Detailed Final Project Report

P-glycoprotein (Pgp, ABCB1), multidrug resistance associated protein 1 (MRP1, ABCC1) and breast cancer resistance protein (BCRP, ABCG2) are active efflux pumps that are able to extrude a large variety of lipophilic chemotherapeutic drugs from the cells, causing the phenomenon of multidrug resistance. The intimate association of the transporters with the plasma membrane implies that it may have strong modulatory effects on their catalytic function.

Rafts are isolated from bulk cellular membrane by density gradient centrifugation in the presence of the non-ionic detergents e.g. Triton X-100 (TX-100) or Brij 98. It has been proposed that lipid rafts are composed of a highly ordered, TX-100-insoluable core region, rich in sphingolipids and cholesterol, which is surrounded by a less structured shell (resistant to milder detergents, e.g. Brij 98) and then pass into a bulk liquid disordered membrane phase. Pgp and ABCG2 molecules reside in partially different membrane territories. Most of the cell surface ABCG2 molecules (>80 %) reside in the Brij 98 and TX-100 resistant core region of the rafts. However, only 10-40 % of Pgp molecules are found in the TX-100 resistant rafts and/or caveolae in various cell lines including NIH 3T3 MDR1, A431 and MDCK cells, while the rest of Pgps are localized in the TX-100 soluble, but Brij 98 resistant shell region of rafts ***(Goda et al. 2009, Current Cancer Drug Target)***.

Topological heterogeneity of cell surface Pgps determines their functional state

P-glycoprotein (Pgp), is present in two subpopulations in the plasma membrane of many cell types, distinguished by a conformation sensitive antibody: Pool I is recognized by the UIC2 mAb even in the absence of substrates/modulators, while pool II Pgps bind this mAb only in their presence. However, when we permeabilize the cells by *Staphylococcus aureus* alpha toxin and wash out their ATP content, or deplete the ATP of intact cells by Na-azide and deoxy-glucose, all the cell surface Pgps become UIC2 mAb reactive. At the same time the catalytically inactive double Walker A mutant Pgps are recognized by the UIC2 mAb in the absence of substrates/modulators and without ATP depletion. These data suggest that pool I Pgps are in an ATP-free state. In addition,

we measured strong co-localization between the Pgp substrate vinblastine-BPY and UIC2-bound pool I Pgps in the membrane, suggesting that the pool I Pgps are in a drug binding state. The raft associated Pgps have about 7 fold lower affinities to ATP compared to the non-raft Pgps. The two Pgp pools can also be distinguished in terms of their membrane microdomain localization and intracellular molecular neighbours, since pool I Pgps are highly associated with the TX-100 resistant rafts and the cytoskeleton. Endocytosis of Pgps proceeds mainly from the dominantly raft associated pool I and involves caveolae and the actin microfilament system. Depletion of ATP switches pool II Pgps from the UIC2 *non*-reactive conformation to the UIC2 reactive conformation, but it does not affect their raft and cytoskeleton association. While restitution of normal intracellular ATP level switches pool II Pgps back to the UIC2 non-reactive state. Thus we propose that the raft associated pool I Pgps have too high K_M for ATP to become saturated at physiological ATP concentrations and they preferentially engaged in trafficking processes, while pool II Pgps are saturated with ATP and involved in substrate transport. (The manuscript will be submitted soon.)

Molecular interaction partners of the transporters

We aimed to identify interaction partners of the examined transporters in co-immunoprecipitation experiments. The co-precipitated proteins were separated by SDS PAGE and the excised protein bands were analysed by MALDI. When we used whole cell lysates to immunoprecipitate Pgp the majority of the co-precipitated proteins were cytoskeleton associated and probably involved in the caveolae-mediated endocytosis of Pgp in addition to some membrane proteins (e.g. ERGIC 53, stim1, Aspartyl/asparaginyl beta-hydroxylase, delta-1-pyrroline-5-carboxylate synthetase, synaptotagmin-1) and a heat sock protein (hsp71).

In further experiments Pgp and ABCG2 proteins were immunoprecipitated in RIPA buffer from a plasma-membrane rich fraction of cells. In these experiments hsp71 and myosin-9 were co-immunoprecipitated with both Pgp and ABCG2. Since these proteins may potentially affect the expression or function of the examined transporters we examine this issue by silencing hsp71 and myosin-9.

Interactions of retinoids with P-glycoprotein, ABCG2 and MRP1

We examined the effect of several retinoids (e.g. retinol, all-trans-retinoic acid, all-trans-4-oxo-retinoic acid, 9-cis-retinoic acid, 9-cis-4-oxo-retinoic acid, 13-cis-retinoic acid and 13-cis-4-oxo retinoic acid and retinyl-acetate) on the transport and ATPase activity of Pgp, ABCG2 and MRP1. For the measurement of ATPase activity we used isolated cell membranes derived from MDCK (dog kidney epithelia) and Sf9 (Spodoptera frugiperda) cells expressing human Pgp, ABCG2 or MRP1 at high levels. We found that only 9-cis-4-oxo-retinoic acid has a weak stimulatory effect on the basal ATPase activity of ABCG2 at high concentrations. Retinol, 13-cis-retinoic acid, 13-cis-4-oxo-retinoic acid and retinyl-acetate inhibit the basal- and substrate-stimulated ATPase activity of Pgp, ABCG2 and MRP1 in a micromolar concentration range. Interestingly, 13-cis-retinoic acid inhibits the transporters, while 9-cis-retinoic acid and all-trans-retinoic acid do not have any effect, besides that they are stereo-isomers, suggesting that stereo-chemical differences also affect the interactions between the retinoids and the transporters.

Since the physiological tissue and blood concentration of the different retinoids is in the nanomolar range (1-20 nM), it is unlikely that they interfere with the transport activity of the examined ABC transporters. However, retinoid therapy or retinol supplementation may cause high enough local retinol concentrations in the intestinal lumen that inhibits Pgp and ABCG2 expressed in the intestinal epithelium and consequently may result in intestinal absorption of their toxic substrates. (We have prepared a manuscript from the above results and submit it to the Eur. J Pharmacology)

Elucidation of the catalytic mechanism of P-glycoprotein (Pgp):

According to three-dimensional models based on crystal structures of ABC proteins the TMDs are always in outward facing conformation in the nucleotide-bound form of the protein, as opposed to the inward facing nucleotide-free form, where the bundle of trans-membrane helices is closed on the extracellular side. Despite accumulating structural and functional data, it is still unknown how ATP binding and hydrolysis are connected to the conformational changes that allow trans-membrane

transport. The discontinuous extracellular epitope of the UIC2 mAb is sensitive to the switch between the above conformational states, as reflected by antibody binding. It was previously shown by Druley et al. (2001) that nucleotide binding to the ABC domains decreases the UIC2 reactivity of Pgp in a concentration dependent manner in cells permeabilized by *Staphylococcus aureus* alpha toxin. Following the same strategy, we have studied how substrates, hydrolysable and non-hydrolysable nucleotides and phosphate analogues affect the formation of the above catalytic intermediates discriminated on the basis of UIC2 reactivity. To elucidate partial catalytic reactions, we studied Pgp variants carrying mutations in the conserved Walker A region (K433M and K1076M) of either the N-terminal or C-terminal ABC domains or both. Although mutation of these key residues have been shown to abolish ATPase and transport activity, we found that single mutants possessed a residual drug efflux activity (the double mutant variant was indeed inactive).

We have found that nucleotide binding itself is sufficient to switch Pgp from the inward facing (UIC2 binding) to the outward facing (UIC2-nonbinding) TMD conformation in both wild-type and single Walker A mutant Pgp variants. Similarly to wild-type Pgp, single Walker A mutants can also be trapped in the post-hydrolysis state in the presence of ATP and vanadate (V_i) or BeF_x, suggesting that these variants are capable of ATP hydrolysis. Formation of the BeF_x-trapped complex is facilitated by substrates both in the wild-type and the single Walker A mutant Pgp variants. Interestingly, cells expressing single or double Walker A mutant Pgp variants accumulate the Pgp substrate vinblastine-bodipy in their plasma membranes. Based on confocal microscopic images vinblastine-bodipy staining strongly co-localized with Pgp molecules and could be prevented by CsA or V_i treatment. The above data support a model in which Pgp molecules seesaw between the nucleotide-free inward facing conformation characterized by high drug affinity and the nucleotide-bound outward facing conformation(s) with low drug affinity. Binding of ATP is sufficient to switch Pgps from the inward facing to the outward facing conformation. Pgps trapped in the post-hydrolysis state by phosphate analogues e.g. V_i are still in the low drug affinity conformation, while release of nucleotides resets them to the high drug affinity conformation.

(Based on these data we presented posters and lectures and now we are preparing a manuscript).

Pgp inhibition by UIC2 antibody can be followed *in vitro* by using tumor-diagnostic radiotracers, ^{99m}Tc-MIBI and ^{18}FDG

Using the UIC2 monoclonal antibody we made some interesting observations which were not included in the project plans. These data may promote the *in vivo* application of the UIC2 mAb for reversal of Pgp-mediated drug resistance after humanization of the antibody.

The UIC2 monoclonal antibody recognizes human Pgp and inhibits its drug transport activity. However, this inhibition is partial, since the majority of Pgps are not accessible to this antibody and they become UIC2 reactive only in the presence of certain substrates or modulators (e.g. cyclosporine A (CsA)). We have shown earlier that the combined application of a class of Pgp modulators (e.g. cyclosporine A and SDZ PSC 833) used at low concentrations and UIC2 antibody specifically and effectively blocks the transport activity of Pgp *in vitro* (Goda et al. 2007). In this project we studied the UIC2 antibody mediated Pgp inhibition in more detail measuring the accumulation of tumor diagnostic radiotracers, 2-[^{18}F]fluoro-2-deoxy-D-glucose (^{18}FDG) and [^{99m}Tc]hexakis-2-methoxybutyl isonitrile (^{99m}Tc-MIBI), into Pgp$^+$ (A2780AD) and Pgp$^-$ (A2780) human ovarian carcinoma cells.

Co-incubation of cells with UIC2 and cyclosporine A (CSA, 2 μM) increased the binding of UIC2 more than 3 fold and reverted the rhodamine 123 (R123), daunorubicin (DNR) and ^{99m}Tc-MIBI accumulation of the Pgp$^+$ 2780AD cells to approx. the same level as observed in Pgp$^-$ cells. Similarly, 50 μM paclitaxel (Pacl) increased UIC2 binding, and consequently reinstated the uptake of R123, DNR and ^{99m}Tc-MIBI into the Pgp$^+$ cells. Blocking Pgp by combined treatments with CSA+UIC2 or Pacl + UIC2 also decreased the glucose metabolic rate of the A2780AD Pgp$^+$ cells measured in ^{18}FDG accumulation experiments suggesting that the maintenance of Pgp activity requires a considerable amount of energy. Similar treatments of the A2780 Pgp$^-$ cells did not result in significant change in the R123, DNR, ^{99m}Tc-MIBI and ^{18}FDG accumulation demonstrating that the above effects are Pgp-specific. Thus, combined treatment with the UIC2 antibody and

Pgp modulators can completely block the function of Pgp in human ovarian carcinoma cells and this effect can be followed *in vitro* by using tumor-diagnostic radiotracers, ^{99m}Tc-MIBI and ^{18}FDG. ***(Krasznai ZT et al. 2010 Eur J Pharm)***.

The strong *in vivo* anti-tumor effect of the UIC2 monoclonal antibody is the combined result of Pgp inhibition and antibody dependent cell-mediated cytotoxicity

We also tested whether the combined treatment with CsA and UIC2 mAb can potentiate the anti-tumor effect of doxorubicin (DOX) in Pgp^+ tumors to achieve clinically relevant reduction in tumor size, applying SCID mice xenotransplanted with Pgp^+ and Pgp^- tumors. Tumor growth was followed by weighing the mass of the tumors in sacrificed animals and also *in vivo* on the basis of ^{18}FDG accumulation. In the latter case a small-animal Positron Emission Tomography (PET) camera was applied to visualize tumors on the basis of their increased rate of glucose metabolism. In xenotransplanted SCID mice co-treated with DOX, UIC2 and CsA the average weight of Pgp^+ tumors was only ~10 % of the untreated control and in 50 % of the animals we could not detect tumors, while DOX treatment alone did not decrease tumor weight. These data were confirmed by visualizing the tumors *in vivo* based on their increased ^{18}FDG accumulation. UIC2 + DOX treatment also decreased tumor size compared to the DOX only treated animals; this result was unexpected, since the UIC2 binding does not affect the EC_{50} value of DOX significantly. In view of the data that UIC2 binding itself does not decrease cell viability, but it triggers cell killing by peripheral blood mononuclear cells, it seems likely that its *in vivo* anti-tumor effect is mediated by antibody-dependent cell-mediated cytotoxicity (ADCC) besides Pgp inhibition. (***The manuscript was submitted to PlosOne.***)

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