

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Investigation of the basal and cholesterol-dependent trafficking of  
P-glycoprotein

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# Investigation of the basal and cholesterol-dependent trafficking of P-glycoprotein

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## 1. Introduction

When chemotherapy was successfully introduced against cancer diseases, clinical obstacles promptly arose as a result of the development of drug-resistance to the chemotherapeutic drug. Interestingly, cancer cells were able to develop resistance to a wide variety of chemotherapeutics at the same time. The phenomenon is known as multidrug resistance (MDR). MDR1/P-glycoprotein/ABCB1, several members of the MRP family (MRP1-9), and BCRP/MXR/ABCG2 were found to be the most important MDR-ATP-binding proteins in the human body. All of these proteins belong to one of the largest and oldest transmembrane protein families with representatives from prokaryotes to humans, the ATP Binding Cassette (ABC) family of transport ATPases. All ABC transporters utilize the energy of ATP hydrolysis. ATP is bound by the highly conserved, characteristic feature of the protein family, the approximately 215-amino acids consensus sequence designated as ATP-binding cassette (ABC) situated within the nucleotide binding domain(s) (NBDs).

P-glycoprotein (Pgp), the MDR protein we are interested in, pumps a wide range of compounds varying in molecular weight (from 200 Da to 1900 Da) and chemical structure. Most of its substrates are weakly amphipathic and relatively hydrophobic compounds; many (but not all) contain aromatic rings and a positively charged tertiary N atom. Several drugs that are used in chemotherapy meet these criteria. Therefore, Pgp, mostly overexpressed in the plasma membrane (PM) of cancer cells, is able to reduce the intracellular concentration of these drugs. Pgp is still considered to be a substantial factor in most clinical cases of multidrug resistance, however it is now clear that the MDR phenotype always involves other factors in addition to Pgp. Besides, Pgp is also physiologically existing in certain tissues that are important in the absorption (epithelial cells of the gut); metabolism-elimination (liver, kidney) of drugs or takes an active part in the protection of vital structures such as the brain, cerebrospinal fluid, testis, ovaries and foetus against toxic compounds.

Functionally, Pgp is envisaged as a “hydrophobic vacuum-cleaner” extracting amphiphilic and hydrophobic compounds embedded in the inner leaf of the PM, and pumping them directly to the external aqueous medium. In this model, substrates could gain access from the PM to their binding sites through “gates” formed between the transmembrane domains. In an alternative proposal, the “flippase” model, substrates are flipped (according to the recent nomenclature flopped) from the inner leaflet of the lipid bilayer to the outer leaflet. Having accumulated in the outer leaflet, they finally diffuse out to the external medium. It is now generally believed that the central pore, formed by the transmembrane helices, represents an expansive binding pocket, which accommodates substrates by interaction with a large complement of amino acid residues. Structurally diverse ligands can be accommodated in the binding cavity by hydrogen bonding and van der Waals interactions, offering an explanation for the polyspecificity of the transporter. In this „substrate induced fit” model, the binding affinity of certain substrates relies on the summation of these interactions. The substrate binding site(s) alternate between high- and low-affinity conformations exposed at alternate faces of the membrane during the transport process, coupled to the kinetics of ATP hydrolysis. Based on previous and recent data of bacterial ABC transporters and on the crystal structure of ABCB1a, Pgp is likely to exhibit an inward-facing conformation (the substrate-binding cavity facing the cytoplasm) in the absence of nucleotides. This conformation favours drug binding, as the substrate-binding chamber is exposed to the cytoplasm and contains a laterally open gap that is accessible from the inner leaflet of the membrane. In the presence of nucleotides, the protein exhibits an outward-facing conformation (the substrate-binding cavity facing the extracellular environment). In this conformation the substrate-binding gap is exposed to the outer leaflet of the bilayer and the external environment.

In accordance with the “hydrophobic vacuum -cleaner” or with the “flippase” model, the lipid environment greatly influences the distribution of Pgp substrates in the membrane, which consequently has an effect on drug binding. At the same time, lipid dependence of basal ATPase activity of the transporter, i.e. ATPase activity in the absence of substrates, confirms the direct effect of these lipids on the catalytic activity. Based on the results of several studies, Pgp resides both in non-raft domains and raft domains, i.e. detergent resistant, cholesterol- and shingolipid-rich membrane domains. These membrane microdomains can be caveolae or any other raft domains. Cholesterol, therefore, is special among the membrane lipids. Besides influencing basal and drug stimulated ATPase activity of Pgp and drug binding, it also plays an active role in preserving the integrity of raft domains. Cholesterol extraction studies on whole cells, carried out by methyl- $\beta$ -cyclodextrins, proved that raft association of Pgp is of functional significance because its modulation markedly affects drug pumping. Cholesterol repletion induced a shift in Pgp localisation back to lipid rafts and restored impaired Pgp-transport capacity. Inhibition of the synthesis of shingolipids, i.e. the other major components of raft domains, also caused a shift in the Pgp localisation to detergent soluble fractions. Depletion of shingolipids disassembled Pgp containing lipid rafts and resulted in the subsequent accumulation of the Pgp substrate rodamin-123. Pgp also segregated out of lipid rafts due to inhibition of the association of the transporter with the CD19 protein. The shift in Pgp localisation had similar functional repercussions.

At first sight, all of these observations suggested that functional Pgps favour raft domains. On the other hand, most of the cell surface Pgps were found out of these raft domains by several workgroups (vide infra previous observations). Therefore, it seems more likely that Pgp is constantly redistributed among raft and non-raft domains and the disruption of this well controlled balance results in functional consequences. This concept would agree with the dynamic structure of raft domains.

The “flippase” model of Pgp and the basal ATPase activity of the transporter allows us to speculate that Pgp might be able to translocate certain lipids between the exofacial and cytofacial leaflets of the PM. Several workgroups aimed at confirming the translocation of phosphatidylethanolamine (PE), phosphatidylcholine (PC), shingomyelin (SM), glucosylceramide (GluCer) and cholesterol by Pgp. On closer examination it was found that clear evidence about the effective translocation of these natural lipids, e.g. cholesterol, by Pgp is still missing. It is still questionable whether Pgp plays a decisive role in the distribution of these lipids between the two leaflets of the PM.

Elevated levels of raft domains and caveolae in MDR cells made it particularly interesting to investigate the translocation of cholesterol and GluCer, used by the cell for the synthesis of further glycosylated shingolipids. Retroviral infection of the MDCK renal cell line with human MDR1 cDNA (encoding Pgp) induced a major accumulation of the glycosphingolipid (GSL), globotriaosylceramide (Gb3), the receptor for verotoxin (VT), to effect an increase in cell sensitivity to VT. Pgp inhibitors, e.g. ketoconazole or cyclosporinA (CsA) prevented the increased Gb3 and verotoxin sensitivity. During the synthesis of GSLs most of the glycosylation steps take place in the lumen of the Golgi. The only byproduct is GluCer, which is synthesized on the cytosolic side. Thus a mechanism for the translocation of GluCer from the cytosolic face to the lumen of the Golgi must exist. The authors proposed that Pgp acts at that point. However, later these drugs were shown to inhibit the GluCer synthase as well. Thus, these observations make it unlikely that Pgp indeed translocates GluCer in the Golgi. Beside many other workgroups, Sharom's group investigated the effect of cholesterol on various functional activities of purified Pgp. Cholesterol modulated the transporter's basal and drug stimulated ATPase activity and the binding of substrates. However, Pgp did not appear

to mediate the flip-flop of a fluorescent cholesterol analogue across the bilayer. Cholesterol probably does not require a transport protein to move between bilayer leaflets at all, as its spontaneous flip-flop rate is very fast. Therefore, it is also unlikely that Pgp translocates cholesterol in the membrane. As a result of these studies Pgp is principally confirmed to be a drug transporter. At the same time, it is possible that Pgp initially was indeed a primitive lipid transporter but became an effective drug transporter during the evolution.

As we previously mentioned, an altered, deviant lipid profile and membrane composition is often observable in MDR cells when compared to sensitive cells. Drug resistant cells possessed elevated levels of cholesterol, complex glycosphingolipids and gangliosides in several cases. Moreover, an increment of cholesterol content, characterized in resistant cells, was directly proportional to the relative resistance to cytotoxic drugs. All these data confirm that the metabolism of certain lipids, e.g. cholesterol is altered in these drug resistant cells. Available data indeed prove that MDR cells (drug selected cell lines) show an increased esterification of excess cholesterol from PM and as a result of this own an intracellular cholesterol pool. This esterified cholesterol pool provides these cells the ability to effectively react on changes of cholesterol, e.g. after cholesterol extraction. In addition, MDR cells possess significantly enhanced cholesterol synthesis as well. All these observations confirm that MDR cells are more proficient in obtaining increased PM cholesterol levels than drug sensitive cells. As a general rule, resistant cells exhibit a higher degree of structural order in the lipid phase of their PMs, which hint that raft domains are upregulated as a result of the altered lipid metabolism in these cells. It seems likely that these membrane composition modifications, independently from the protein expressions, contribute to the MDR phenotype.

Both in polarized and non-polarized cells Pgp follows two different trafficking routes in order to reach the PM. Membrane proteins can traffic to the PM via either the constitutive pathway which involves membrane protein-containing vesicles moving directly to the PM or the endosomal pathway in which protein-containing vesicles are first transported to endosomal compartments to establish the intracellular pool, and then traffic to the PM. In both cases, the cytoskeleton is needed for the traffic of these membrane protein vesicles. Based on the literature early endosomes were confirmed to be such an intracellular reservoir. Several studies suggest that P-gp is constantly trafficking and recycling via different endosomal pathways (early endosome vs. recycling endosome) with the contribution of the actin cytoskeleton. As a result of this 30% of mature Pgps are constantly localized in these intracellular organelles.

Interestingly, sequestration of Pgp substrates in certain intracellular organelles is often observable in MDR cells. This fact prompted several workgroups to presume that functionally active Pgp, localized in the membrane of these organelles, might have a role in it. However, the available data are controversial. Therefore, there is no obvious evidence that Pgp would indeed take part in the intracellular accumulation of these drugs. At the same time, the intracellular localization of Pgp is confirmed in many cellular organelles, including endoplasmic reticulum (ER), Golgi, early endosome, recycling endosome, later endosome, lysosome, and proteasome. These intracellular localizations refer to synthesis (ER), modification (Golgi), traffic/recycling (Golgi and endosomes), and degradation (lysosome and proteasome) sites for P-gp.

Mature PM Pgps (170 kDa) are known to exhibit an exceptionally long half-life (14–17 h). Immature, core-glycosylated Pgps were confirmed to be rapidly degraded by proteases in the lumen of the ER or via the ubiquitin-proteasome degradation pathway. There is no such agreement in the literature regarding the final degradation organelle of matured cell surface

Pgps. The existence of ubiquitinated cell surface transporters suggested that the ubiquitin-proteasome system-mediated degradation could target mature Pgp on the PM beside or instead of the lysosomal dependent degradation pathway. However, the primary targets of the ubiquitin-proteasome pathway-mediated degradation are rather nuclear and cytoplasmic proteins with short half-lives. Taking all data available into consideration, the lysosomal dependent degradation pathway of matured cell surface transporters seems more likely. Beside the known function of ubiquitin to mark cytosolic proteins for proteosomal degradation, it is now evident that it also serves to label membrane proteins for lysosomal destruction. Proteosomal degradation is signalled by polyubiquitin conjugation through lysine residues at position 48 (Lys-48-linked), whereas multiple monoubiquitination and Lys-63-linked polyubiquitination mediate protein degradation in the lysosome by functioning as sorting signals for the „endosomal sorting complex required for transport (ESCRT)” machinery. By means of this machinery membrane proteins are delivered to lysosomes within multivesicular bodies.

Previous observations from our lab:

Several monoclonal antibodies (mAbs) were developed against the external epitopes of Pgp and are used for its detection (e.g. MRK16, MM12.10, UIC2, 15D3, 4E3). Most of these antibodies are able to label all cell surface Pgps. One of these mAbs (UIC2) exhibits conformation-sensitivity as it recognizes the pump only within a window of its catalytic cycle, binding to a composite epitope formed by the 1<sup>st</sup>, 4<sup>th</sup> and 6<sup>th</sup> extracellular loops. UIC2 (or its Fab fragment) applied at saturation binds only to 10-40% of Pgps present in the cell membrane. However, the entire population of Pgp molecules becomes recognized by this antibody in ATP-depleted cells and when it is applied in the presence of certain substrates/modulators (vinblastine, cyclosporineA (CsA) and SDZ PSC 833, but not verapamil). The phenomenon is known as the “UIC2-shift” and has been rather attributed to a greater number of Pgps available for mAb binding, than an increased association constant of the mAb.

Two distinct subpopulations in the PM of multidrug resistant and normal cells, distinguished by binding of the UIC2 mAb. The mAb UIC2 applied at saturation binds 10-40% of Pgps present in the cell membrane, that we refer to as Pool1 Pgps. The rest of Pgp molecules that become recognized by this antibody only in the presence of certain substrates or modulators (e.g. CsA) are referred to as Pool2 Pgps. In the absence of the CsA-type drugs, Pool2 Pgps may also be visualized by sequential labeling with UIC2 and any of a series of antibodies (e.g. MRK16, MM12.10 or 15D3) binding to partially overlapping epitopes.

The conformational heterogeneity of cell surface Pgps must be a general feature of Pgp expressing cells in view of the fact that the UIC2-shift phenomenon has been demonstrated in a wide variety of cell types.

In co-localization experiments performed by confocal microscopy, Pgp molecules of the two “Pools” appear to occupy different territories in the membrane of NIH 3T3-MDR1 cells. The inhomogeneous distribution of the two Pgp Pools may reflect different molecular relationships, suggested by the marked co-localization of Pool1 Pgps with FITC-phalloidin-decorated actin microfilaments. When Pool1 and Pool2 Pgps were selectively labeled with their antibodies, then cells were lysated by TritonX-100 (TX-100) and separated on sucrose density gradients, the low buoyant density raft fractions preferentially contained Pool1 Pgps relative to Pool2 molecules. All these data demonstrate that the two populations of antibody labeled Pgp molecules are present in different membrane microdomains.

Associations of the two Pools with the cytoskeleton, directly and via rafts, were also compared. In the flow-cytometric detergent resistance assay (FCDR), developed by our workgroup, direct cytoskeletal association is reflected by resistance to NP40 treatment, while those Pgp molecules anchored via rafts resist the milder TX-100 detergent. Pool1 Pgps exhibited enhanced cytoskeletal and raft association.

The kinetics of endocytosis of the two Pgp Pools was compared by measuring the loss of primary antibody labeled Pgps from the cell surface using fluorescent dye conjugated secondary antibody. Apparently, Pool1 Pgps are internalized at a significantly higher rate. When all cell surface Pgps are labeled by 15D3 mAb, i.e. in the absence of UIC2, the onset of internalization is delayed. These data demonstrate that Pool1 Pgps appear to become preferentially associated with endocytotic processes, as soon as these Pgps become ligated with UIC2.

Finally, immunoprecipitation studies completed by mass spectroscopic analysis were carried out in order to identify co-precipitating protein partners. These data suggested that the intracellular molecular partners of the two Pgp pools are partially different.

## 2. Aims

During the investigation of Pgp's basal and cholesterol-dependent trafficking, we were facing with some metodological problems:

1. Under in vitro conditions some detergents-most frequently used in membrane raft studies- had an effect on the polymerization properties of actin. This raised the question, how these detergents act on detergent-resistant membranes (DRMs-rafts) in intact cells. Recently, a flow cytometric method was developed by our workgroup for DRM isolation, called FCDR assay. According to the assay raft-associated proteins remain cell associated after detergent solubilization of the whole cells. It was assumed that these membrane proteins could remain on the cell surface because submembrane actin-cytoskeletal connections and associations to raft proteins were kept and this preserved membrane raft proteins. We investigated therefore the actin polymerization properties of these detergents (TX100; NP40; CHAPS; Brij) ex vivo in NIH 3T3-MDR1 cells with the help of flow cytometric and microscopic techniques.
2. In cholesterol extraction studies, carried out by random-methyl- $\alpha$ -cyclodextrin the affinity of the 15D3 monoclonal antibody (used for the detection of total cell surface Pgps) was decreased. We investigated further the phenomenon with the help of  $\alpha$ -cyclodextrin derivatives with different cholesterol solubilizing capacities.

In connection with the conformational and topological heterogeneity of P-glycoprotein we put the following questions in order to investigate trafficking processes of the transporter:

3. Do Pool1 (mainly raft-associated) and Pool2 Pgps (less raft-associated) exhibit differences in their trafficking?
  - a, Is the endocytosis of raft-associated Pool1Pgps indeed faster?
  - b, Is there any difference between the two Pools in the final degradation organelle?
  - c, Do internalization processes exhibit temperature and ATP dependence?
  - d, How do exocytotic trafficking processes change in the presence of the protein synthesis inhibitor, cycloheximide?
4. Is there any difference between the cholesterol-dependent trafficking of the two Pgp conformers, Pool1 and Pool2 Pgps?

In our cholesterol extraction experiments, carried out by cyclodextrins, it came as a surprise to notice that certain exocytotic processes were enhanced. Recent studies reported that cholesterol lowering cyclodextrin treatments stimulated lysosomal exocytosis. The role of lysosomal exocytosis is a well-known phenomenon in membrane repair, where damaged plasma membrane parts are removed and replenished by normal trajectories of exocytosis. These facts led us to hypothesize that the previously observed exocytotic events were likely to be only part of this complex mechanism. According to this the following questions arose that did not closely relate to the trafficking processes of Pgp:

5. We were interested in investigating whether lysosomal exocytosis also takes place in our experimental system?
6. We aimed at comparing the stability of lysosomes in NIH 3T3 and NIH 3T3-MDR1 cells, as recent studies reported that cholesterol can greatly influence it.



7. Additionally, we were interested in studying the consequences of the found lysosomal differences, namely we compared cell viabilities in case of the two cell lines.

### **3. Materials and methods**

#### **3.1 Cell culture**

The human *mdr1* gene-transfected NIH 3T3 mouse fibroblast cell line (NIH 3T3-MDR1 G185, gift from M. Gottesman) was used in most of the experiments. This line is stably transfected by the gene in a retroviral vector. Cells were cultured at 37 °C in a humidified gas incubator containing 5 % CO<sub>2</sub> and subdivided every third day in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % heat-inactivated fetal calf serum, 2 mM L-glutamine, 25 µg/ml gentamycin, 670 nM doxorubicin. Two-three days before the experiments, the medium was replaced by doxorubicin free medium. NIH 3T3 mouse fibroblast cells, as the non-transfected parental counterpart of Pgp expressing cells, were also used in some of the experiments. Cells were tested for mycoplasma regularly. Cells were mainly trypsinized before the experiments.

#### **3.2 Cholesterol modulation of the cell membrane**

Cells were trypsinized and washed with phosphate-buffered saline supplemented with 8 mM glucose (glucPBS). The pellets were suspended at a concentration of  $1 \times 10^6$  cells/ml and incubated with an increasing concentration of randomly methylated  $\beta$ -cyclodextrin (CD), or randomly methylated  $\beta$ -cyclodextrin cholesterol complex (chol-CD; cholesterol content 3.0–5.1%, as determined by high performance liquid chromatography), with other cyclodextrin derivatives, or as a control sample without it for 20 min at 37°C and washed twice again with glucPBS at room temperature.

#### **3.3 Cell surface labeling of Pgp molecules with monoclonal antibodies (mAb)**

UIC2 (IgG2a) and 15D3 (IgG1) mouse monoclonal antibodies (mAb) produced against human P-glycoprotein were isolated from supernatant of their hybridomas and purified using protein A affinity chromatography. mAb preparations were >97 % pure, checked by SDS-polyacrylamide gel electrophoresis. The mAbs were conjugated with different dyes [Alexa488, Alexa647, pHrodo]. The unbound dyes were removed with the help of Sephadex-G50 gel-filtration chromatography. The dye/protein ratio was determined with spectrophotometer based on absorbance. As the extinction molar coefficient of the pHrodo dye was missing, the dye/protein ratio was determined with the help of a concentration curve in this case. CsA (10 µM) was applied at 37°C for 10 minutes before the UIC2 mAb labeling.

#### **3.4 Kd and Bmax values of antibodies (UIC2 and 15D3)**

After the previously described cholesterol modulation of the cell membrane, cells were labeled with various concentrations of 15D3 mAb tagged with A647 (15D3-A647) or UIC2 mAb labeled with A647 (UIC2-A647) for 30 minutes at 37°C followed by two final washes of ice-cold glucPBS. Samples were measured by flow cytometry (BD FACSAArray, Budapest Flow Soft Kft.) in the presence of propidium iodide (PI) in order to exclude dead cells. Experiments were repeated at least 3 times. Curve fitting was done by Sigma Plot version 10.0 software (Systat Software, Inc. San Jose, CA). Dissociation constants (Kd) and the fluorescence of total antigen binding capacities of antibodies (Bmax) were calculated according to the saturation model of ligand binding to one site corrected for non-specific binding using the  $f = \frac{B_{max} * abs(x)}{(Kd + abs(x)) + Ns * x}$  equation.

### **3.5 Endocytosis measurements of cell surface Pgp molecules**

After modulation of cell membrane cholesterol, cells were incubated in DMEM supplemented with 1 % heat-inactivated fetal calf serum, 2 mM L-glutamine, and 25 µg/ml gentamycin and 10 mM Hepes, pH 7.4 for different time periods. Medium was replaced for glucPBS and cells were divided in two aliquots. In one, the surface-bound antibody was removed by acidic treatment (0.1 M glycine, 0.5 M NaCl, pH 2.5) for 3 minutes at RT, while the other aliquot was kept in glucPBS. Finally, cells were washed twice, 5 µg/ml PI was added and assayed using flow cytometer (BD FACSAarray, Budapest Flow Soft Kft.). PI was excited at 542 nm and detected in the Yellow channel (585/42 nm), while A647 was excited at 635 nm and measured in the Red channel (661/16 nm). Data analyses: First, PI-positive fraction was excluded, and values were corrected for nonspecific background. Acid-wash treated cells provided estimates of the amount of internalized Pgps, and glucPBS treated cells provided estimates for total surface bound and internalized transporters. The degree of internalization was calculated as the ratio of the antibody labeling of the acid-treated and untreated cells as a percentage.

### **3.6 Temperature and ATP dependence of internalization**

After labeling of cell surface Pgp molecules cells were washed twice with glucose free or with glucPBS. Cells were further incubated for 30 min in glucose free or in glucPBS. During the incubation time cells were kept on 0 °C (ice); on 37 °C or in the third case on 0 °C and 37 °C with 2 min intervals. Cells were washed with the 0 or 37 °C buffer and labeled with the GamIg-A647 mAb for 30 min on ice. Cell surface transporters were determined by flow cytometry in the presence of PI.

### **3.7 Measurement of the degradation end point of internalized Pgp molecules with the help of pH sensitive dye, pHrodo**

Cells were labeled with pHrodo conjugated mAbs as usual. Cells were washed and fixed with 2% PFA. pH of the buffer containing labeled cells were changed in order to determine pH sensitivity. Different samples were prepared for kinetic measurements. Cells were labeled with UIC2-pHrodo (100 µg/ml; 37 °C; 30 min) with or without CsA preincubation or with 15D3-pHrodo (100 µg/ml; 37 °C; 30 min) with or without preincubation with unlabeled UIC2 mAb. Cells were washed twice with glucPBS. For further incubations cells were kept in the previously described DMEM solution supplemented with 10 mM HEPES. Fluorescence intensities were determined at certain incubation time points by flow cytometry (FACSAarray) (Becton Dickinson, Franklin Lakes, NJ, USA). Samples were excited at 532 nm and detected with the help of a 585/42 band pass filter. Every sample was corrected with background intensity, which was determined on UIC2-pHrodo and 15D3-pHrodo labeled samples with preincubation of unlabeled mAbs, respectively.

### **3.8 Confocal laser scanning microscopy**

Confocal microscopy pictures of cells labeled with pH sensitive dye conjugated mAbs were taken at certain incubation time points (0 min, 3 hour) with the LSM 510 (Zeiss, Jena, Germany) confocal laser scanning microscopy.

### **3.9 Exocytosis of the UIC2 reactive fraction**

Cells were incubated in glucPBS with unlabeled UIC2 mAb (100 µg/ml) for 30 min at 37 °C and washed twice with ice-cold glucPBS in order to pre-cover cell surface Pgps with unlabeled mAb. Then cholesterol modulations were applied and subsequently cells were incubated in DMEM (supplemented as described above) for different times (30, 25, 20, 15, 10, 5 and 2 minutes) in the presence of A647 conjugated UIC2 mAb (100 µg/ml) in order to monitor the newly upcoming Pgps. Cells were washed twice with ice-cold glucPBS and

measured by flow cytometry. Data analyses: PI positive population was excluded. In order to calculate the kinetics of exocytosis, the half labeling time of the UIC2-A647 mAb were taken into consideration with regard to short labeling times in some samples.

### **3.10 Inhibition of exocytosis**

Cells were pre-incubated with Exo 1 (2-(4-Fluorobenzoylamino)-benzoic acid methyl ester, 400  $\mu$ M) or Brefeldin-A (360  $\mu$ M) for 2 hours in glucPBS at 37  $\text{^\circ}$ C. Then cells were treated for cholesterol modulations and the UIC2 reactive cell surface Pgps were labeled with 100  $\mu$ g/ml UIC2-A647 for 5 minutes, washed once with ice-cold glucPBS and measured in the presence of PI. To decrease the labeling time, a 5 minutes pulse label was applied, which labels 91.7 $\pm$ 3.7 % (mean  $\pm$  SD) of Pgps compared to 30 minutes labeling in untreated samples.

### **3.11 Cell surface appearance of LAMP1 and LAMP2 after cholesterol extraction**

Cell monolayers of the NIH 3T3 mouse fibroblast cell line and its human mdr1-transfected counterpart were harvested as described previously and washed either with phosphate-buffered saline (PBS) or with Hank's Balanced Salt Solution (HBSS containing 1 mM  $\text{CaCl}_2$ ) supplemented with 8 mM glucose. In case of the LAMP2 samples, experiments were also carried out with glucPBS supplemented with 1 mM EDTA. The cell pellets were suspended and incubated with or without CD. Next cells were labeled with 2.5  $\mu$ g/ml anti-mouse LAMP1-PE (CD107a-PE) or 10  $\mu$ g/ml LAMP2-Alexa488 (CD107b) for 30 min at 37  $\text{^\circ}$ C, washed and immediately fixed with freshly prepared 2 % paraformaldehyde (PFA) for 1h at RT. In order to determine the total LAMP levels cells were fixed first with 2 % PFA for 20 min at RT. Washed and then quenched with 1.5 mg glycine/ml solution for 10 min. Anti-mouse LAMP1-PE antibody or LAMP2-Alexa488 was added for 1h at RT in 0.5 % saponin/ 1% BSA buffer. Cells were washed extensively with 0.5 % saponin/ 1% BSA buffer and resuspended in glucPBS 1% BSA and measured immediately by flow cytometer (BD FACSAria III). Cell surface anti mouse LAMP fluorescence intensities are expressed as percentage of the total LAMP fluorescence in a cell.

### **3.12 Measurement of cellular cholesterol levels and cell viability of MDR1 transfected and non-transfected cell lines**

The NIH 3T3 mouse fibroblast cell line and its human mdr1-transfected counterpart were used in these experiments. Cells were treated with different concentrations of CD or chol-CD as usual and washed twice with glucPBS. Cells were either labeled with PI (5  $\mu$ g/ml) and measured immediately by flow cytometer or fixed with freshly prepared 3 % PFA for 1h at RT for further procedure. Fixed cells were washed twice with glucPBS and incubated with 1.5 mg glycine/ml glucPBS for 10 min at RT. Cells were finally stained with 125  $\mu$ g/ml filipin in glucPBS / 10 % FBS for 1 h at RT in dark and measured by flow cytometer (BD FACSAria III). PI fluorescence signals were taken at 561 nm excitation with 561 LP filter, while 450/40 BP filter was used with 375 nm excitation to detect filipin signals. Measured cholesterol contents were corrected for cell volume. Cell viability curves were calculated according to sigmoidal dose-response ligand binding using the  $f = \text{min} + (\text{max} - \text{min}) / (1 + 10^{(\log \text{EC}_{50} - X)})$  equation with Sigma Plot software.

### **3.13 Determination of lysosome content of cells**

The lysosomatropic chromatic dye neutral red (NR) staining was used for quantitative imaging cytometry. For single cell analyses, cell nuclei were stained by Hoechst 33342 and lysosomes were loaded with NR. Cells were recognized and contoured by a total cellular signal adding together the Hoechst 33342 (excited with 405 nm violet laser and detected via 430-470 nm bandpass filter) and NR fluorescence (excited with 488 nm blue laser and

detected via a 650 nm longpass filter) and the light absorbance of NR (illuminated at 488 nm). Single cells were selected by area of the summed signal and subsequently by DNA content using the Hoechst 33342 signal. Finally, lysosomal content of single cells was determined by the NR chromatic intensity of identified cells. Measurements and analyses were made applying the fluoro-chromatic option of our iCys laser-scanning imaging cytometer and its iNovator Applications Development Toolkit software (Thorlabs Imaging Systems, Sterling, VA, USA, formerly known as CompuCyte).

### **3.14 Measurements of photo-destruction of lysosomes**

Acridine orange (AO) sensitized light induced photo-destruction of lysosomes was investigated by laser-scanning cytometry (iCys). Cells were cultured in 8-well tissue culture plates in DMEM and incubated until they reached confluency. Cells were stained with 1  $\mu\text{g/ml}$  AO in glucPBS for 30 minutes at 37 degree and washed twice with glucPBS. AO loaded cells were then illuminated by a solid-state 488-nm blue laser in an area repeatedly in 50 cycles by 40x objective (Olympus, UIS2 40x plan fluorite, NA 0.75). The area was set to be covered by 2x2 scanning field windows using 0.25 micrometer step size (one scanning field window is 1024x768 steps). Fluorescence intensity of red (565-595, 580/30 nm) and green (515-545, 530/30 nm) ranges of light emission was measured at each time point. Data was exported from the iCys software to Microsoft Excel spreadsheet program and calculations were done with it. The high concentration of stacked AO dye molecules accumulating in lysosomes fluoresces red. During blue light illumination, AO-sensitized lysosomal membrane ruptures and dye molecules are released from the acidic milieu, consequently red fluorescence decreases. Continuous release of AO dye to the cytosol and its binding to DNA in the nucleus increases green fluorescence. The time constant at which the green fluorescence is half of the total green fluorescence intensity of cells was determined by fitting sigmoid curve to data applying an online curve-fitting tool (<http://zunzun.com/>). Mean and SD of the time constant in seconds of the half-maximum-fluorescence were calculated from 15 and 10 cells. Cholesterol dependence of photo-destruction of lysosomes was investigated as described hereinafter. The 20 min acute cyclodextrin (CD; chol-CD) treatment of adherent cells was followed by the 30 min acridine orange (AO) incubation, and then a field of view of cells was exposed to a continuous blue light irradiation (FITC filter set) using the epifluorescence Hg-lamp illumination of the iCys system (an Olympus IX-71 fluorescent microscope). Microscopic videos were recorded by an Olympus DP71 (Olympus, Budapest, Hungary) high resolution color digital camera from the beginning of irradiation. Events of lysosomal rupture, corresponding to fluorescence flashes in the green channel were recorded over time.

Analysis of image series:

The detection of exocytotic events was performed using an automated program based on what had been used for the analysis of calcium sparks on time series confocal images (Rodriguez, Lefebvre et al. 2014). In the first step, intermean thresholding with a correction factor of 1.65 was performed on the first frame in each series to create a mask of pixels belonging to cells. Morphological closing was applied on this mask to eliminate features smaller than 5 pixels in diameter. A background area completely free of cells was manually selected and the average intensity of this area was subtracted from all pixel intensity values.  $F/F_0$  series were calculated for each pixel, where intensities below mean + 1.8 SD of the time series data for the given pixel were considered baseline ( $F_0$ ). Events were identified on each frame as contagious groups of pixels with intensity values above mean + 1.12 SD containing at least one pixel above mean + 1.18 SD within the cell area mask. Events containing less than 45 pixels were dropped. The diameter of this group of pixels was measured in the X and Y direction and the arithmetic mean of these two numbers was used to describe the size of each exocytotic event. To determine the event frequency for a given condition, the number of events on all images was counted. Events that occurred on consecutive images at the same

location were counted as a single event. Numbers were normalized to the cell number. Irradiation times required to destroy every lysosome were also presented for both cell-lines.

### **3.15 Cholesterol extraction effect on lysosomatropic dye accumulation**

For dye accumulation studies cells were cultured in 96-well tissue culture plates at a density of  $1 \times 10^5$  cells/well in DMEM. Cells were incubated at previously described adequate conditions for 24 h. This incubation period allows for cell recovery and adherence and for exponential growth according to previous observations (Repetto, del Peso et al. 2008). After gently replacing the medium, cells were incubated with different concentrations of CD solution and 1  $\mu$ g/ml acridine orange solution, and fluorescence intensity measurements were taken with a Synergy HT reader (Bio-Tek Instruments, Winooski, VT, USA) at 460 nm excitation and 645 nm emission for red fluorescence intensities and at 485 nm excitation and 528 nm emission for green one.

### **3.16 Fluorescent Labeling of Actin**

To characterize the process of polymerization, actin was labeled with N-(1-pyrene) iodoacetamide; 1 mg/ml F-actin (in buffer A without DTT, supplemented with 100 mM KCl and 2 mM MgCl<sub>2</sub>) was incubated with 1.1-fold of pyrene iodoacetamide at room temperature for 18 h. Labeling was terminated with ultracentrifugation of the sample for 45 min at 328,000g and the pellet was incubated in buffer A for 2 h and then gently homogenized with a homogenizer. The homogenized sample was dialyzed overnight against buffer A at 4°C. The concentration of the fluorescent dye in the protein solution was determined by using the absorption coefficient of 45,000 M<sup>-1</sup> cm<sup>-1</sup> at 344 nm for actin-bound pyrene iodoacetamide. The extent of labeling was 0.7 mol/mol of actin monomer.

### **3.17 Flow cytometric detergent resistance assay (FCDR)**

After cell surface labeling of Pgp (15D3-A647 or UIC2-A647) and CD treatment, samples were divided in half. TX-100 was added to one-half of the sample at a final concentration of 0.5%, and the other one-half of the sample was mock treated. During the 15-minute treatment, cells were kept on ice and fixed with freshly prepared 2% paraformaldehyde. Fluorescence intensity of cells was measured in flow cytometer (BD FACSArray, Budapest Flow Soft Kft.). The percentage of the detergent resistant fraction was calculated as described previously (Bacso, Nagy et al. 2004; Gombos, Bacso et al. 2004).

### **3.18 Statistical analysis**

Statistical analysis was performed with GraphPad Prism software (6.01 version) by unpaired, two-tail t-test. Differences were considered to be significant at  $p < 0.05$ . Exact p values are also displayed.

## **4. Results and discussion**

### **4.1 Observations and methodological problems related to our experiments.**

#### **4.1.1 The effects of detergents on the polymerization properties of actin.**

Under in vitro conditions some detergents-most frequently used in membrane raft studies- had an effect on the polymerization properties of actin. In subsequent ex vivo experiments we studied how these modification effects, primarily polymerization, are manifested in cells. These observations were quite important, as we developed a flow cytometric method for detergent resistant membrane isolation, called flow cytometric detergent resistance assay

(FCDR) assay. In general, every detergent begins actin depolymerization around its critical micellar concentration. NP40, TX100, and CHAPS has 0.0105 (w/v)%, 0.0149 (w/v)%, and 0.1476–0.6150 (w/v)% CMC, respectively. However, Brij98, which has the lowest micellar concentration [0.0029 (w/v)%] and had a slight actin polymerization effect in vitro, was out of this tendency obtained for the other detergents in flow cytometric experiments. Due to their filamentous actin content under the cell pernatant, cells kept such degree of cellular morphology, even with the highest amount of detergents applied, that their total cellular filamentous actin content was quantifiable by flow cytometry, such as its pattern by confocal laser scanning microscopy. The observed actin depolymerization property of TX100 might query the amount of the raft associated fraction of Pgp labeled by UIC2 or 15D3. But it probably did not influence their relative raft association compared to each other.

#### **4.1.2 Cholesterol-dependent conformational changes of P-glycoprotein are detected by the 15D3 monoclonal antibody**

While studying the cholesterol dependent trafficking of Pgp we found that binding affinity of the 15D3 monoclonal antibody showed great cholesterol dependence. We made further experiments with the help of  $\beta$ -cyclodextrin derivatives with different cholesterol solubilizing capacities. We investigated cholesterol content of NIH 3T3-MDR1 cells using filipin staining. Cellular cholesterol levels were reduced after treatment with different CD derivatives, and data exhibited a negative linear correlation ( $r = -0.97$ ) with the cholesterol-solubilizing capacities of CDs. This confirmed that CD derivatives are appropriate tools for extracting different amount of PM cholesterol. The binding affinity of the 15D3 mAb showed strong linear correlation ( $r=0.98$ ) with cholesterol content of cells. Our results and further data from the literature confirm that 15D3 mAb binds to a conformation sensitive epitope of Pgp that was responsive to PM cholesterol levels in single cells.

#### **4.2 UIC2 labeled Pool1 Pgps exhibit enhanced internalization**

The kinetics of endocytosis of the two cell surface Pgp Pools was previously compared by measuring the loss of primary antibody labeled Pgps from the cell surface using fluorescent dye conjugated secondary antibody. Pool I Pgps are internalized at a significantly higher rate. In order to confirm the enhanced internalization of Pool1 we carried out further experiments by directly measuring the fraction of internalized Pgps after removal of antibodies exposed on the cell surface by acidic washes. After 10 minutes 20.9 $\pm$ 0.8% of UIC2 labeled Pool1 Pgps and 8.5 $\pm$ 1.0% of CsA+UIC2 labeled Pgps located intracellularly. When all cell surface Pgps are labeled by 15D3 mAb or CsA+15D3, i.e. in the absence of UIC2, onset of internalization is delayed, as the internalized fraction was only 1.8 $\pm$ 0.5% and 2.2 $\pm$ 0.3%. After 45 minutes the internalized amount was 42.6 $\pm$ 4.0% for UIC2 labeled Pool1 Pgps and 18.2 $\pm$ 0.9% for CsA+UIC2 labeled Pgps compared to samples labeled by 15D3 (Pool1+2) or CsA+15D3 (4.1-5.2%). These results were reconcilable with previous results and demonstrated that UIC2 labeled Pool1 Pgps exhibit and increased internalization compared to Pool2 Pgps. However, if we compare the CsA+15D3 labeled and the CsA+UIC2 labeled samples it is apparent that the UIC2 monoclonal antibody plays an important role in this internalization, as Pool 1 appears to become preferentially associated with endocytotic processes, as soon as these Pgps become ligated with UIC2.

UIC2 applied at saturation binds only to 10-40% of Pgps (Pool1 Pgps) present in the cell membrane. At the same time we determined the exact amount of Pool1 Pgps in every experiment, therefore we were able to compare the absolute amount of internalized Pgps in case of the UIC2 and CsA+UIC2 labeled samples. In the first 10 minutes 6.4 $\pm$ 1.1% (UIC2 labeled) and 8.5 $\pm$ 1.0% (CsA+UIC2 labeled) of the cell surface Pgps were intracellular. After 30 minutes these internalized amounts were 10.4 $\pm$ 2.2% and 12.7 $\pm$ 1.59%. These quite similar quantities in the early incubation times in case of the UIC2 labeled and CsA+UIC2 labeled

samples made likely that original Pool1 Pgps are internalized first. However the endocytosis of induced Pool1 Pgps, i.e. in the presence of CsA sequentially follows the internalization process.

#### **4.3 The internalization of UIC2 labeled Pool1 Pgps show temperature and ATP dependence**

In order to confirm that internalization of Pool1 Pgps is an active process we set different ATP levels and temperatures in our experiments. In the absence of ATP the endocytosis of the UIC2 labeled Pool1 Pgps stopped and it exhibited clear temperature dependence in the presence of ATP. Therefore the internalization of Pool1 Pgps seem to be an active process.

#### **4.4 Lysosome organelles are candidates for the degradation of Pool1 Pgps**

In order to determine the organelle for final degradation of Pgp, we made further experiments with the help of our antibodies conjugated with a pH sensitive dye (UIC2-pHrodo and 15D3-pHrodo). First, we confirmed the pH sensitivity of the antibody conjugated dyes on fixed cellular samples. With the help of these dye conjugated antibodies we have followed the appearance of the different Pgp Pools in the acidic compartment in kinetic measurements. The greatest fluorescence increment was observed for the UIC2-pHrodo (Pool1) sample. So as to control the absolute amounts of Pgps in the acidic compartments we carried out endpoint measurements. The initial relative fluorescence ( $36.3 \pm 1.2\%$ ) of UIC2-pHrodo (Pool1) increased to  $98.2 \pm 14.1\%$  at the end of the 180 minutes incubation. In case of the CsA+UIC2-pHrodo (Pool1+Pool2) and 15D3-pHrodo (Pool1+Pool2) samples, the fluorescence increment was also observable (from  $100.0 \pm 0.4\%$  to  $136.4 \pm 11.5\%$ ), (from  $100.0 \pm 0.2\%$  to  $153.1 \pm 13.2\%$ ). However in case of the UIC2 prelabeled and 15D3-pHrodo labeled sample (Pool2) the fluorescence increment was much smaller (from  $104.7 \pm 0.9\%$  to  $116.5 \pm 11.6\%$ -ra). In most cases the fluorescence increment was not noticeable.

Our data demonstrate that the protein, most likely the UIC2 labeled Pool1 conformer, enters the lysosomal degradation pathway in accordance with the internalization experiments.

#### **4.5 Pool1 Pgps recycle to the cell surface**

We would have liked to examine the level of cell surface Pgps after UIC2 monoclonal antibody induced internalization. Do initial Pgp levels recover, and if yes, on what time scale? We have monitored the newly upcoming Pgps to the cell surface with fluorescently labeled UIC2-A647 and 15D3-A647 after precovering initial cell surface ones with the help of UIC2 and CsA+UIC2 preincubation. All cell surface Pgps are covered, and based on previous results are internalized with the application of CsA+UIC2 preincubation. With the help of UIC2 preincubation only original Pool1 conformers are covered. After covering original Pool1 Pgps with the help of UIC2 prelabeling, we have monitored cell surface Pgps with the help of UIC2-A647 and 15D3-A647. The initial Pool1 fraction restored for the first incubation time point (30 minutes). However only a fraction of initial cell surface Pgps can be detected on the cell surface after covering all cell surface Pgps with the help of CsA+UIC2. The percentage of this fraction was reconcilable with the initial Pool1 fraction. Restoration of total cell surface Pgp levels required longer incubations (24 hours).

After covering original Pool1 Pgps (UIC2 prelabeling) in the presence of cycloheximid (inhibition of protein synthesis) both Pool1 and total cell surface Pgp levels restored for the first incubation time point (30 minutes). However, after the first hour of incubation total cell surface Pgp levels gradually decreased till the last incubation time point (24 hour). These results were completely compatible with the half life time (14-17 hour) of the protein. After covering all cell surface Pgps (CsA+UIC2) in the presence of cycloheximid, initial Pgp levels failed to restore and even after longer incubation times only a certain fraction could be detected, that amount was reconcilable with the original Pool1 fraction.

All these results suggest that a certain amount of Pgp is localized intracellularly and accordingly the appearance of these Pgps on the cell surface is independent from de novo protein synthesis. From these experiments it seems quite likely that Pool1 Pgps are continuously internalize and recycle to the cell surface.

#### **4.6 Cholesterol extraction increased the amount of raft-associated, UIC2-binding conformer of Pgp in cell surface membranes**

It seems quite likely that Pgp Pools located in different membrane microdomains exhibit different cholesterol dependent trafficking as well. First we asked whether alterations of cholesterol levels of the PM affect cell surface level of different Pgp conformers. We have determined the fluorescence of total antigen binding capacities (Bmax) of anti-Pgp mAbs (UIC2 and 15D3), applying them at various concentrations in the presence of cyclodextrin (CD) treatments; CD to extract cholesterol from cell membrane and chol-CD to increase cholesterol levels.

An increase in cell surface Pgp was observed, detected using either the UIC2 (Pool1) or the 15D3(Pool1+2) mAbs after cholesterol depletion. When cholesterol levels were increased, cell surface levels of Pgp transporters were decreased, demonstrating the cholesterol dependence of the phenomenon. In order to confirm that elevations in antibody binding did not originate from affinity changes, dissociation constants ( $K_d$ ) of anti-Pgp mAbs were also determined. Affinity alterations were either not significant (UIC2), or had a tendency to fall (15D3) when cholesterol levels were reduced. Therefore, the measured elevation of antibody binding cannot originate from changes in binding affinity. These data indicated that changes in expression of Pgp conformers on the PM were inversely proportional to cholesterol levels. In addition, it must be highlighted that these changes in protein levels affected the raft-associated UIC2 reactive conformer, which also manifested in total cell surface Pgp level alterations in the 15D3 reactive conformers.

#### **4.7 Cholesterol depletion induced rise in the UIC2-binding Pgp conformer is the result of increased exocytosis and not decreased endocytosis**

We demonstrated previously that raft associated, UIC2-binding Pgp was internalized significantly faster, than non-raft associated Pgp molecules. Thus, we hypothesized that increased cell surface amounts of UIC2-binding Pgp may result from cholesterol dependent trafficking. We examined the cholesterol dependent internalization process in acidic wash experiments. The rapid internalization of UIC2-binding transporters was further enhanced when cholesterol level was increased by chol-CD. The endocytosed amount of UIC2-binding Pgp was  $39 \pm 5$  % higher than the control, 15 minutes after the chol-CD treatment. Surprisingly, cholesterol depletion did not change the internalized fraction of UIC2-binding Pgps significantly: it was  $103 \pm 2$  % compared to the  $100 \pm 2$  % control sample. We made similar observation for total Pgp changes applying the 15D3 mAb. Thus, the increased cell surface amount of UIC2 reactive Pgps could not be explained by altered internalization. We also examined the kinetics of exocytosis, which behaved in the opposite manner. The rate of exocytosis was increased when the cell membrane was depleted in cholesterol and was reduced after the PM was cholesterol enriched, as the amount of UIC2 labeled transporters was increased to  $250 \pm 48$  % for the CD treated sample and decreased to  $55 \pm 21$  % for the chol-CD treated sample compared to the untreated control ( $100 \pm 28$  %), 15 minutes after cholesterol modulation. These data indicate that alterations in cholesterol content of the PM extensively modified the exocytosis of the raft-associated fraction of this transmembrane protein.



#### **4.8 Inhibition of exocytosis prevents increase of UIC2 bound Pgp caused by cholesterol depletion**

To confirm these exocytotic events, we examined the cholesterol-modulated effects in the presence of drugs known to inhibit exocytosis. Exo1 decreased the cell surface level of UIC2 bound Pgps in every case. Cell surface levels were decreased by  $38 \pm 6\%$  for CD treated cells;  $29 \pm 7\%$  for control cells and  $6 \pm 5\%$  for the chol-CD treated cells. The pattern of inhibition was compatible with the concept of a major role for exocytosis in this process. The effect of brefeldin-A (BFA) was less pronounced in case of cholesterol depleted cells, and in case of cholesterol supplementation or at undisturbed cholesterol contents, the cell surface level of the UIC2 reactive conformer was increased. Exo1 inhibits ARF1 function in an ARF-GAP-dependent manner in the Golgi. Morphologically, Exo1 induces rapid redistribution of the Golgi content back to the ER by transforming the protein and lipid biosynthetic vesicular system into a tubular form. BFA, is a noncompetitive inhibitor of the ARF1-GDP/GTP exchange factor and therefore inhibits many downstream cytosolic effectors of ARF1. With the inhibition of these complexes, BFA affects vesicular transport not only at the secretory but at the endocytic and recycling pathways as well. The different targets of these drugs reflected in our results. Based on the literature perturbation of PM cholesterol levels, initiates several compensatory mechanisms. Our exocytotic events were completely reconcilable with the kinetics of these compensatory mechanisms. From these observations, we concluded that UIC2 binding conformers showed trafficking with raft domains in line with the compensatory cholesterol and shingolipid export.

#### **4.9 Exocytosis of LAMP1 and LAMP2 were enhanced after cyclodextrin treatment as well in a calcium and cholesterol dependent manner**

Recent studies showed that cholesterol lowering CD treatments stimulated lysosomal exocytosis. The role of lysosomal exocytosis in a calcium-dependent manner is a well-appreciated phenomenon in membrane repair, where damaged PM parts are removed and replenished by normal trajectories of exocytosis. It was supposed that cholesterol extraction permeabilizes the PM to small ions present in the extracellular milieu, such as  $\text{Ca}^{2+}$ , and triggers lysosomal exocytosis. These facts led us to hypothesize that the enhanced exocytosis of Pgp was likely to be only part of this complex mechanism. To proof our idea, we measured the cell surface level of the lysosome-associated membrane protein 1 (LAMP1), a marker for lysosomal exocytosis. LAMP1 increased proportionally with decreasing cholesterol by applying increasing CD concentrations in the presence of extracellular  $\text{Ca}^{2+}$  in Pgp overexpressing NIH 3T3-MDR1 cells. The effect was more pronounced in the presence of extracellular  $\text{Ca}^{2+}$ , but it was evident without  $\text{Ca}^{2+}$  as well. Fusion events require  $\text{Ca}^{2+}$ , with  $\text{Ca}^{2+}$  being released from the lumen of the fusing organelles. In case of lysosomal exocytosis, the two involved “organelles” are the PM and lysosomes. This means that the increase of intracellular  $\text{Ca}^{2+}$ , which is requisite for lysosome fusion with the PM, may derive from the EC milieu or from the lysosome itself. Recently, an inwardly rectifying cation channel, the mucolipin transient receptor potential (TRP) channel 1 (TRPML1) has been confirmed to be responsible for intra-lysosomal- $\text{Ca}^{2+}$  release and regulation of lysosomal exocytosis. Interestingly, the channel demonstrated to be under significant lipid control.

Another report that many MDR cell lines secrete increased amounts of lysosomal enzymes, prompted us to further investigate the process. We have compared the NIH 3T3-MDR1 cell line stably expressing Pgp to its parental non-transfected NIH 3T3 counterpart with respect to LAMP1 expression. An increased proportion of LAMP1 was present on the surface of NIH 3T3-MDR1 cells (7.8 %) as compared to non-transfected cells (2.8 %) in the absence of added  $\text{Ca}^{2+}$  and in the presence of  $\text{Ca}^{2+}$  (9.4 % compared to 4.7 %) added extracellularly.

Cholesterol depletion enhanced cell surface levels from 7.8 % to 14.3 % in the absence of added  $\text{Ca}^{2+}$  and from 9.4 % to 18.4 % when  $\text{Ca}^{2+}$  was present in the buffer. In parental NIH 3T3 cells, a smaller enhancement was observed. Observations carried out with LAMP2 were reconcilable with these results. These data suggest increased recycling between lysosomes and the PM in Pgp overexpressing MDR cells. Moreover, lysosomal exocytosis, which takes part in membrane repair, was further enhanced in Pgp overexpressing cells after cholesterol depletion of the PM. Our data indicate that not only extracellular calcium, as it was known earlier, but also PM cholesterol, as a new observation, has a role in membrane repair.

#### **4.10 Enhanced number and increased stability of lysosomes in NIH 3T3-MDR1 cells**

In a membrane repair process, that relies on the fusion of lysosomes with the PM, the number and the stability of lysosomes are important factors. First we compared the amount of acidic vesicles in Pgp-transfected and non-transfected cell lines by measuring accumulation of a lysosomatropic weakly basic chromatic dye, neutral red (NR). NIH 3T3-MDR1 cells showed more intense NR staining by light microscopy. Next, we measured NR intensity by quantitative imaging cytometry. NIH 3T3-MDR1 cells accumulated 1.5 times more NR than NIH 3T3 cells. We also determined light side scatter (SSC) intensities of these cells by flow cytometry. This measurement also indicated that NIH 3T3-MDR1 cells have more granularity. All of these data signify more numerous and functional endo-lysosomes in NIH 3T3-MDR1 cells than in its parent NIH 3T3 cell line. Then we examined the stability of the lysosomes by measuring photo-oxidation of another weakly basic lysosomatropic dye, acridine orange (AO), using laser-scanning cytometry. AO, which is excited by blue light and acts as a metachromatic dye, emits red fluorescence when highly concentrated inside endo-lysosomes. AO fluorescences in green when it dilutes into the cytosol as a result of rupture of lysosomal membranes induced by the blue light-activated AO caused photo-oxidative damage of the endo-lysosomal membranes. The loss of lysosomal integrity was monitored by measuring the time required to reach the half maximum of the green fluorescence intensity from the beginning of the blue 488-nm laser illumination. We found that NIH 3T3-MDR1 cells exhibited longer half-maximum time ( $409 \pm 184$  sec) compared to NIH 3T3 cells ( $296 \pm 135$  sec). These laser-scanning cytometric measurements indicated more stable lysosomes in MDR cells. Our data provided evidence for the increased number and stability of endo-lysosomes in NIH 3T3-MDR1 cells, which data further suggest that NIH 3T3-MDR1 cells might have enhanced membrane repair and consequently less sensitivity to membrane damage than NIH 3T3 cells.

#### **4.11 Lysosomes of NIH 3T3-MDR1 cells were more resistant to photolysis than lysosomes of parental NIH 3T3 cells, even after cholesterol depletion**

PM cholesterol, that is often increased in MDR cells, likely plays an important role in the maintenance of lysosomal membrane integrity. In a recent study, it was suggested that cholesterol depletion with CD decreased lysosomal membrane stability. Therefore we further examined the lysosomal membrane stability of our two cell lines at the earliest times following cholesterol modulation using live-cell video microscopy. Lysosome stability was affected by cholesterol modulation in both cell lines, with cholesterol depletion reducing the exposure times needed for photolysis and added cholesterol increasing it (NIH 3T3 cells CD: 29 sec; control: 46 sec; cholCD: 60 sec) (NIH 3T3-MDR1 cells CD: 40 sec; control: 65 sec; cholCD: 79 sec). However the number of lysosome rupture events after cholesterol depletion greatly reduced only in the 3T3 cell line, suggesting the disruption of these organelles prior to photolysis. To determine the concentration dependence of cholesterol depletion more precisely, we examined lysosomal photolysis using a spectrofluorometric plate reader. CD treatment sensitized more NIH 3T3 cells to AO, as they exhibited steeper decline in red fluorescence and enhanced green fluorescence with increasing levels of cholesterol depletion.

This corresponds to disappearance of red acidic granules and conversion of the dye into the monomeric green form in cytoplasmic and nuclear compartments. A different pattern was observed with NIH 3T3-MDR1 cells with a slight decrease in both red and green fluorescence, a sign of reduction in the number of acidic organelles. Whereas the changes in fluorescence were indicative of intracellular rupture of lysosomes in NIH 3T3 cells, the alterations in NIH 3T3-MDR1 cells indicated a different mechanism for the loss of lysosomes. Based on our LAMP1 and LAMP2 observations, the latter was likely the result of lysosomal exocytosis. These data reverberate the notion that NIH 3T3-MDR1 cells have more efficient membrane repair than NIH 3T3 cells.

#### **4.12 The more effective repair mechanisms of NIH 3T3-MDR1 cells result in higher viability after cholesterol modulation**

Better membrane repair should lead to better cell survival after cholesterol modulation. We therefore compared our Pgp expressing cell line with its parental line focusing on sensitivity to cholesterol perturbations. Cholesterol depletion reduced cell viability of the NIH 3T3 control cells by  $46.4 \pm 3.5$  % following treatment with 5 mM CD. This is compared to (less than half of)  $20.6 \pm 4.3$  % decrease of the Pgp expressing line. Interestingly, addition of cholesterol to the PM resulted in similar decreases in cell viability. We measured the cholesterol content of these cells by filipin staining and flow cytometry. The Pgp overexpressing line had higher levels of cellular cholesterol ( $216.4 \pm 13.3$  %) compared to its counterpart ( $100.0 \pm 6.2$  %). The observed different PM cholesterol content could already explain cell viability disparities after cholesterol extraction, though similar cell viability tendencies were observed after cholesterol saturation as well. Moreover, when monitored by filipin, similar pattern of the cholesterol content was detected in the two cell lines after cholesterol modulations, compared to their initial cholesterol set points (filipin stained control samples without cholesterol modulation). These almost parallel curves indicated more efficient membrane repair and cholesterol compensatory mechanisms in the Pgp positive cells after cholesterol extraction and cholesterol loading of the PM as well. We assumed that membrane repair of NIH 3T3-MDR1 cells after cholesterol depletion might carry more rafts, which has excess of cholesterol, to the cell surface according to the increased exocytosis of the raft conformer Pgp. In case of cholesterol overload, absorption of the surplus cholesterol in rafts from the cell surface is managed by the endocytotic traffic to intracellular cholesterol reservoir vesicles.

Our acute cholesterol depletion experiments demonstrated that lysosome stability quickly responds to PM cholesterol levels. Therefore, it can be assumed that, if a cell effectively controls PM cholesterol, it might influence the level of cholesterol in lysosomes as well. Our results show that the extent of loss of cholesterol from lysosomes might decide between life and death, whether it acts as an initial feedback for membrane repair or enhances suicidal membrane permeability of lysosomes. MDR cells have more effective cholesterol compensatory mechanisms, including increased uptake and esterification of excess cholesterol from PM and enhanced cholesterol synthesis. The enhanced internalization of raft domains constitutes another cholesterol pool. Moreover the higher number of raft domains that contain most of the cholesterol with low “escape tendency” in the PM, also give MDR cells the ability to react to relatively small changes of cholesterol level. Our cell viability measurements following cholesterol addition also show that MDR cells are more proficient in managing increased PM cholesterol levels. The more effective cholesterol homeostatic and membrane repair process, observed in Pgp overexpressing MDR cells, includes re-formation of raft domains to the PM indicated by the rise of the raft-associated conformer of Pgp.

## 5. Summary

1. We have confirmed that detergents, which are most frequently used in membrane raft studies, have an effect on the polymerization of actin *ex vivo* as well. The depolymerization effect of most detergents, applied vitally, was first observed at around their critical micellar concentrations.

2. Cholesterol modification of PM influenced the binding affinity of 15D3 mAb. 15D3 mAb binds to a conformation sensitive epitope of Pgp that was responsive to PM cholesterol levels.

3.a, We have confirmed that UIC2 labeled Pool1 Pgps show enhanced internalization compared to Pool2 Pgps.

b, We have confirmed that internalized Pool1 Pgps more likely choose the late endosomal, lysosomal degradation pathway. At the same time we have recognized that binding of the UIC2 mAb promotes internalization.

c, We have confirmed that uptake of UIC2 labeled Pgps is an energetically active process.

d, We have confirmed that Pool1 Pgps recycle to the cell surface on a time scale of 30 minutes. At the same time, recovery of Pgp expression to its original level requires intact protein synthesis and much longer time after internalization of the total amount of cell surface Pgp.

4. Alterations in cholesterol content of the PM modified the trafficking of raft-associated Pool1 Pgps. Cholesterol surplus increased the endocytosis and decreased the exocytosis of Pool1 Pgps. Cholesterol depletion induced the exocytosis of Pool1 Pgps. The timing of the process was closely linked to the timing of the compensatory cholesterol and sphingolipid export via the secretory pathway.

5. Cholesterol extraction by CD triggers lysosomal exocytosis in NIH 3T3 and NIH 3T3-MDR1 cells as well. Data indicate that not only extracellular calcium, as it was known earlier, but also PM cholesterol, as our new observation, has a role in this membrane repair process.

6. Pgp-overexpressing drug resistant NIH 3T3-MDR1 cells possess more numerous and stable lysosomes compared to drug sensitive parental NIH 3T3 cells without and even after cholesterol modulations.

7. Compared to parental cells, NIH 3T3-MDR1 cells were more resistant to cholesterol perturbations of the PM. This was reflected in significantly better viabilities.

## **6. Publications**



Registry number: DEENK/211/2016.PL  
Subject: PhD Publikációs Lista

Candidate: Zsuzsanna Gutay-Tóth  
Neptun ID: H5EJ0Q  
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

### List of publications related to the dissertation

1. **Gutay-Tóth, Z.**, Fenyvesi, F., Bársony, O., Szente, L., Goda, K., Szabó, G., Bacsó, Z.:  
Cholesterol-dependent conformational changes of P-glycoprotein are detected by the 15D3 monoclonal antibody.  
*Biochim. Biophys. Acta Mol. Cell Biol. Lipids.* 1861 (3), 188-195, 2016.  
DOI: <http://dx.doi.org/10.1016/j.bbalip.2015.12.007>  
IF: 4.779 (2015)
2. Újfalusi-Pozsonyi, K., Hild, G., Gróf, P., **Gutay-Tóth, Z.**, Bacsó, Z., Nyitrai, M.: The effects of detergents on the polymerization properties of actin.  
*Cytometry A.* 77 (5), 447-456, 2010.  
DOI: <http://dx.doi.org/10.1002/cyto.a.20855>  
IF: 3.749





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### List of other publications

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**Total IF of journals (all publications): 16,99**

**Total IF of journals (publications related to the dissertation): 8,528**

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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