SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
(Ph.D.)

STUDYING THE TRANSPORT MECHANISM OF HUMAN P-GLYCOPROTEIN (ABCB1) AND BREAST CANCER RESISTANCE PROTEIN (ABCG2)

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The PhD defense will take place in a virtual setting using Zoom at 28th of May 2021, 2PM.
Participation requires registration. For registration and further information write an e-mail to tarapcsakszabolcs@gmail.com until the 27th of May 2021, 2PM.

1. Introduction

Cancer is undoubtedly one of the leading causes of death worldwide. Numerous therapeutic modalities are available for the routine therapy of tumors including radiotherapy, immunotherapy, chemotherapy along with others. During chemotherapy, clinical oncologists often face the emergence of drug resistance: following the promising results of the initial phase of chemotherapy cancers gradually become resistant to several anti-neoplastic agents, including drugs they have never been exposed to. Numerous cellular processes can lead to resistance. Since anticancer drugs often target proteins involved in cell cycle regulation or apoptosis, alterations of these mechanisms may result in chemotherapy resistance.

However, frequently the reason for cellular drug resistance is the over-expression of certain members of the ATP-binding cassette (ABC) transporter superfamily in tumor cells including P-glycoprotein (Pgp, ABCB1, MDR1), Multidrug Resistance Protein 1 (ABCC1, MRP1) and Breast Cancer Resistance Protein (ABCG2, BCRP). These multidrug transporter proteins have exceptionally wide substrate specificity, they can recognize and export the majority of anticancer drugs from the tumor cells leading to chemotherapy resistance. The phenomenon when tumor cells acquire cross-resistance against multiple agents is termed *multidrug resistance* (MDR).

ABC proteins form one of the largest and the most versatile protein families. Members of the ABC protein family are present in all domains of life, from bacteria to humans. Members of the ABC protein family show enormous functional diversity. They are involved in several physiological processes, however, the majority of them are transmembrane proteins that function as channels, channel regulators or active transporters. The human genome encodes 48 ABC proteins that are classified into seven subfamilies from ABCA to ABCG based on their sequence similarity. Molecular details of the transport cycle of multidrug transporters like Pgp and ABCG2 are still elusive and are in the focus of intense research. Understanding the details of their transport cycle might result in new therapeutical approaches to overcome multidrug resistance in cancer.

1.1. Structure of Pgp and ABCG2

P-glycoprotein is a 1280 amino acid long single polypeptide chain consisting of two transmembrane domains (TMD), each containing 6 transmembrane helices, and two nucleotide-binding domains (NBD). As opposed to Pgp, ABCG2 is a 655 amino acid long half-transporter consisting of only one TMD with 6 transmembrane helices and one NBD with a reversed NBD-TMD orientation compared to Pgp.

Based on available crystal structures of ABC transporters and indicated by biochemical and biophysical experiments eukaryotic ABC transporters seem to share common major conformational states including the "inward-facing" and "outward-facing" conformations. In the inward-facing conformation Pgp and ABCG2 form an inverted "V"-shape, with a large cavity in the center that is accessible from the intracellular space. The inward-facing conformation allows substrates to enter and bind to the drug-binding cavity lined by TMD helices. The "outward-facing" conformer is characterized by the close contact of the NBDs and opening of the central cavity to the extracellular space. The proposed role of the outward-facing conformation is the release of substrates to the extracellular space.

The TMDs of Pgp and ABCG2 are responsible for the binding of substrates molecules and their transmembrane transport. Both halves of Pgp (each containing 6 TMD helices) contribute in a pseudo-symmetric fashion to substrate binding. TMDs mostly contain aromatic and hydrophobic residues but there are also polar or charged side chains on the surface of the substrate binding cavity. On the other hand, the homodimer ABCG2 shows complete axial rotational symmetry. As opposed to Pgp, the substrate binding cavity of ABCG2 is narrower and more hydrophobic. Moreover, a second smaller drug-binding cavity (cavity 2) close to the extracellular gate of the translocation apparatus can be observed.

NBDs of ABC transporters are the functional units responsible for ATP binding and hydrolysis. Both NBDs of ABC proteins contain a RecA-like domain and an α-helical domain. The RecA-like subdomain is formed by the evolutionary conserved Walker A and Walker B sequences and it is present in many ATPases and molecular motors. The α-helical domain contains the signature sequence (LSGGQ motif), which is characteristic for the NBDs of ABC transporters. In all ABC transporters the two NBDs collectively form two nucleotide-binding sites (NBS1 and NBS2). Upon ATP binding the ATP molecules are sandwiched between the Walker A and Walker B motifs of one NBD and the signature motif of the contralateral NBD. Beside the Walker A, Walker B and signature motifs, NBSs of Pgp and ABCG2 are characterized by a series of additional evolutionary conserved sequence elements (e.g., A-loop). In accordance with their crucial role in ATP binding and hydrolysis, mutations of highly conserved residues within these sequence motifs significantly reduce or completely abolish ATPase and transport activity, as it was highlighted in many mutagenesis studies using heterologous expression systems or purified proteins.

1.2. Catalytic cycle of Pgp and asymmetry in the NBDs

Characterization of the catalytic cycle of Pgp and ABCG2 is extremely challenging. Partly it is due to experimental and instrumental limitations of

membrane protein research, e.g., difficulties related to the purification and crystallization of large membrane proteins. On the other hand, computational limitations of molecular dynamics simulations make *in silico* analysis restricted to only certain events of the catalytic cycle.

However, recent developments in structural biology and spectroscopic techniques helped to better understand the mechanism of these enigmatic transporters. Models describing the molecular details of the catalytic cycle of Pgp share similar steps including: (1) binding of two ATP molecules to the inward-facing high-affinity conformer, (2) NBD-NBD sandwich dimer formation, (3) conformational remodeling of TMDs leading to the low substrate-binding affinity outward-facing conformation, (4) nucleotide hydrolysis at the NBDs, (5) ADP and inorganic phosphate (Pi) release and (6) resulting conformational changes in the TMDs and resetting the cycle.

Interestingly, prevalent catalytic cycle models agreed on that the functional activity of both catalytic centers is needed for the conformational changes and the transport activity of Pgp. As a corroborating evidence it has been shown that phosphate analogues (e.g., vanadate (Vi)) can replace the cleaved gamma phosphate forming a stable Pgp-ADP-Vi complex at a single NBS and lead to the inactivation of Pgp. In addition, several studies demonstrated that point mutations in the highly conserved key residues of a single NBS also completely inactivate Pgp. However, our group previously demonstrated that single Walker A mutant Pgp molecules show significant residual transport and ATPase activity when they are studied in their natural plasma membrane environment.

In about half of the human ABC transporters both NBDs are fully functional and either identical such as in ABCG2 or highly similar such as in Pgp. Interestingly, in the other half of the human ABC transporters the sequence of NBS1 has diverged from the consensus sequence. In several ABC proteins, like the main bile acid transporter ABCB11 (BSEP) the glutamate residue in the Walker B sequence, often termed as “catalytic glutamate” is replaced with a “non-canonical” amino acid. Moreover, ABCB11 has three additional substitutions in the Q-loop and in the signature sequence. Interestingly, ABCB11 shows a remarkable sequence similarity to Pgp and the NBD-NBD interfaces of the two proteins differ only in the above mentioned four amino acids. However, mutation of the “catalytic glutamate” (E556) in the NBS1 of Pgp, as opposed to ABCB11, renders it transport-incompetent.

1.3. Retinoids and ABC transporters

Pgp and ABCG2 possess extremely wide and partially overlapping substrate spectra including structurally diverse hydrophobic or amphiphilic compounds in the

molecular weight range of 300 to 2000 Da. Interestingly, despite the extremely wide substrate spectrum of Pgp and ABCG2, only a few endogenous substrates have been identified for ABCG2 and there are no well-characterized physiological Pgp substrates identified.

Retinoids are a large group of biomolecules derived from vitamin A. A series of major cellular processes including cell survival, proliferation or differentiation is regulated by retinoids. Since retinoids are hydrophobic compounds with an average molecular mass of 300 Da, they are potential Pgp and/or ABCG2 substrates. Retinol is present in the human plasma at 1-2 μM concentration under physiological circumstances, while its natural metabolites e.g., retinyl-esters, all-*trans*-retinoic acid (ATRA), 13-*cis*-retinoic acid or 9-*cis*-retinoic acid are present at significantly lower (pM to nM) concentrations. Transcriptionally active retinoids regulate the activation of nuclear receptors including RAR-RXR (retinoic acid - retinoid X receptor) heterodimer nuclear receptors, PPARs (peroxisome proliferator activated receptors) and VDRs (vitamin D receptor, calcitriol receptor). Because of their major role in the regulation of cell proliferation and differentiation retinoids are routinely applied in chemotherapy at supra-physiological concentrations (app. 10-20 μM final plasma concentrations). Retinyl-esters, like retinyl-acetate and retinyl-palmitate are frequently used food additives and they are also applied as components of anti-aging cosmetics. Furthermore, retinyl-acetate showed promising results in the treatment of certain degenerative diseases of the retina.

Nonetheless, direct interactions of retinoids with Pgp or ABCG2 have not been studied so far. According to literature data, several retinoid derivatives exhibit uneven distribution between the placenta and the developing embryo raising the possibility that active transporters might be involved in the transport of these compounds. Since both Pgp and ABCG2 are expressed in the placental syncytiotrophoblasts, together with several other ABC transporters, they might have roles in the transport of retinoids and consequently in the protection of the fetus from their teratogenic effects by maintaining significantly lower retinoid concentrations in the embryonic tissues.

2. Aims of the study

Despite the large number of substrates that are recognized by Pgp and ABCG2 only a few endogenous Pgp or ABCG2 substrates were identified so far. Retinoids are potential Pgp and/or ABCG2 substrates/modulators because of their highly hydrophobic character and molecular weight of app. 300 Da. In view of their physiological and clinical significance we have studied their interaction with Pgp and ABCG2 and aimed to answer the following questions:

- Whether retinoids can interact with Pgp and ABCG2 as substrates or inhibitors?
- Whether stereo-chemical differences between retinoid derivatives can affect their interaction with Pgp and ABCG2?
- Do retinoids affect the function of Pgp and ABCG2 directly or they act indirectly by changing the membrane environment?

In the second part of our work, we investigated various aspects of the functional collaboration of the NBSs upon the catalytic cycle of Pgp studying mutant Pgp variants with one intact NBS. We tried to answer the following questions:

- Whether human Pgp molecules with one intact and one ABCB11-like degenerate NBS are functional?
- Whether mutant Pgp variants that possess a single point mutation in the A-loop (Y401A or Y1044A) or in the Walker B sequence (D555N or D1200N) are transport competent?
- Whether the transport deficient mutants have any partial activity including capability for nucleotide binding, nucleotide hydrolysis and concomitant conformation changes?

3. Materials and methods

3.1. Cell lines

For our experiments we used NIH 3T3 mouse fibroblast cells and its wild-type human Pgp expressing counterpart (NIH 3T3 MDR1 G185), MDCK II (Madin-Darby canine kidney) cells and its ABCG2 transfected counterpart. All cell lines were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM). Cell culture media were supplemented with heat-inactivated fetal calf serum, L-glutamine and penicillin-streptomycin cocktail. Cells were checked regularly for mycoplasma infection by the MycoAlert mycoplasma detection kit and were found to be negative.

3.2. Substrate accumulation tests

For fluorescent drug accumulation studies, we used calcein-AM and Hoechst 33342 to assess transport activity of wild-type and different NBD mutant Pgp molecules. For ABCG2 transport activity measurements we used mitoxantrone as fluorescent substrate. Cells were harvested and washed three times in PBS containing glucose (gl-PBS) or HEPES buffer (in case of Hoechst 33342). Cells were pre-incubated in the presence or absence of the tested retinoids at different concentrations or specific inhibitors of Pgp (CsA or tariquidar) and ABCG2 (Ko143) for 20 min at 37 °C and then stained with mitoxantrone for 40 min, calcein-AM for 20 min or Hoechst 33342 for 30 min at different concentrations at 37 °C. Cells were washed and were stored on ice until flow cytometric measurement. Transport activity factors were calculated from fluorescence intensity values of inhibitor treated and untreated samples.

3.3. Western blot analysis

Sf9 or NIH 3T3 cell membrane samples (2.5 or 5 μg/slot) were subjected to SDS-polyacrylamide gel electrophoresis on 8% polyacrylamide gel and were electroblotted to 0.45 μm pore size nitrocellulose membrane. Pgp expression was detected using monoclonal anti-Pgp mAb while ABCG2 was detected by a monoclonal anti-ABCG2 mAb at 1:5,000 dilution. As a secondary antibody a goat anti-mouse HRP-conjugated IgG mAb was applied at 1:5,000 dilution.

3.4. Membrane preparations

For ATPase activity measurements we used membrane samples derived from Sf9 (*Spodoptera frugiperda*) insect ovarian cells expressing human Pgp or ABCG2 and membrane preparations from NIH 3T3 cells expressing wild-type- or different mutant Pgps. Sf9 or NIH 3T3 cells were harvested and cell membranes were isolated by differential centrifugation. Membrane pellets were re-suspended in TMEP solution. The ABCG2 expressing Sf9 membrane samples were loaded with cholesterol by cholesterol-methyl-beta-cyclodextrin complex (Chol-RAMEB).

3.5. ATPase activity measurements

The vanadate-sensitive ATPase activity of wild-type and mutant Pgps and ABCG2 was determined by a colorimetric assay. Specific ATPase activity of the transporters was calculated from the amount of released inorganic phosphate. The ATPase reaction was started with the addition of MgATP. After 25 min incubation at 37 °C, the reaction was stopped by 5% SDS, then the samples were incubated with color reagent at room temperature for 30 min. The absorbance of the samples was measured at 700 nm using a BioTek Synergy HT plate reader. Half-inhibitory doses (IC_{50}) of retinoids were determined by fitting the dose-response relationships with a four-parameter sigmoidal curve.

3.6. Cellular uptake of retinoids and transporter inhibitors

NIH 3T3 cells in gl-PBS containing 1% BSA were incubated with retinoids or transporter ligands (quercetin, cyclosporine A, Ko143) applied at different concentrations for 30 min at 37 °C. After incubation the cells were pelleted by centrifugation and supernatants were collected. The cellular uptake of the examined compounds was determined by calculating the ratio of the absorbances measured in the supernatants at their absorption maximum before and after incubation with cells. Absorbances were determined using a NanoDrop 1000 UV/VIS Spectrophotometer.

3.7. Fluorescence anisotropy measurements

NIH 3T3 cells in Hank's buffer were pre-treated with various retinoid derivatives for 10 min at 37 °C and then further incubated with DPH or TMA-DPH at room temperature, in dark for 20 min. Steady-state fluorescence anisotropy measurements were carried out at 37 °C using a Horiba Jobin Yvon Fluorolog-3 spectrofluorometer equipped with a thermostated cell holder. The fluorescence of DPH and TMA-DPH was excited at 358 nm and their emission was measured at 427 nm.

3.8. Cytotoxicity assays using Alamar Blue

Pgp-negative, wild-type or mutant Pgp expressing NIH 3T3 cells were seeded in 96-well plates. 24 hours later retinyl-acetate or vinblastine was added to the wells at different concentrations and the plates were further incubated for 72 h at 37ºC. The cell viability was determined using the Alamar Blue assay measuring the 530/590 nm fluorescence intensity of the dye in an automated micro-plate reader. The fluorescence intensities of the samples were normalized to the fluorescence of the untreated control samples and were plotted as a function of retinyl-acetate or vinblastine concentrations.

3.9. Cell permeabilization with streptolysin-O

Cell suspensions were treated with streptolysin-O (SLO) in PBS in the presence of BSA, DTT and Protease Inhibitor Cocktail at 37 °C for 30 min in PBS

(allowing permeabilization of 50–70% of cells). The reaction was stopped with 37 °C PBS and the cells were centrifuged at room temperature. Unbound toxin was removed by washing the cells 3 times with PBS and the cell pellet was re-suspended.

3.10. Measurements of the apparent ATP-binding affinity of mutant Pgps

SLO permeabilized NIH 3T3 cells expressing wild-type or mutant Pgp variants were pre-incubated with nucleotides added in a broad concentration range for 20 min in the presence or absence of vanadate (Vi) and then further incubated with A647-conjugated UIC2 monoclonal antibody for another 30 min (all treatments were carried out at 37 °C). UIC2 mAb binding to Pgp is a reversible reaction in the presence of MgATP, thus, UIC2 was applied at quazi saturating concentrations. The UIC2-A647 fluorescence intensity of the cells was measured by flow cytometry and plotted as a function of the nucleotide concentration. To determine the apparent affinity of Pgp to nucleotides (K_A) data points were fitted with the four-parameter Hill function.

3.11. UIC2-reactivity assay

Intact wild-type, E556M and quadruple mutant Pgp expressing NIH 3T3 cells were treated with CsA in gl-PBS for 30 min at 37°C. Alternatively, cells were ATP depleted by Na-azide and 2-deoxy-D-glucose treatment for 30 min at 37°C in glucose-free PBS. UIC2-A647 or 15D3-A647 mAbs were added directly without washing step to untreated, CsA-treated and ATP-depleted cells and samples were further incubated for 30 min at 37°C. Samples were washed and re-suspended in ice cold gl-PBS before flow cytometric analysis. UIC2-reactivity was calculated as a ratio of the F/P-corrected UIC2 and 15D3 signals.

3.12. Measurement of the kinetics of UIC2 dissociation

Wild-type and mutant Pgp expressing NIH 3T3 cells were harvested and washed. Subsequently, cells were permeabilized with SLO toxin for 30 min at 37 ºC and were washed three times with ice-cold PBS. Permeabilized cells were labeled with A647-conjugated UIC2-Fab for 20 min at 37 ºC and after washing twice with excess volume of PBS they were kept on ice. Before measurement, cells were mixed with pre-warmed 37 ºC PBS containing different concentrations of MgATP and/or verapamil. Fluorescence intensities were measured continuously at 37ºC for 15 min with a Becton Dickinson FACSAria III Cell Sorter.

3.13. Flow cytometry

Calcein-AM, mitoxantrone and Hoechst 33342 accumulation measurements were carried out on a Becton Dickinson FACSAria III Cell Sorter. Calcein was excited by the 488 nm line of a solid-state laser and the emitted light was detected using a 502 nm dichroic mirror and a 530/30 nm band-pass filter. Mitoxantrone was excited with a 631 nm solid state laser end emitted fluorescence intensity was

detected using a 685/15 band-pass filter. Hoechst 33342 was excited with a 365 nm UV laser and the blue intensity of the dye was recorded using a 445/40 band pass filter. PI was excited by the 562 nm line of a solid-state laser and the emitted light was detected applying a 590 nm dichroic mirror and a 595/50 nm band-pass filter. UIC2-A647 labeling of permeabilized cells was measured by using a Becton Dickinson FACSArray flow cytometer. A 635 nm laser was used for the excitation of A647 dye and fluorescence values were detected in the red channel (661/16 nm) while the 532 nm laser was used for the excitation of PI (detected at 585/42 nm). UIC2-dissociation was measured with the Becton Dickinson FACSAria III Cell Sorter. Alexa647 conjugated UIC2 was excited with a 631 nm solid state laser and fluorescence intensities were detected using a 660/20 band pass filter while PI was excited with a 562 laser and emitted light was detected applying a 590 nm dichroic mirror and a 595/50 nm band-pass filter. For the continuous monitoring of UIC2 dissociation the sample injection chamber was kept at 37 °C.

In all flow-cytometric measurements cell debris was excluded from the analysis on the basis of FSC and SSC signals. Cytofluorimetric data were analyzed by using FCS Express 4 Research Edition.

3.14. Statistical analysis

Data were analyzed using SigmaStat and are presented as means ± SD. Comparison of two groups was carried out by unpaired t-test, while in the case of three or more groups statistical significance was assessed using analysis of variance (ANOVA). For post hoc pair-wise comparison of the treatment groups with identical variances the Holm-Sidak multiple comparison test was applied, while groups with unequal variances were analyzed by Dunnett T3 post hoc test. Differences were considered significant at $P < 0.05$. Dose-response curves were fitted using SigmaPlot 12.0.

4. Results

I. Interaction of retinoids with human Pgp and ABCG2

4.1. Retinoid derivatives inhibit Pgp- and ABCG2 mediated substrate transport

We have studied the effects of retinoid derivatives on the drug transport activity of Pgp and ABCG2 using the NIH 3T3/NIH 3T3 MDR1 and MDCK/MDCK ABCG2 cell line pairs. Resistant cell lines expressed wild-type human Pgp and ABCG2 at high levels as it was demonstrated by immunofluorescent staining.

Calcein-AM and mitoxantrone accumulation assays were used to study the transport function of Pgp and ABCG2, respectively. Our experimental data showed that from the applied retinoids retinol, 13-*cis*-retinoic acid and retinyl-acetate increased the cellular accumulation of fluorescent transporter substrates in a concentration dependent manner in both transporter-expressing cell lines. Applied at super-physiological concentrations (25-100 μM), the effect of retinoid derivatives retinol, 13-*cis*-retinoic acid and retinyl-acetate was comparable to that of the specific transporter inhibitors CsA or Ko143. Inhibitory effect is not attributed to the cytotoxic effect of retinoid derivatives as treatment of transporter non-expressing NIH 3T3 and MDCK cells with 50 μM of different retinoid derivatives did not have any significant impact on the viability of the cells.

4.2. Retinoids inhibit Pgp- and ABCG2 mediated basal- and substrate-stimulated ATPase activity

In further experiments we applied membrane preparations derived from Sf9 insect cells expressing Pgp and ABCG2 at high levels. We measured the effect of retinoids on the basal- and the substrate-stimulated ATPase activities of the transporters in a sensitive ATPase assay.

Retinyl-acetate slightly stimulated the basal ATPase activity of Pgp indicating that it is probably transported by Pgp. Although, in Alamar Blue based cytotoxicity assays we were unable to show a significant difference in the cytotoxic effect of retinyl-acetate between sensitive and resistant NIH 3T3 cells.

In good agreement with the findings of the calcein-AM and mitoxantrone accumulation studies retinyl-acetate had inhibitory effect on the substrate-stimulated ATPase activity of both Pgp and ABCG2 at higher concentrations. Additionally, retinol and 13-*cis*-retinoic acid had a pronounced inhibitory effect on both the basal- and the substrate-stimulated ATPase activity of the transporters in a concentration-dependent manner.

Interestingly, we found stereo-specific differences between the effects of certain retinoids (e.g., 13-*cis*-retinoic acid 9-*cis*-retinoic acid). We supposed that a

possible explanation of this difference might be the differential membrane partitioning of the stereoisomers. We estimated their cellular accumulation by measuring the absorbance of the retinoids in the supernatant before and after incubation with Pgp negative NIH 3T3 cells. In our experiments we observed a significant correlation between the octanol/water partition coefficient ($LogP_{ow}$) values and the intracellular/membrane accumulation of particular retinoids but did not see stereospecific differences in their cellular accumulation.

4.3. Inhibitory retinoids decrease membrane fluidity in the acyl-chain region of the membrane

In view of the intimate association of Pgp and ABCG2 with their membrane environment we examined the effects of retinoids on the fluidity and packing order of the membrane. In order to delineate their effects on the membrane fluidity we carried out DPH and TMA-DPH fluorescence anisotropy measurements. DPH accumulates in the inner hydrophobic region of the membrane while its cationic derivative TMA-DPH accumulates at the polar head group region of the lipid bilayer therefore allowing us to monitor changes in the membrane fluidity at different depths of the membrane.

Pgp and ABCG2 expressing and non-expressing NIH 3T3 and MDCK cells were treated with DPH and TMA-DPH. The fluorescence anisotropy value of DPH was in the range of 0.14 and 0.18 under steady-state conditions indicative of high structural order in the core of the membrane, while the fluorescence anisotropy of TMA-DPH varied between 0.28 and 0.30. The measured anisotropy values were found to be similar in sensitive and resistant cell lines in agreement with previous data. Interestingly, retinol, 13-*cis*-retinoic acid and retinyl-acetate, the retinoids that proved capable of transporter inhibition in ATPase and drug transport assays, increased DPH fluorescence anisotropy values significantly in both NIH 3T3 and MDCK cells while treatment of cells with other examined retinoids did not alter DPH anisotropies significantly. Interestingly, retinyl-acetate had significantly lower effect on the DPH fluorescence anisotropy of Pgp expressing NIH 3T3 MDR1 cells compared to sensitive cells supposedly because of its Pgp-mediated transport.

4.4. Retinoids hamper substrate stimulation of Pgp and ABCG2 ATPase activity through mixed-type inhibition

In order to shed light on the action of retinoids at the molecular level we analyzed how the kinetic parameters of the substrate stimulation of ATPase activity are influenced by retinol and 13-*cis*-retinoic acid. ATPase activity of Pgp and ABCG2 was stimulated by increasing concentrations of verapamil and quercetin in the presence of different concentrations of retinol and 13-*cis*-retinoic acid. Dose-response curves showed that retinol increased the K_M and decreased the v_{max} values

of transporter stimulation in case of both transporters supporting a mixed-type transporter inhibition.

Similarly, 13-*cis*-retinoic acid treatment of ABCG2-expressing membrane preparations resulted in a mixed-type inhibition of quercetin-stimulation. However, in case of Pgp we showed that v_{max} of verapamil stimulation significantly decreased in the presence of 13-*cis*-retinoic acid, but we did not observe significant increase in K_M indicating that 13-*cis*-retinoic acid inhibits Pgp-stimulation non-competitively.

II. Studying the catalytic cycle of Pgp using ATP-binding site mutants

4.5. Quadruple mutant Pgp with a non-canonical NBS1 regain conformational flexibility

In several ABC proteins including the main bile acid transporter ABCB11 the glutamate residue in the Walker B sequence, often termed as "catalytic glutamate" is replaced with other "non-canonical" amino acids. Pgp has two canonical NBSs, and mutation of the "catalytic glutamate" (E556) in NBS1 makes it transport-incompetent. The NBD-NBD interfaces of Pgp and ABCB11, including the "catalytic glutamate" differ in four amino acid residues, all within NBS1. To study the role of the four non-canonical amino acids in the degenerate NBS1 of ABCB11 we have introduced the corresponding mutations into the NBS1 of human Pgp. We engineered a Pgp variant containing the E556M mutation of the "catalytic glutamate" and we generated a "quadruple mutant" that also contains the three additional diverging amino acid residues (S474E, G1178R and Q1180E).

To evaluate the conformational flexibility of single and quadruple mutant Pgps we performed UIC2 staining experiments in the presence of a Pgp inhibitor CsA or following ATP depletion of cells. Wild-type Pgps showed low UIC2-reactivity in untreated cells as only a minor fraction (app. 20%) of cell surface Pgps were labeled with the conformation-sensitive UIC2 antibody, however, depletion of ATP resulted in UIC2 labeling of all cell surface Pgp molecules similarly to CsA treatment. Single E556M mutant Pgps showed a remarkably different behavior: in untreated cells only app. 5% of cell surface Pgps were in a UIC2-reactive conformation and CsA treatment could only moderately increase their UIC2-reactivity. Interestingly, quadruple mutant Pgps showed very similar UIC2-reactivity pattern to wild-type as in untreated cells app. 20% of cell surface Pgps were in the UIC2-reactive conformation and practically the whole cell surface Pgp pool switched to the inward-facing UIC2-reactive conformation in response to CsA treatment. These results strongly suggest that quadruple mutants can avoid the conformational lock that is observed in single E556M mutant Pgps.

Using SLO-permeabilized cells we measured the apparent ATP-binding affinity (K_A) of wild-type and mutant Pgps in the presence and absence of Vi. Wild-type and quadruple mutant Pgps exhibited similar apparent ATP-binding affinities and Vi treatment resulted in 2-3 orders of magnitude increase in their affinity suggesting that quadruple mutant Pgps are capable to hydrolyze ATP. As opposed to wild-type and quadruple mutants, E556M variant exhibited similarly high apparent affinities for ATP in the absence and presence of Vi.

4.6. Quadruple mutant Pgps regain their transport and ATPase activity

In the following experiments, we aimed to examine the functional activity of mutant Pgps applying ATPase activity and drug accumulation measurements. Membranes isolated from NIH 3T3 cells expressing wild-type Pgp showed the expected basal ATPase activity, which was stimulated about 4-fold by the addition of verapamil. Strikingly, the quadruple mutant showed a low steady state ATPase activity that could be stimulated by verapamil. Although the basal ATPase activity was significantly reduced, the degree of stimulation by verapamil was almost identical to wild-type. In contrast, the "catalytic glutamate" mutant (E556M) showed no significant basal or verapamil-stimulated ATPase activity.

To answer the question whether quadruple mutant Pgps are able to couple their ATPase activity to uphill drug transport we performed calcein and Hoechst 33342 accumulation experiments using intact cells. As expected, the transport activity factor (TAF) value was close to 0 in cells devoid of Pgp, while expression of wild-type Pgp effectively prevented the intracellular accumulation of Hoechst 33342 and calcein. The E556M mutant did not show any transport activity in agreement with the loss of steady state ATPase activity and the reduced conformational flexibility. In sharp contrast, the quadruple mutant was able to limit Hoechst 33342 and calcein accumulation, though not as efficiently as wild-type Pgp. These data clearly demonstrate that despite the absence of the NBS1 "catalytic glutamate", the quadruple mutant regained its ability to efflux substrates.

4.7. Vanadate increases apparent ATP-binding affinity of single A-loop and Walker B mutants

Previous studies performed on purified proteins or heterologous expression systems demonstrated that point mutations introduced into key residues of the A-loop (Y401A and/or Y1044A) or Walker B sequences (D555N and/or D1200N) inactivate Pgp either present in one or both NBSs. To study the conformational activity of these Pgp variants we carried out UIC2-labeling experiments using intact mammalian cells.

In good accordance with literature data, wild-type Pgps preferred the UIC2-nonreactive conformation(s) and switched to the UIC2-reactive inward-facing conformation in the presence of CsA or in response to ATP depletion. In contrast to

it, the A-loop tyrosine and Walker B aspartate mutants exhibited high UIC2-reactivity, which was not increased further by ATP depletion or CsA treatment. These results support the idea that the above mutations favor the ATP-free UIC2-reactive conformation of Pgp in live cells.

To further study the effects of A-loop and Walker B mutations on ATP binding to Pgp we determined the K_A of the mutant Pgp variants measuring the ATP-dependence of UIC2-reactivity in the presence or absence of Vi. We proved that single A-loop mutant (Y401A or Y1044A) and single Walker B mutant (D555N or D1200N) Pgps exhibited significantly higher K_A values compared to wild-type in accordance with literature data. Interestingly, Vi treatment decreased the K_A values of single A-loop and Walker B mutants suggesting that they are capable of ATP hydrolysis at least at their intact NBS. On the other hand, we did not observe any change in response to Vi treatment in case of double mutants (Y401A/Y1044A and D555N/D1200N, respectively) suggesting that they are really unable to hydrolyze ATP.

4.8. Single A-loop and Walker B mutant Pgps can hydrolyze ATP and transport substrates

As a next step, ATPase activity measurements were carried out using membrane preparations. We measured the effects of the mutations on the basal and the substrate-stimulated ATPase activity of the transporters. NIH 3T3 cell membrane preparations derived from wild-type Pgp expressing cells exhibited weak basal ATPase activity in the absence of verapamil, while a 3-fold stimulation was observed in the presence of verapamil. Y401A and Y1044A single A-loop mutant Pgps and D555N, D1200N single Walker B mutant Pgps demonstrated weak basal ATPase activity in the absence of a Pgp substrate verapamil. Addition of 40 μM verapamil brought about a two-fold stimulation in the ATPase activity of Y401A mutant.

Furthermore, we performed calcein-AM accumulation studies and Alamar Blue-based vinblastine cytotoxicity assays on NIH 3T3 cells expressing different single and double Walker B and A-loop mutant Pgps. By measuring intracellular calcein accumulation and vinblastine-induced cytotoxicity we revealed that Y401A or Y1044A single A-loop mutant Pgps showed significant transport activity.

4.9. Single A-loop and Walker B mutant Pgps are able to pass on several conformational cycles

According to our previous results UIC2 binding to Pgp is reversible and the dissociation of the antibody can be stimulated by MgATP and Pgp substrates. In the following experiments we analyzed the kinetics of UIC2 dissociation using UIC2-Fab fragments and SLO toxin permeabilized NIH 3T3 cells expressing wild-type and mutant Pgp variants. In the absence of MgATP we did not observe

significant UIC2 dissociation. However, in response to MgATP treatment we measured significant UIC2-Fab dissociation in case of single A-loop mutants and Walker B mutants similarly to wild-type Pgps. In accordance with the substrate stimulation of Pgp activity verapamil treatment significantly increased the rate of UIC2-Fab dissociation in case of the wild-type and the transport competent mutant Pgp variants. In case of the quadruple mutant, we also observed elevated UIC2-Fab dissociation in the presence of verapamil. Of note, the ATPase- and transport-function deficient double Walker B and A-loop mutants did not show significant UIC2-Fab dissociation. Although, Pgp can bind ATP even in the absence of Mg^{2+} ions and can switch into a UIC2-dim state, ATP-hydrolysis is completely inhibited, and the catalytic cycle is blocked at this condition. Interestingly, in the absence of Mg^{2+} addition of ATP did not induce significant UIC2-Fab dissociation in wild-type Pgp expressing cells suggesting that Pgps should hydrolyze ATP and pass on many conformational cycles to lose the bound UIC2-Fab.

5. Discussion

5.1. Interaction of retinoids with human Pgp and ABCG2

We have studied the possible interactions between Pgp and ABCG2 and several physiologically relevant retinoid derivatives including all-*trans*-retinoic acid, 13-*cis*-retinoic acid, retinol and different retinoid metabolites.

We have proved that retinol, 13-*cis*-retinoic acid and retinyl-acetate are effective inhibitors of Pgp- and ABCG2-mediated substrate transport. The highly lipophilic character of the examined retinoid derivatives as well as their possible bioconversion by cellular enzymes renders the assessing of binding and transport of retinoids by membrane transporters difficult in live cells. Therefore, we carried out ATPase activity measurements using Pgp and ABCG2 expressing Sf9 cell membrane preparations. ATPase activity measurements confirmed the inhibitory effect of retinol, 13-*cis*-retinoic acid and retinyl-acetate. Interestingly, these experiments also demonstrated that retinyl-acetate had a weak stimulatory effect on the basal ATPase activity of Pgp at app. 10 μM concentration indicating that retinyl-acetate might be a substrate of Pgp. Our DPH anisotropy measurements also indicated altered membrane distribution of retinyl-acetate in Pgp-positive cells. Nevertheless, we observed identical cytotoxicity profile of retinyl-acetate in Pgp-positive and negative cells suggesting that even if it is really transported by Pgp its active efflux is negligible compared to its passive membrane permeation.

Interestingly enough, 13-*cis*-retinoic acid had a pronounced inhibitory effect on the ATPase and transport activity of both Pgp and ABCG2, while its stereoisomers ATRA (all-*trans*-retinoic acid) and 9-*cis*-retinoic acid did not modify the functional activity of the transporters. Considering the wide substrate specificity of Pgp and ABCG2 this stereospecific interaction is striking and might provide previously unknown details of drug recognition and binding by Pgp and ABCG2 and probably by other multidrug transporters. In fact, it has been shown in several publications that stereo-selective differences can be observed in the interaction of certain modulators with Pgp. High-resolution X-ray crystal structures of mouse Pgp demonstrated that stereoisomers of a cyclic oligopeptide QZ59-RRR and QZ59-SSS interact with Pgp differently: QZ59-RRR binds to Pgp at only one site located at the center of the drug-binding pocket, while QZ59-SSS binds at two distinct sites. Similarly, *cis*- and *trans*-stereoisomers of flupentixol have been shown to have stereochemical differences in their Pgp modulatory effects. Although both flupentixol stereoisomers inhibit Pgp-mediated drug transport and reverse drug resistance, they have opposite effects on the rate of ATP hydrolysis and photo-affinity labeling of Pgp with the substrate analogue [125I]IAAP. Taken together, previous studies observed stereospecific differences between ligands in their mode

of interaction with Pgp. In contrast, we observed that the recognition of certain retinoids by Pgp and ABCG2 is stereo-selective.

Stereo-selective recognition of the ligands may occur at the level of the drug-binding site(s) or allosteric site(s) of the transporter or alternatively, at the level of the plasma membrane from where substrates and modulators can reach the drug binding pocket. We observed high cellular accumulation of the tested retinoids in NIH 3T3 cells that exhibited strong correlation with their $LogP_{ow}$ values. Since the retinoid stereoisomers showed similar cellular accumulation and $LogP_{ow}$ values suggesting similar extent of membrane partitioning, it seems likely that distinct intramembrane localization of the stereoisomers can explain their different behavior. In agreement with this hypothesis *cis* and *tran*s isomers of zeaxanthin have been observed to have different orientations in dimyristoyl phosphatidylcholine bilayer membranes. Interestingly, these stereoisomers modified the biophysical properties of the membrane including hydrophobicity, membrane order and membrane fluidity at different depths of the membrane. In agreement with this finding, we observed that retinol, 13-*cis*-retinoic acid and retinyl-acetate selectively decreased the membrane fluidity and increased the packing density of the inner hydrophobic region of the membrane as it was indicated by DPH anisotropy measurements. On the other hand, non-inhibitory retinoids e.g., ATRA or 9-*cis*-retinoic acid did not alter DPH anisotropy. These results imply that the trans-membrane orientation of the retinoic acid stereoisomers is different. Differences in the net length of their isoprene tail depending on the presence and position of the kink introduced by the *cis* double bond might explain their different orientation.

To analyze the interactions of retinol and 13-*cis*-retinoic acid with the transporters in more detail we studied their effects of on the kinetic parameters (K_M and v_{max}) of the substrate-stimulated ATPase activity of Pgp and ABCG2. For stimulation of ATPase activity, we applied verapamil and quercetin that are well-known transported substrates of Pgp and ABCG2, respectively. Therefore, changes in the kinetic parameters of ATPase stimulation in response to retinoids truly represent alterations in substrate binding/transport by the transporters. The apparent increase of the K_M values of verapamil and quercetin in the presence of increasing concentrations of retinol seems to indicate direct interactions of retinol with both transporters. 13-*cis*-retinoic acid increased the K_M of quercetin stimulation in ABCG2 while leaving the K_M of verapamil stimulation unaltered in Pgp. In addition, retinol and 13-*cis*-retinoic acid decreased the v_{max} value of both transporters, that can be attributed to a non-competitive inhibition of the transporters or alternatively to a decreased effective concentration of the substrates (verapamil/quercetin) in the plasma membrane.

As it was highlighted previously retinol and its natural derivatives reach nano-molar concentrations under physiological circumstances in human tissues, however in our study half-inhibitory concentrations of retinoids were found to be between 20-70 μM. This remarkable difference makes it unlikely that retinoids inhibit Pgp and ABCG2 *in vivo* under physiological conditions. However, retinoid therapy or retinol supplementation can significantly increase the concentration of retinoids in the blood or in case of oral administration in the intestinal epithelium that may inhibit Pgp or ABCG2 expressed in physiological barriers. Inhibition of Pgp and ABCG2 caused by retinoid therapy may also affect the pharmacokinetics of other co-administered chemotherapeutic drugs. The above effects should be considered upon therapeutic application of retinoids to avoid the possibility of drug-drug interactions occurring at the level of Pgp or ABCG2.

In conclusion, the different intramembrane orientation of certain retinoid stereoisomers might be important in their selective recognition by Pgp and ABCG2.

5.2. Studying the catalytic cycle of Pgp using ATP-binding site mutants

Biochemical and biophysical experiments as well as X-ray crystallography structures and recent cryo-EM data demonstrated that Pgp undergoes a series of conformation changes in an ATP binding and hydrolysis dependent manner to transport its substrates. ATP hydrolysis by the NBDs converts the chemical energy stored in the phosphate bonds of ATP into NBD motions that are propagated to the TMDs through a conformational cross-talk. Evidence for the inter-domain cross-talk come from i) crystal structures showing correlated conformational changes in TMDs and NBDs; ii) substrate-stimulated ATPase activity, revealing that substrate-induced conformational changes in the TMDs are propagated to the NBDs; iii) biochemical experiments showing that substrate binding to the TMDs results in conformational changes of the NBDs. Our observations support that nucleotides are the main determinants of the Pgp conformation, as nucleotide binding (ATP, ADP or ADP + Vi) shapes the transporter conformation in a mutation dependent manner, while all Pgp variants were equally well-recognized by the UIC2 antibody in the absence of nucleotides. ATP is the primary nucleotide controlling Pgp conformation in well-energized cells, because its intracellular concentration is an order of magnitude higher than the ADP concentration and typically 10-fold above the K_D of ATP for Pgp.

Pgp has two canonical ATP-binding sites each being capable to bind and hydrolyze ATP to fuel the transport cycle. Opposed to Pgp, several ABC proteins including CFTR, SUR1 and ABCB11 possess only one canonical ATP-binding site, while in their NBS1 the “catalytic glutamate” necessary for efficient ATP

hydrolysis is missing. The exact role of the "catalytic glutamate" in ATP hydrolysis is still not completely understood. A direct contact between the negatively charged "catalytic glutamate" and the negatively charged phosphate moieties of ATP seems unlikely, rather it may have an indirect role in ATP hydrolysis. A nucleophilic attack on the phosphate atoms leading to bond breakage between the β and γ-phosphate of ATP may be mediated by an activated OH^- nucleophile generated with the contribution from residue E556. When we have replaced the "catalytic glutamate" in the NBS1 of human Pgp with methionine similarly to ABCB11 we have found that Pgp became conformationally trapped, transport incompetent and ATP hydrolysis deficient, confirming earlier findings.

The NBD-NBD interface of ABC transporters exhibit a remarkable level of amino acid sequence conservation demonstrating the critical role of the conserved NBD sequences in ATP-binding and hydrolysis. Sequence alignment of ABCB11 and Pgp revealed that besides the "catalytic glutamate" three other amino acid residues of the NBD-NBD interfaces differ between Pgp and ABCB11 and all of them belong to NBS1. Generating a "quadruple mutant" Pgp we have mimicked the complete non-canonical NBS1 of ABCB11 and studied it in the context of Pgp. Strikingly, in case of the "quadruple mutant" Pgp we have observed restored conformational dynamics, substrate-stimulated ATPase activity and transport function. The above data suggest that "quadruple mutant" Pgps can avoid the high-affinity ATP binding and trapping observed in the E556M "catalytic glutamate" mutant Pgp variant. Binding of ATP to the "quadruple mutant" was weaker, as hydration of the phosphate atoms increased, and the NBD separation was more pronounced as it was demonstrated in molecular dynamic simulations (data not shown). We can infer from these data that the ABCB11-like NBS1 of the "quadruple mutant" allows for ATP binding, but it prevents occlusion through altered interactions, thereby avoiding strong binding of ATP.

In our further experiments we studied single and double Walker B aspartate and A-loop tyrosine mutant Pgp variants and portrayed their conformational dynamics, transport and ATPase activity.

It has been proposed in several studies that mutation of the Walker B aspartate or the A-loop tyrosine sequences lead to the inactivation of the transporter either the mutation is present in one or in both NBSs. Our results proved that double mutant Pgps (Y401A/Y1044A or D555N/D1200N) did not show any transport and ATPase activity and they are locked in a UIC2-reactive conformation either in the presence or absence of MgATP and substrates.

Interestingly, single A-loop tyrosine mutant Pgp variants showed significant verapamil-stimulated ATPase activity and transport activity, while single Walker B aspartate mutants did not show any measurable transport or ATPase activity,

probably because of their very weak ATP-binding affinity. Further studying the conformational activity of single Walker B aspartate mutants, we have found that they are not conformationally locked, they can switch into a UIC2-dim outward-facing conformation in an MgATP concentration dependent manner, although at much higher ATP concentrations compared to wild-type. In addition, Vi treatment increased their K_A about 5 to 10-fold supporting that they can hydrolyze ATP at least at their intact NBD. Similar to wild-type Pgp and the transport competent mutant variants, they also showed ATP hydrolysis dependent UIC2 dissociation in permeabilized NIH 3T3 cells further supporting that they can pass on several conformational cycles, albeit at a low turnover rate, not sufficient for measurable drug transport.

ABC transporters are sensitive to their membrane microenvironment and changes in membrane properties can have a significant effect on their functional activity. In previous studies reporting the inactivating effect of single Walker B and A-loop mutations, heterologous expression systems (e.g., Sf9 or *Saccharomyces cerevisiae* cells) or purified and reconstituted proteins were used for the functional characterization. In contrast we have targeted the different Pgp variants in their natural plasma membrane environment in mammalian cells. Insect- or lower eukaryotic organism cell membranes contain significantly lower amounts of cholesterol compared to mammalian cell membranes. ABC transporters are enormously sensitive to the membrane cholesterol content and therefore inactivation of A-loop and Walker B mutants in heterologous expression systems might be due to lower cholesterol levels in these membrane environments.

Our results regarding the functional activity of "quadruple mutant" Pgp challenge models assuming strictly alternating catalysis, proposing the continuous switching of ATP hydrolysis between NBS1 and NBS2 of Pgp. Assuming strict alternation between NBS1 and NBS2, in Pgp molecules with a single intact NBS (e.g., the "quadruple mutant" or Y401A A-loop mutant) every second ATP-hydrolysis event would occur at the inactive, mutant NBS and would lead to the inhibition of the catalytic cycle. Our data also argue against a model which predicts that a second ATP hydrolysis event in the second NBS is needed to reset Pgp to start a new transport cycle, because in the "quadruple mutant" only NBS2 is catalytically active at a high turnover. Taken together, the functional activity of mutant Pgp variants with one intact catalytic center including A-loop mutants (Y401A or Y1044A), Walker A mutants (K433M or K1076M) and "quadruple mutant" supports random recruitment of the two catalytic centers for ATP hydrolysis in Pgp.

6. Summary

In the first part of our work, we studied the effects of various retinoids on the transport- and ATPase activity of Pgp and ABCG2. We have made the following statements:

1. Certain retinoids including retinol, 13-*cis*-retinoic acid and retinyl-acetate inhibit the ATPase and the transport activity of Pgp and ABCG2 in a concentration-dependent manner.

2. Retinoids that inhibit Pgp preferentially accumulate within the hydrophobic core of the membrane and decrease the membrane fluidity based on our DPH and TMA-DPH fluorescence anisotropy measurements.

3. Intramembrane distribution might be an important factor in the recognition of retinoids by ABC transporters.

In the second part of our work, we investigated the functional cooperation between the nucleotide-binding sites of Pgp studying the functional and conformational activity of Pgp variants carrying mutations in one or both catalytic sites. We came to the following conclusions:

1. Simultaneous introduction of three other amino acid to the catalytically inactive E556M "catalytic glutamate" variant, resulting in an ABCB11-like NBS1, leads to rescue of conformational response and ATPase and transport function of Pgp.

2. Simultaneous mutation of the A-loop tyrosine in both NBSs of Pgp leads to a catalytically dead protein showing no conformational and functional activity, while single A-loop mutants are functionally active.

3. Simultaneous mutation of the Walker B aspartate in both NBSs of Pgp results in a completely dead protein, while single mutants can possess ATP binding and hydrolysis dependent conformational cycles at low turnover rate, not sufficient for measurable drug transport.

4. Catalytic activity of single A-loop mutant Pgps and Pgp molecules harboring an ABCB11-like degenerate NBS1 suggests that a single intact ATP-binding site might be sufficient for maintaining steady state ATPase activity and uphill substrate transport in Pgp, supporting the random recruitment of the two catalytic centers for ATP hydrolysis in Pgp.

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List of publications related to the dissertation

1. Goda, K., Dönmez-Cakil, Y., **Tarapcsák, S.**, Szalóki, G., Szöllősi, D., Parveen, Z., Türk, D., Szakács, G., Chiba, P., Stockner, T.: Human ABCB1 with an ABCB11-like degenerate nucleotide binding site maintains transport activity by avoiding nucleotide occlusion.
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