THESIS FOR THE DEGREE OF DOCTOR OF PHILPSOPHY (Ph.D.)

Inhibition of P-glycoprotein transport function by modulating its conformational and topological states

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1. Introduction

P-glycoprotein (Pgp) is a transmembrane protein that transports a broad range of chemically diverse hydrophobic compounds, including chemotherapeutics, conferring multidrug resistance on cells.

Pgp belongs to the family of ATP-binding cassette (ABC) transporters, it is comprised of two homologous halves, each containing 6 transmembrane α-helices and an ATP binding site characterized by an "ABC signature" element, in addition to Walker A and B sequence motives. The α-helices form a pore-like structure allowing the passage of a wide range of hydrophobic substrates against their concentration gradient, governed by ATP fueled conformational changes of the protein.

Cell surface Pgp molecules are partially raft associated, with 10-40% (depending on the cell type and detergent used) of all cell surface Pgps localized in the detergent-insoluble low-density membrane domains. Pgp has a special relationship with its membrane environment since it recognizes its substrates within the lipid bilayer. Furthermore, Pgp may be involved in the relocation of cholesterol from the cytosolic to the exoplasmic leaflet of the plasma membrane and in the stabilization of the cholesterol-rich microdomains. The ATPase activity of Pgp measured in the presence and absence (basal ATPase activity) of Pgp substrates and modulators exhibit a strong dependence on the amount of cholesterol incorporated into native membrane vesicles or proteoliposomes. Cholesterol depletion by methyl-β-cyclodextrin decreases its ATPase activity. Acute cholesterol depletion or saturation in different cell lines inhibited transport activity, and in certain cell types cholesterol saturation enhanced active drug efflux. Thus, modulation of membrane cholesterol content can significantly alter Pgp function, but the relationship between the cholesterol content and Pgp function may be complex.

Numerous studies suggest that the principal physiological role of Pgp is to protect the organism from toxic substances, since it is expressed mostly in tissues having barrier functions, e.g. in capillary endothelial cells comprising the blood-brain barrier, placental trophoblasts and in polarized endothelial cells of several organs, like the guts, the liver, or the kidneys. Tumors derived from these tissues are intrinsically resistant to chemotherapy, while other malignancies may express Pgp or other ABC transporters during later stages of disease progression or in response to chemotherapy.

In view of the great medical importance of overcoming mdr especially in cancer chemotherapy, search for effective and specific reversal strategies continues. These tools include the co-administration of reversing agents (mdr modulators) with the disease specific drugs to overcome their efflux mediated by the pumps. Concerning Pgp, its antagonists may hinder drug extrusion competitively (e.g. CsA, FK506) or allosterically (e.g. XR9576, SR33557, cis-(Z)-flupentixol).

Several monoclonal antibodies recognizing discontinuous extracellular epitopes of Pgp have been developed. A few of them (e.g. MRK16, MRK17, MC57, HYB-241 and UIC2 in particular) appear to partially inhibit Pgp mediated drug export *in vitro* or *in vivo.* Unfortunately, the modulatory effect of the antibodies is partial and extremely variable, moreover it depends on the type of the transported substrate; thus the feasibility of antibody based mdr reversal strategies *in vivo* has been highly questionable.

The following observations and methods served as the preamble to the experiments performed in the context of my Ph.D. work.

The UIC2 conformation sensitive mAb doesn't bind to all of the cell surface Pgps, unless certain substrates/modulators are co-administered or after incubation of the cells with ATP-depleting agents (UIC2-shift). Thus, cell surface Pgps exhibit a conformational heterogeneity that calls for explanation.

Saturation of its binding sites with UIC2 in the absence of substrates/modulators hardly affect the binding of another mAb (MM12.10, MM6.15 etc.) specific to an overlapping Pgp epitope. However, in the presence of certain modulators (such as cyclosporine A, vinblastin, herein referred to as "ACT-positive" agents) completely abolishes the binding of the second mAb. Another group of modulators (e.g. verapamil, "ACT-negative" agents) has a minor effect, i.e. the binding of second mAb is not inhibited by UIC2 pre-incubation. Applying this assay (Antibody Competition Test, "*ACT*"), two distinct classes of Pgp modulators have been identified.

The raft-association of cell surface Pgps can be examined by detergents. Triton X-100 (TX-100) is a week, non-ionic detergent that is able to solubilize the membrane except for the lipid microdomains that are rich in cholesterol and sphingolipids, the rafts. The majority of cytoskeleton associated prtoteins remain immobilized in the cell membrane after TX-100 treatment. Those proteins that are not anchored to cytoskeleton directly or via rafts are eluted from the cell. Extracting cholesterol, one of the main components of rafts, by cyclodextrin

disrupts rafts and a subsequent TX-100 treatment leaves just the directly cytoskeleton anchored proteins in the cell remnants. The extent of raft and cytoskeleton association of membrane proteins can be examined in the flow cytometer by this method (Flow cytometric Detergent Resistance test, FCDR).

Open questions

The heterogeneity of cell surface Pgp molecules raise important questions. It is not clear up till now why just a fraction of cell surface Pgps is available for UIC2 labeling in the absence of substrates, while the remaining molecules are accessible only in the presence of certain drugs. An intriguing explanation would be if those Pgps that are readily recognized by the mAb take part in the transport of an endogenous substrate.

Moreover, the UIC2 accessible fraction ("pool I") and the fraction that can be labeled with other anti-Pgp mAbs (e.g. MRK16; "pool II") are spatially separated, i.e. they are not co-localized in the resolution of confocal microscopy: pool I molecules are concentrated in small patches, while pool II is dispersed in the membrane. The relation of conformational changes and topological heterogeneity is unclear.

The pump can have a bidirectional interaction with its immediate lipid environment, and this interaction may be especially crucial in view of the above topological heterogeneity.

Hereafter I list those aspects of these open questions that could, at least partially, be answered during my work, also leading closer to the resolution of the above more fundamental problems.

a. The dichotomy of substrates/modulators previously was just an impression obtained from the behaviour a limited number of drugs tested in the ACT assay. We were interested to examine weather it is a general feature of many substrates/modulators. A definitive answer to this question might help us identify new, highly efficient inhibitors.
b. We had only partial information concerning the reasons of increased UIC2 reactivity in the presence of Pgp substrates/modulators. It was not clear if it is the result of an increase in the number of binding sites or of a changing dissociation constant? This is not just a theoretical

problem, since the partial inhibition of the transporter by the antibody could be augmented if the fraction of mAb bound Pgps were increased.

c. An augmented inhibition of Pgp by UIC2 *in vitro* implies the perspective of its *in vivo* application, provided the reasons of varied inhibition are understood.

d. An UIC2-evoked near-complete *in vitro* inhibition calls for an appropriate animal model to test the feasibility of Pgp inhibition *in vivo,* for therapeutical purposes.

To answer the above questions the following aims were drafted.

2. Aims

1. Testing the dichotomy exhibited by certain substrates/modulators in the ACT test in the case of numerous other drugs.
2. Examination of the inhibitory effect of the UIC2 mAb on Pgp transport function in the presence of ACT positive and ACT negative agents *in vitro*.
3. Examination of the binding affinity and number of binding sites for the UIC2 mAb on cells expressing the pump, in the presence and absence of substrates/modulators.
4. Testing "UIC2 inhibition" in tumor xenografts *in vivo*.
5. Topological heterogeneity and the lipid domain structure of the cell membrane: the effects of cyclodextrins.
6. Inhibition of Pgp transport function by modulating the membrane's lipid domain structure: the effects of cyclodextrins.

3. Materials and methods

3.1. Cell culture

The NIH 3T3 mouse fibroblast cell line and its human mdr1-transfected counterpart (NIH 3T3 MDR1 G185) were used in most of the experiments. The NIH 3T3 MDR1 cells were cultured in the presence of 670 nM doxorubicin. The cells were grown as monolayer cultures and were trypsinized 2-3 days prior to the experiments and maintained without doxorubicin until use. In some measurements the A2780 (Pgp^-) / 2780^{AD} (Pgp^+; maintained with 2 μM doxorubicin human ovarian carcinoma cell pair was used.

3.2. Modulation of membrane cholesterol level

Cell suspensions were pre-incubated with heptakis(2,6-di-O-methyl)- β-cyklodextrin (DIMEB) or randomly-methylated-β-cyklodextrin (RAMEB), to decrease, or with their cholesterol inclusion complex (Chol-DIMEB and Chol-RAMEB, respectively) to increase the membrane cholesterol level, at 37^{o}C, for 20 min. After cyclodextrin treatment, the cells were washed twice and resuspended for subsequent measurements.

3.3. Antibody labeling of cell surface Pgps

MM6.15 and MM12.10 mAbs were obtaind from M. Cianfriglia (Istituto Superiore di Sanita, Roma). UIC2 and 15D3 mAb preparations (ATCC#: HB-11342) were isolated from hybridoma supernatant and were >97% pure.

For flow cytometric measurements the mAbs were conjugated with FITC, Cy5 and Alexa 488 dyes.

To measure UIC2 binding, the cells were pre-incubated with cyclosporine A (CSA) for 10 min, then further incubated with the UIC2 mAb (10 μg/ml) at 37 ºC for 30 min. Cell bound UIC2 molecules were labeled with FITC-, or Alexa 647 conjugated rabbit anti-mouse IgG (RAMIG) (100 μg/ml) on ice for 40 min. After two washing steps the fluorescence intensity distributions of the samples were measured in flow cytometer. The dissociation constant (K_d) and binding sites (B_{max}) of UIC2 were determined according to the same protocol, using different UIC2 mAb concentrations (0 μg/ml- 75 μg/ml).

The antibody competition test (ACT) was performed as follows. About $1x10^6$ cells in 1 ml NaCl/P_i supplemented with 8mM glucose were pre-incubated in the absence or presence of drugs/modulators at 37 ºC for 15 min. The drugs examined in my studies: colhicine (100 µM), etoposide (200 µM), galangin (50 µM), Hoechst 33342 (50 µM), ketoconazole (50 µM), nonidet P-40 ($6x10^{-4}$ V/V%), oligomycin (5 µM), progesterone (10 µM), propranolol (500 µM), quercetin (200 µM), rapamycin (20 µM), reserpine (40 µM), tween 20 ($1,4x10^{-3}$ V/V %), cyclosporin A (10 µM). After pre-incubation with any of these drugs, the first mAb, UIC2 (10µg/ml) was added, without washing the cells. After further 30 min incubation at 37 ºC, the FITC conjugated MM6.15 or MM12.10 mAb (8µg/ml) was added and incubation was continued at 37 ºC for another 30 min. Then the samples were washed twice and analyzed by flow cytometry. The extent of competition between mAbs UIC2 and FITC-MM12.10 (or MM6.15) was expressed as $R_{competition}$, the difference of mean fluorescence intensities of cell-bound FITC-MM12.10 in the absence (F1) and in the presence of UIC2 (F2), divided by the fluorescence intensity obtained in the absence of UIC2 (F1), i.e. $R_{competition} = (F1-F2)/F1$.

3.4. Assay of Flow Cytometric Detergent Resistance (FCDR)

3×10^5 cells were resuspended in 0.5 ml glucPBS and labeled with Alexa Fluor 488 (A488) conjugated 15D3 mAb (15 µg/ml) at 37°C for 30 min. Cells were washed twice with PBS and then treated with DIMEB or Chol-DIMEB at 37°C for 20 min. The samples were washed twice and the fluorescence intensity of cell-bound A488-15D3 was measured immediately in the flow cytometer. Then the samples were treated with TX-100 at a final concentration of 0.5 % on ice for 30 min. The fluorescence intensities were measured again and the extent of raft association of Pgp molecules was expressed as the percentage of the mean fluorescence intensity of the cells after TX-100 treatment, compared to the fluorescence intensity before treatment.

3.5. Measurement of Pgp internalization

The percentage of endocytosed Pgps labeled with A488-15D3 mAb was determined after modulation of membrane cholesterol levels by 5 mM RAMEB or Chol-RAMEB (used in these experiments instead of the similar DIMEB and Chol-DIMEB, respectively). The antibodies still exposed on the cell surface after 20 min incubation were removed by washing the cells with a low pH buffer (0.5 M NaCl, 0.1 M glicin, pH=2.5. The fluorescence intensity

of the acid treated and non-treated samples were determined in the flow cytometer and the fraction resistant to the treatment was used to calculate the percentage of internalized receptor-antibody complexes.

3.6. Drug accumulation studies

Calcein accumulation was measured as follows. Cells were pre-incubated with Pgp substrates/modulators for 10 min (10 μM CSA, 10 μM valinomycin, 75 μM vinblastin, 8 μM SDZ PSC 833, 75 μM verapamil, 20 μM quinine, 125 μM nifedipin) and than further incubated with UIC2 mAb (10 μg/ml) at 37 ºC for 30 min. Then the samples were divided into two parts. Pgp substrates/modulators were removed from one of the aliquots by washing with 1 % BSA-PBS and twice with PBS, while the other aliquot was kept at room temperature. Finally, samples were stained with 0.25 μM calcein for 15 min, washed twice and measured in flow cytometer. Dead cells staining with 2 μg/ml PI were exluded from the analysis. In some experiments cell bound UIC2 molecules were labeled with Alexa 647 conjugated GAMIG following the calcein accumulation assay.

Cyclodextrin-treated cells were stained by calcein in the same conditions. The extent of intracellular calcein accumulation was expressed as relative calcein accumulation, calculated as a ratio of the mean calcein fluorescence intensities measured in the cyclodextrin-treated and untreated cells, corrected for background fluorescence.

3.7. Membrane lipid packing density measurement

To examine the changes of lipid packing after cholesterol depletion or saturation, about 3×10^5 cyclodextrin-treated cells were resuspended in 100 μl PBS. MC540 stock solution (1 mg/ml in 50% ethanol) was freshly diluted with water to 0.5 mg/ml, and 2 μl of this solution was added to the cell suspensions. The samples were stained at room temperature for 10 min, diluted with PBS to 0.5 ml and measured immediately in the flow cytometer. The extent of MC540 staining was expressed in relative units, calculated as a ratio of the mean MC540 fluorescence intensities of the cyclodextrin-treated and untreated cells, corrected for background fluorescence.

3.8. Flow cytometry

Two- or three-color cytofluorimetric analysis was performed by using the Becton Dickinson FACScan or FACSCalibur flow cytometers (Mountain View, CA, USA), respectively. Dead cells stained with PI were excluded from the analysis. Fluorescence signals were collected in logarithmic mode and the cytofluorimetric data were analyzed by the BDIS CELLQUEST (Becton Dickinson) and the WinMDI 2.8 (written by Joseph Trotter; http://facs.scripps.edu/software.html) software.

3.9. ATP assay

Aliquots of the cyclodextrin-treated, washed cell samples were frozen and kept under liquid nitrogen (LN_2) until measurement. The ATP content of the cells was determined using the Bioluminescent Somatic Cell Assay Kit according to the manufacturer instructions, in a NovoStar microplate reader (BMG Labtech, Offenburg, Germany). To determine the average ATP content of the viable cells, their number was determined in each sample. Aliquots from the cyclodextrin-treated and washed samples were also frozen after addition of EDTA to 100 mM final concentration. These aliquots were thawed, stained with DAPI at a final concentration of 1 μg/ml, at room temperature for 15 min, transferred into black 96-well plates and analyzed with a FLUOstar OPTIMA microplate reader (BMG Labtech, Offenburg, Germany). The DAPI stock solution of 1 mM concentration was prepared in water and kept in the dark at 4^{o}C. Using a calibration curve with known cell numbers, the total cell number/sample could be determined based on the DAPI fluorescence intensities of the samples. To calculate the living cell number/sample, the total cell number in each sample was multiplied by the ratio of PI negative cells in the same samples, determined by flow cytometry after cyclodextrin treatment. The measured ATP content was then divided by the viable cell number in each sample, yielding the ATP content/viable cell.

3.10. Cell viability tests

3.10.1. Flow cytometric cell viability test

2 µg/ml PI was added to the samples prior the measurement to determine the viable cell ratio.

3.10.2. *In vitro* cytotoxicity assay

The MTT assay was used to study the cytotoxic effect of daunorubicin. The cells were seeded in 96-well plates at a cell density of 1×10^4 cells/well. 24 hours later daunorubicin was added at different concentrations, with SDZ PSC 833 and/or UIC2 mAb or without the modulator, and the plates were further incubated for 72 h at 37ºC. After removing the medium, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide solution (0.5 mg/ml in DMEM without phenol red) was added and the cells were further incubated for 4 h at 37ºC. After removing the supernatant, the formazan crystals were completely solubilized in isopropanol:1N HCl (25:1) and the absorbance at 570 nm was measured using an automated microplate reader (BMG Fluostar Optima, Germany).

3.11. Measurement of cellular cholesterol levels

The cholesterol content of the cyclodextrin-treated cell samples was determined using the cholesterol oxidase method by HPLC measurement. The measurement was carried out by our collaboration partner (Prof. Tamás Janáky, Szeged).

3.12. Studies on tumor xenografts

Twenty adult (10 to 12 week-old), pathogen-free B-17 SCID (severe combined immunodeficiency) mice were used in this study. The „Principles of laboratory animal care" (NIH) were strictly followed and the experimental protocol was approved by the Laboratory

Animal Care and Use Committee of the University of Debrecen. The NIH 3T3 and NIH 3T3 MDR1 cells (4×10^6 cells in 300 µl serum-free Dulbecco's MEM) were injected subcutaneously into opposite flanks of the mice. The tumors were grown for 10-12 days. Animals were pre-treated with 10 mg/kg CsA (Sandimmun, Novartis, Basel, Switzerland) intraperitoneally and / or UIC2 mAb (5 mg/kg, added intravenously) 4 hours before the administration of daunorubicin (5 mg/kg, i.v.). The animals were killed 4 hours after the addition of daunorubicin by cervical dislocation and the tumors were dissected and kept in liquid nitrogen until further use. 8-10 pieces of consecutive 6-µm thick cryosections were prepared from the tumor samples to compare nearby sections for their daunorubicin accumulation, UIC2 binding and tissue morphology. The morphology of the tissue sections was routinely checked by conventional hematoxylin-eosin staining after paraffin embedding, using an Olympus CX31 epifluorescent microscope equipped with a 7.1 mega pixel C7070 wide zoom Camedia camera (Olympus Hungary, Budapest).

UIC2 binding was visualized by indirect immunofluorescence. The cryosections were blocked with 10 % goat serum for 20 min and labeled with GAMIG-Alexa 488 (5 µg/ml in PBS containing 2 % goat serum) for 1 hour. Then the slides were washed twice with 1 % BSA-PBS and twice with PBS; the cover slips were mounted on slides using Prolong antifade (Molecular Probes, Eugene, Oregon, USA) and confocal images were immediately recorded.

3.13. Confocal laser scanning microscopy and laser scanning cytometry

The daunorubicin accumulation as well as UIC2 binding of the cryosections prepared from the tumors were measured by confocal laser scanning microscopy (LSM 510, Zeiss, Jena, Germany) and laser scanning cytometry (iCys, CompuCyte, Cambridge, MA, USA).

The 488-nm line of an argon-ion laser was used in confocal microscopy experiments. Fluorescence intensities were detected through a 505-550-nm bandpass filter (for Alexa 488 dye) and a >580 nm longpass filter (for daunorubicin). Images were collected through a Plan-Apochromat 63× oil-immersion objective (numerical aperture=1.4). The pinhole was totally open. The images were always recorded at the same laser intensities and detection parameters, making the comparison of the different samples possible.

The tumor specimens were also analyzed with a laser scanning cytometer in one experiment to obtain quantitative results of whole sections. The 488 nm wavelength of the

argon-ion laser was used for excitation and the fluorescence was detected in the green channel (emission: 530±15 nm). For fast setup of scan areas, the "scout" or low resolution-scanning feature of the “Tissue Scan” input module of the iNovator toolkit was applied. The scout scan was used to find the boundaries of tissue samples based on the fluorescence intensity detected in the green channel, and then a high-resolution scan was conducted, to analyze only the defined areas. The 20× objective was used for the high resolution scan and the phantom contouring feature of the iCys software was applied to characterize the fluorescence intensity distribution in large areas of the sections. In these experiments, the highest possible numbers of phantom contours were arranged randomly throughout the scan area, with the radius of each contour set to 10 μm and with no overlap allowed between phantom contours. The integral fluorescence (the sum of the pixel intensities inside a contour) of each contour was used to characterize the fluorescence intensities and contour maps were created with Sigma Plot 8.0 (SPSS Inc., Chicago, IL, USA) for each section.

3.14. Statistical analysis

Data have been analyzed using SigmaStat (version 3.1, SPSS Inc., Chicago, IL, USA) and are presented as means ± SD. Comparison of two groups was performed by unpaired *t*-test, while in the case of three or more groups statistical significance was assessed using analysis of variance (ANOVA), applying Bonferroni’s multiple comparison test for post hoc pairwise comparison of the results. Differences were considered significant at $p<0.05$.

4. Results

4.1. Dichotomous behaviour of Pgp substrates/modulators

Based on the extent of mAb competition the ACT test can uniquely distinguish two types of Pgp substrates/modulators: the CSA-like ACT-positive and verapamil-like ACT-negative agents. We extended these observations to a panel of well known or potential Pgp substrates to examine the generality of this phenomenon. Among the newly tested drugs rapamycin was classified into the group of ACT-positive agents, progesterone, galangin, propranolol, quercetin, reserpine, Tween-20, ketoconazole and NP-40 into the group of ACT-

negative agents. In the case of colhicine, ologomycin, etoposide and Hoechst 33342 we observed weak mAb competition as well as weak Pgp inhibition. The ACT+/- groups of Pgp modulators can be clearly distinguished by the ACT test and this phenomena seems to be general for numerous drugs.

4.2. Inhibition of Pgp function by the UIC2 mAb in the presence of ACT+ and ACT- agents *in vitro*

4.2.1. Calcein-accumulation studies

The ACT phenomenon suggested to us that the augmented binding of UIC2 in the presence of "ACT-positive" agents may also potentiate the pump inhibitory effect of this mAb. UIC2 strongly increases calcein accumulation in NIH 3T3 MDR1 cells prelabeled with the mAb in the presence either of CsA, vinblastine, SDZ PSC 833, or valinomycin. This Pgp inhibitory effect of UIC2 is preserved after removal of the drugs by washing the cells with 1 % BSA-PBS. All the "ACT-positive" agents could be effectively extracted from the cells by this washing protocol, except for valinomycin. At the same time, the "ACT-negative" verapamil, quinine and nifedipine did not elicit an inhibitory binding of UIC2 to Pgp molecules, demonstrated by the fact that calcein accumulation decreased to the control level after removal of these agents. In accordance with the above, verapamil, quinine and nifedipine did not change significantly the number of UIC2 reactive Pgps, while CsA and other "ACT-positive" agents increased UIC2 binding 2-3 times.

When Pgp inhibition was measured as a function of the concentration of CsA, a biologically significant (>10x) increment in intracellular calcein levels occurred at ≥0.2 μM concentration of the modulator when used in combination with UIC2, while approx. 20x higher CsA concentration was just as effective when the modulator was applied alone. Thus, an inhibitory binding of UIC2 is brought about at much lower concentrations of CsA than what is necessary for blocking transport by the drug acting as a competitive inhibitor. At ≥0.2 μM CsA concentration, UIC2 labeled about ≥70 % of all cell surface Pgps, as detected by indirect immunofluorescence in parallel samples. A large (25x) increase of calcein accumulation was achieved also in response to a combined treatment of NIH 3T3 MDR1 cells with UIC2 and 10 nM SDZ PSC833; when applied alone, the modulator was completely ineffective at this concentration, and the same increment was achieved only at its 10 times higher concentration.

4.2.2. Cell viability tests

2780^{AD} (Pgp^+) and 2780 (Pgp^-) cells were treated with 15 nM SDZ PSC 833 and 20 µg/ml UIC2 mAb in the presence of different daunorubicin concentrations, for 72 hours, then cell viability was analyzed by the MTT test. Neither the UIC2 mAb, nor the modulator alone was able to decrease the IC_{50} value of daunorubicin (1 µM). The combined addition of UIC2 and low concentrations of SDZ PSC 833 significantly increased the cytotoxic effects of daunorubicin and decreased the IC_{50} value (100 nM).

4.3. Determination of the dissociation constant (K_d) and binding sites (B_{max}) of UIC2

UIC2 binds only to 10-40 % of all Pgps present in the cell membrane. Incubation of cells with ACT-positive agents increases the reactivity of UIC2 to Pgp to a large extent: close to 100 % of all cell surface Pgp molecules become labeled, unlike the Pgps of the cells that have not been treated or incubated with ACT-negative agents, like verapamil.

In the case of verapamil treatment the number of binding sites (B_{max}= 53,17 ± 1,39 %) increased slightly compared to the untreated control (B_{max}= 42,69 ± 0,788 %), while in the case of CSA treatment all the cell surface binding sites were available for the mAb.

The dissociation constant exhibited a slight decrease upon treatment with modulators (verapamil: K_d= 2,49 ± 0,265 µg/ml, CSA: K_d= 3,49 ± 0,38 µg/ml), compared to the untreated control (K_d= 6,49 ± 0,38 µg/ml).

4.4. UIC2 inibiton *in vivo*, in tumor xenografts

SCID mice were injected in their two opposite flanks with NIH 3T3 MDR1 Pgp^+ and NIH 3T3 Pgp^- cells, respectively. Palpable subcutaneous tumors developed in 10-12 days. Then these mice were treated with CsA and/or UIC2 mAb, followed by the administration of daunorubicin, and the accumulation of this chemotherapeutic agent, as well as UIC2 binding were measured in cryosections of the tumors, both by confocal microscopy and laser scanning cytometry. Nuclear accumulation of daunorubicin was observed deep inside the tumor tissue. The combined application of 10 mg/kg CsA and UIC2 increased daunorubicin accumulation of the Pgp^+ tumor approx. to the level of the Pgp^- tumor in the same animal. At the same time, daunorubicin accumulation did not increase significantly in the Pgp^+ tumors treated with the antibody or 10 mg/kg CsA alone, as compared to the untreated mice; 5 times higher CsA

concentration was required to reach effective pump inhibition without co-administration of the antibody. Quantitative evaluation of the changes was performed in one of the experiments by laser scanning cytometry. The mean daunorubicin fluorescence intensity in a section of the Pgp^+ tumor of the mouse treated with 10 mg/kg CsA *and* the antibody was 3.35×10^6 (CV = 26.87 %); the mean daunorubicin fluorescence of the Pgp^- tumor of the same mouse was 2.98×10^6 (CV = 20.43 %), compared with the 1.64×10^6 (CV = 18.12 %) mean value measured in the Pgp^+ tumor of the mouse treated only with UIC2 in the same experiment.

UIC2 applied together with CsA, could readily penetrate into the compact solid tumors, intensively staining cell surface Pgps. The antibody binding was specific, as no significant labeling of the Pgp^- tumors was detected. Quantitative tissue section analysis demonstrated that the whole tumor section was labeled by UIC2 in the presence of CsA, while it barely labeled the cells when added without CsA. The localization of strong UIC2 binding correlates with the distribution pattern of the daunorubicin fluorescence intensity in the specimen. Hematoxylin-eosin staining of the adjacent sections showed typical tumor tissue histology. In the central tumor areas of lower cell density we also observed mostly intact cells with strong cell surface Pgp staining at higher magnification, excluding the possibility that nonspecific binding of the antibody to necrotizing regions have been observed.

4.5. The effect of cholesterol on Pgp transport function

4.5.1. Cell membrane cholesterol modulation

Cellular cholesterol depletion by 5 mM DIMEB decreased the cholesterol level of the cells by about 50 % (8,34±2,35 µg cholesterol/10^6 db cell), compared to the untreated control (16,97±0,65 µg cholesterol/10^6 db cell), while cholesterol saturation carried out by Chol-DIMEB treatment increased cellular cholesterol content (27,83±7,01 µg cholesterol/10^6 db cell).

The altered lipid composition changed the overall physicochemical properties of the membrane also. Cholesterol depletion by DIMEB gave rise to an augmented staining by MC540, reflecting decreased lipid packing density. Chol-DIMEB could efficiently load the cell membrane with cholesterol, as shown by the increment in membrane packing density shown by a decreased MC540 staining

4.5.2. Membrane cholesterol modulation affects raft association of Pgp

Cholesterol depletion by DIMEB significantly decreased, cholesterol saturation by Chol-DIMEB increased the fraction of raft associated Pgp molecules. Thus, changes in overall membrane cholesterol level also involve perturbation of the membrane microenvironment of the transporter molecules.

4.5.3. Membrane cholesterol modulation alters Pgp function

Extraction of cholesterol from the cell membrane with DIMEB increased the intracellular calcein accumulation in viable Pgp^+ cells (gating on the PI negative population), as a function of DIMEB concentration. Unexpectedly, cholesterol saturation also enhanced the accumulation of this Pgp substrate, although to a lesser degree. The calcein accumulation was slightly increased also in the Pgp^- cells, due to nonspecific membrane permeabilization.

Modulation of the cholesterol content of the cell membrane may influence Pgp mediated drug transport also by affecting the number of transport competent Pgp molecules. Indeed, cholesterol saturation increased internalization of Pgp by about 2.5 times, after 20 min incubation; cholesterol depletion did not have a significant effect.

4.5.4. Membrane cholesterol modulation by DIMEB or Chol-DIMEB decreases viability without effecting ATP leakage in the live fraction of cells

Cell viability decreased as a function of DIMEB or Chol-DIMEB concentration, as detected by PI staining. Cholesterol depletion caused membrane damage and cell death at a lower molar concentration of cyclodextrins, as compared to the effect of cholesterol saturation. Toxicity apparently correlated with the influence on calcein accumulation, as reflected by the increased number of non-viable, PI positive cells and the simultaneous loss of fluorescein-retaining cells in the FDA test after cyclodextrin treatments. To exclude the possibility that Pgp modulation is the result of toxicity accompanied by membrane permeabilization undetected by dye exclusion, we also measured the ATP content in the PI-negative fraction of the cells. Neither DIMEB nor Chol-DIMEB treatment altered the cellular ATP content in Pgp^+ viable cells. In contrast, incubation of the Pgp^+ cells in glucose-free PBS with verapamil that markedly stimulates the ATPase activity of the transporter significantly decreased the ATP content of the cells.

5. Discussion

Using the ACT test, worked out previously by our group, the agents studied by ourselves also fall into sharply distinguishable categories in spite of their diverse chemical structures. Agents with high, 0,9-1 $R_{competition}$ (ACT) values (e.g. CSA) are able to completely inhibit the function of Pgp (ACT-positive agents). The effect of ACT-positive agents on Pgp conformation is indistinguishable from that of ATP depletion, suggesting that these agents block the pump in the same or a very similar conformational state that is part of the catalytic cycle. The ACT values of verapamil-like (ACT-negativ) agents are between 0,2-0,7. These agents are not able to induce such conformational/topological states of cell surface Pgps that would allow UIC2 binding even after prolonged incubation with the mAb, showing that the two states, one without treatment or after treatment with ACT-negative comounds, and that elicited by ACT-positive agents, are distinct.

Although the tested drugs have diverse chemical structures, some common features can be observed. The ACT-positive agents are usually relatively large-size molecules and rich in groups that are able to form hydrogen bonds. This could result in stronger substrate binding and slower transport, leading to the conformational/topological states typical of ACT-positive agents. However, this interpretation wouldn't reconcile the catalytic cycle related conformational changes with the apparent topological heterogeneity. The transport of small substrates requires that "cholesterol fills in" the Pgp's binding pocket, while substrates bigger than 1000 Da are transported alone by Pgp. These observations help outline a model in which the UIC2 un-recognized („pool II") Pgps might be in a „stand by", ATP-bound state in the absence of ACT-positive, large substrates, while the other, raft-associated pool („pool I") may have a permanent basal catalytic activity, and the presence of cholesterol here may make the transport of small-size substrates possible.

This model is in accordance with the finding that ACT-positive substrates increased the number of binding sites to a great extent in contrast with the verapamil-like agents, while both had but a small effect on binding affinity. We propose that the increase in the binding sites dominates over the changing in the binding affinity and this mechanism causes the increased UIC2 reactivity in the presence of ACT-positive substrates.

When the cells are incubated with the UIC2 mAb in the presence of "ACT-positive" modulators/substrates, an enhanced and inhibitory binding of the antibody is observed, and this inhibited state of Pgp is preserved after the removal of the modulators. At the same time,

"ACT-negative" agents do not induce an inhibitory binding of UIC2, even if they elicit a mild increase in UIC2 reactivity. As calcein accumulation was increased significantly only at ≥60-70 % ligation of cell surface Pgps with UIC2, a prominent inhibition of drug transport is not expected when UIC2 binds to only 20-50 % of all cell surface Pgps in the absence of modulators, or in the presence of "ACT-negative" drugs. These data are best interpreted in terms of the conformational/topological changes of the transporter elicited by the "ACT-positive" drugs that make all cell surface Pgps UIC2-reactive.

Co-incubation of Pgp^+ cells with CsA or SDZ PSC 833 and UIC2 leads to the inhibitory binding of the antibody to most cell surface Pgps at ~20x lower modulator concentration than what is necessary for the complete blocking of transport by the modulator acting merely as a competitive inhibitor. We propose that a rather low concentration of "ACT-positive" agents (under the K_M of their transport) is sufficient to initiate the catalytic cycle so that all cell surface Pgps gradually enter and become trapped in a UIC2 reactive conformational state.

These results were confirmed by cytotoxicity tests, in which the combined UIC2 - low dose modulator treatment could decrease mdr cell viability significantly.

To our knowledge, our data represent the first successful attempt to achieve a near-complete antibody-mediated Pgp inhibition in *in vivo* conditions. We have demonstrated in the SCID mouse model that (*I*) the UIC2 mAb reaches its cell surface Pgp targets deeply buried in solid tumors; (*II*) daunorubicin, a fluorescent anthracyclin anticancer agent, readily enters Pgp expressing cells when inhibited by UIC2; (*III*) there is a positive correlation between the extent of UIC2 binding and the increment in daunorubicin uptake; (*IV*) CsA augments the inhibitory UIC2 binding at a ≥5 times lower concentration, than its effective concentration for the competitive inhibition of Pgp function.

Success of mdr reversal strategies has been seriously limited in clinical practice by the side-effects of the modulators applied. At the lower concentrations of the ("ACT-positive") modulator to be used in our protocol, the side-effects could be significantly decreased. Furthermore, since inhibition of Pgp is achieved by the mAb, the effect will be restricted to this transporter. The inhibited state of Pgp molecules persists even after the removal of modulators in *in vitro* experiments, suggesting that the cell-bound UIC2 molecules exert their pump inhibitory effect even after the plasma concentration of the modulator has declined.

The transport mechanism of Pgp is not fully understood. The pore-like structure formed by α-helices is open toward the cell membrane and also allows the passage of substrates to the extracellular space. Therefore it is not surprising that Pgp function is so sensitive to

modulation of its immediate membrane environment that may play an important role in the development of substrate-Pgp interactions and substrate recognition.

The cell membrane cholesterol content can be effectively modulated by derivatives of β-cyclodextrin. Partial removal of cholesterol from the membrane by DIMEB has lead to a decrease in the packing density of membrane lipids, while an increased cholesterol content brought about by Chol-DIMEB has lead to higher packing densities, reflected by MC540 staining.

To analyze the effect of cholesterol modulation on Pgp's membrane microenvironment, we studied the changes of its raft association using the FCDR test. In this assay, the TX-100 resistant fraction of all cell surface Pgps is determined by flow cytometry. Modulation of membrane cholesterol levels were reflected by concomitant changes in raft association, revealing that changes in the membrane microenvironment of the pump have been elicited by the treatments. Thus, treatment with cyclodextrins leads to changes both in the overall physicochemical features of the membrane and in Pgp's immediate environment, as reflected by the MC540 staining and the FCDR assay, respectively.

These modifications of the membrane structure were also accompanied by changes of Pgp transport function. DIMEB increased calcein accumulation in the Pgp^+ cells much more than in Pgp^- cells, suggesting that Pgp inhibition is mostly responsible for the effect in Pgp^+ cells. A possible mechanism of this inhibitory effect is the extraction of cholesterol or phospholipids from the cell membrane.

Chol-DIMEB treatment also increased intracellular calcein accumulation, but to a lesser degree. These data are difficult to interpret either in terms of the possible cholesterol requirement for proper functioning or in terms of the assumption that Pgp may play a role in cholesterol redistribution within the membrane. Another aspect of cyclodextrin treatments is that the modification of the membrane cholesterol level may also influence intracellular trafficking of certain membrane proteins, perhaps changing the level of functional Pgp molecules in the cell membrane. In support of this possibility, cholesterol saturation increased the internalization of Pgp molecules in the cell membrane, while cholesterol depletion did not have any significant effect.

In another scenario, membrane lipid constituents other than cholesterol might also play a role in the above effects. Certain lipids might be co-extracted together with cholesterol, and an exchange of lipids might also take place between the cell membrane and the cyclodextrin ring that has already delivered its cholesterol content. The altered lipid composition itself, or the simultaneous membrane structure alterations, could both affect Pgp function, involving

modulation of k_M or v_{max}. However, these effects may also come about via interference with other pathways of drug transport, or may be due to ATP depletion as a result of toxicity. The first possibility may play a less significant role, since cyclodextrin treatment only slightly increased calcein accumulation in the Pgp^- cells. The possibility that Pgp may be inhibited as a result of toxic side effects of the treatments was investigated measuring intracellular ATP levels. Yet, permeabilization may be a gradual process exhibiting different stages and cyclodextrin treatment could induce the leakage of ATP, from the cell even before the characteristic changes in PI staining become detectable. ATP depletion would also inhibit transport activity. Our data demonstrate that in spite of the conspicuous alterations in the membrane structure by cholesterol depletion or saturation, and in the presence of high percentages of non-viable cells, the intracellular ATP content of the PI^-, non-permeable, i.e. apparently viable cells, does not change. Our data suggest that alteration of substrate accumulation caused by DIMEB and Chol-DIMEB treatment are mainly Pgp-related effects, due to perturbation of the lipid environment of the pump, in viable cells with physiological ATP content. It remains to be seen if these effects are related to the cooperation of cholesterol with other drugs of appropriate size to fill in its binding site (see above), or to cholesterol-dependent linkages of Pgp to its membrane environment.

My results have contributed to a model that attempts to reconcile and decipher the entangled relations between topological heterogenity, conformational changes accompanying the catalytic cycle and the dichotomy of substrates/modulators. Potential practical benefits appear to stem from my work yielding the protocol of reproducible UIC2 elicited pump inhibition and the establishment of an animal model for testing the conditions for its theraputical application *in vivo*.

The thesis is based on the following publications:

1. **Fenyvesi F**, Fenyvesi É, Szente L, Goda K, Bacsó Zs, Bácskay I, Váradi J, Kiss T, Molnár É, Janáky T, Szabó G, Vecsernyés M. (2008) P-glycoprotein inhibition by membrane cholesterol modulation. Eur. J. Pharm. Sci. 34(4-5): 236-242. **IF: 3,127**

2. Goda K*, **Fenyvesi F***, Bacso Zs, Nagy H, Marian T, Megyeri A, Krasznai Z, Juhasz I, Vecsrnyes M, Szabo G Jr. (2007) Complete inhibition of P-glycoprotein by simultaneous treatment with a distinct class of modulators and the UIC2 monoclonal antibody. J. Pharmacol. Exp. Ther. 320 (1): 81-88. **IF: 4,003**
***G.K. and F.F. contributed equally to this study**

3. Nagy H, Goda K, **Fenyvesi F**, Bacso Zs, Szilasi M, Kappelmayer J, Lustyik G, Cianfriglia M, Szabo G Jr. (2004) Distinct groups of multidrug resistance modulating agents are distinguished by competition of P-glycoprotein-specific antibodies. Biochem. Biophys. Res. Commun. 315 (4): 942-949. **IF: 2,904**

List of other publications

1. Szilagyi A, **Fenyvesi F**, Majercsik O, Pelyvas IF, Bacskay I, Feher P, Varadi J, Vecsernyes M, Herczegh P. (2006) Synthesis and cytotoxicity of leinamycin antibiotic analogues. J Med Chem. 49 (18): 5626-5630. **IF: 5,115**

2. Bacso Zs, Nagy H, Goda K, Bene L, **Fenyvesi F**, Matko J, Szabo G. (2004) Raft and cytoskeleton associations of an ABC transporter: P-glycoprotein. Cytometry A. 61(2): 105-116. **IF: 1, 601**

3. Kiss T., **Fenyvesi F**., Pasztor N., Feher P., Varadi J., Kocsan R., Szente L., Fenyvesi É., Szabo G., Vecsernyes M., Bacskay I. (2007) Cytotoxicity of different types of methylated β-cyclodextrins and ionic derivatives, Die Pharmazie. 62(7): 557-558. **IF: 0,606**

4. Kiss T, **Fenyvesi F**, Bácskay I, Fehér P, Kocsán R, Váradi J, Szente L, Fenyvesi É, Iványi R, Vecsernyés M, (2007) Cytotoxic examinations of various cyclodextrin derivatives on Caco-2 cells, Acta Pharmaceutica Hungarica 77(2): 150-154.

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Basckay, I., Kocsan, R., **Fenyvesi, F**., Feher, P., Sipos, T., Varadi, J., Vecsernyes, M. (2005) Cytotoxicity of different types of surfactants on HeLa cells. Eur. J. Pharm. Sci. 25: S46-S47.

Fenyvesi, F., Goda, K., Bacso, Z., Vecsernyes, M., Juhasz, I., Marian, T., Tron, l., Krasznai, Z., Szabo, G. (2005) Conformational and topological states of P-glycoprotein, an ABC transporter involved in multidrug resistance: novel assays of transporter modulation. Eur. J. Pharm. Sci. 25: S16-S16.

Posters and lectures

Nagy, H., **Fenyvesi, F.,** Goda, K., Bacsó, Zs., Cianfriglia, M., Kappelmayer, J., Lustyik, Gy., Szabó, G.: Two groups of multidrug resistance modulators are sharply distinguished both by competition of UIC2 with other anti-Pgp mAbs its ability to completely inhibit pumping. FEBS Advenced Lecture Course: „ATP-Binding Cassette (ABC) Proteins: From Genetic Disease to Multidrug Resistance" Gosau, Austria 2003. *(poster)*

Bacsó, Zs., Nagy, H., **Fenyvesi, F**., Goda, K., Bene, L., Rychly, J., Lustyik, Gy., Matkó, J., Szabó, G.: Raft- and cytoskeleton-assotiation of the P-glycoprotein. FEBS Advenced Lecture Course: „ATP-Binding Cassette (ABC) Proteins: From Genetic Disease to Multidrug Resistance" Gosau, Austria 2003. *(poster)*

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