja E, Herédi Szabó K, Nagy T, Méhn D, Krajcsi P (2009) Mouse Bsep ATPase assay: a nonradioactive tool for assessment of the cholestatic potential of drugs. *J Biomol Screen* 14(1):10-5.
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Ann Rheum Dis 68(7):1201-7.
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IF:3.743

Other publications:

Jani M, Makai I, **Kis E**, Szabó P, Nagy T, Krajcsi P, Lespine A (2010) Ivermectin interacts with human ABCG2. *J Pharm Sci* 2010 Jun 22. [Epub ahead of print]
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