(Patho)physiological function of the Multidrug Resistance protein MRP1
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Chapter 3

Transport of glutathione-conjugates into secretory vesicles is mediated by the multidrug resistance protein 1

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Abstract

Intracellular glutathione-conjugate transport was evaluated in the human small cell lung carcinoma cell line GLC4 with low multidrug resistance protein (MRP1) expression and its 300x doxorubicin resistant, MRP1 overexpressing, GLC4/Adr subline. Transport of non toxic concentrations of monochlorobimane and 5-chloro-methyl fluorescein diacetate was evaluated using fluorescence microscopy. Fluorescence was, after exposure to these compounds, especially observed in intracellular vesicles in GLC4/Adr. Immunotransmission electron microscopy showed that MRP1 was present in the vesicle membranes and plasma membrane, while inside the vesicles the glutathione-conjugate of 1-chloro 2,4-dinitrobenzene could be detected. Experiments with brefeldin A, which induces arrest in vesicle release from the Golgi complex, indicated that these vesicles may originate from the trans Golgi network. In GLC4/Adr cells doxorubicin was also transported in vesicles, with also an arrest in vesicle release from the Golgi complex. These results indicate that MRP1 functions as a glutathione-conjugate transporter not only at the plasma membrane, but also in intracellular secretory vesicles.
Introduction

Tumour cells selected in vitro for resistance against natural products such as anthracyclines, epipodophyllotoxins and vinca-alkaloids, are often cross resistant to a range of other drugs with different chemical structures and cellular targets. This type of resistance is the so-called multidrug resistance (MDR). A major cause for MDR is the overexpression of the MDR1 gene. This gene encodes for the plasma membrane glycoprotein MDR1 P-glycoprotein (MDR1), that induces MDR by an increased export of drugs from the cell resulting in lower cellular drug concentrations.¹ Cole et al. discovered another membrane transporter gene that can confer MDR, namely MRP1 in a doxorubicin resistant small cell lung carcinoma cell line.² MRP1 is a member of the ATP-binding cassette transporter gene superfamily. Many, but not all,² MRP1 overexpressing cells have an increased drug efflux.³⁴ Transfection with the MRP1 gene resulted in resistance to many MDR drugs⁴⁻⁵ and an increased efflux of daunorubicin.⁴ It was recently shown with inside-out vesicles that MRP1 is a glutathione-conjugate carrier.¹⁶ This transporter has been named alternatively as GS-X pump, the Multispecific Organic Anion Transporter (MOAT) or leukotriene C4 transporter. Other multivalent anionic conjugates act as competing substrates of this pump.⁷⁻¹ The function has been shown to be present not only in tumour cells but also in normal cells in the human body such as hepatocytes, red blood cells, cardiac cells, T-lymphocytes, mast cells and lung cells.¹⁸ This MRP is now named MRP1 since the discovery of MRP2, another pump with the same function as MRP1.⁹⁻¹⁰ Recent immunocytochemical studies have provided evidence that MRP1 in tumour cells is predominantly localized at the cellular plasma membrane.¹¹ In tumour cells with high MRP1 overexpression, staining was however also detected in the Golgi region and in the cytoplasm.¹¹⁻¹⁴ For cultured hepatocytes it has been suggested that transport of organic anions and glutathione-conjugates occurs through the plasma membrane but, in addition, also into intracellular vesicles.¹⁵

The aim of this study was to analyze whether MRP1 functions as ATP-dependent carrier for organic anions inside the cell. We therefore analyzed with fluorescence microscopy, confocal scanning laser microscopy and (immuno)-transmission electron microscopy, the transport of glutathione-conjugates as well as doxorubicin. The experiments were performed with monochlorobimane (MCB) and 5-chloro-methyl fluorescein diacetate (CMFDA) that form rapidly fluorescent glutathione-conjugates inside the cells. In addition, 1-chloro 2,4-dinitrobenzene (CDNB) was used because its glutathione-conjugate could be detected with a specific antibody. The experiments were performed in the small cell lung cancer cell GLC₄, and its doxorubicin resistant counterpart GLC₄/Adr which strongly overexpresses MRP1.¹⁶,¹⁷ The exact localisation of MRP1 and its association with glutathione conjugates and possible involvement of the Golgi apparatus was analyzed.
Materials and Methods

Cell lines
The human small cell lung carcinoma cell line GLC4, the human ovarian carcinoma cell line A2780, as well as their in vitro-acquired 300x doxorubicin resistant, MDR1 negative subline GLC4/Adr and 100x doxorubicin-resistant MDR1 positive subline A2780AD were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% heat-inactivated foetal calf serum (FCS), (both Life Technologies, Paisley, UK) in humidified conditions at 37°C, 5% CO2. The GLC4/Adr cell line strongly overexpresses MRP1,16,18 while MRP2 expression analyzed with RT-PCR using specific primers for human MRP2 is negative. The doxorubicin resistant lines were cultured continuously with 1.2 µM and 2 µM doxorubicin (Pharmacia, Milan, Italy) added twice a week to assure optimal resistance. Before experiments, these cells were cultured without doxorubicin for respectively 21 and 14 days.

Immunocytochemistry and Western blotting
Immunocytochemistry was performed on cytospins, with indirect immunoperoxidase staining for MRP1 with the monoclonal antibody MRPm6.11 In addition, GLC4 and GLC4/Adr cells were incubated at 37°C with 50 µM CDNB (Sigma, St.Louis, MD) for 0, 5, 10 and 20 min. Thereafter cytospin preparations were prepared and stained with a guinea pig antibody against CDNB followed with a second antibody a mouse anti guinea pig label and peroxidase conjugated strepavidin (Dako, Glostrup, Denmark). Western blot was performed as described earlier.16

Fluorescence microscopy
MCB (Calbiochem-Novobiochem Corporation, La Jolla, CA) effects were analyzed in GLC4, GLC4/Adr, A2780 and A2780AD. Cells were incubated at 37°C 20 min with 50 µM MCB, followed by a wash step. Cells were pelleted by centrifugation for 10 min at 180 x g and resuspended in RPMI 1640 and 10% FCS. Vesicle transport was evaluated immediately thereafter and 30, 60, and 90 min after MCB exposure.

Vesicle transport was in GLC4 and GLC4/Adr also monitored after combined incubation for 20 min of MCB (50 µM) plus doxorubicin (10 µM), vincristine (25 µM, Eli Lilly, St Cloud, France) or topotecan (25 µM, gift SmithKline Beecham Pharmaceuticals, King of Prussia, PA) as competitive substrates. Similarly, the kinetics of doxorubicin, using the intrinsic fluorescence of doxorubicin, were followed in GLC4 and GLC4/Adr. Incubation with 20 min 50 µM doxorubicin at 37°C was followed by a washing step and vesicle transport was evaluated before, immediately after, and 30, 60 and 90 min after doxorubicin exposure. All experiments were performed three times.

Confocal scanning laser microscopy
Single-cell suspensions of 5x10^5 GLC4 and GLC4/Adr cell/ml were allowed to attach overnight in 5 ml RPMI/FCS medium at 37°C on glass coverslips in petri dishes. Then cells were incubated 15 min at 37°C with 5 µM CMFDA (Molecular Probes Inc, Eugene,
OR) or 10 µM doxorubicin. After a quick rinse with Hanks balanced salt solution (Life Technologies, Paisley, UK) without phenol red, coverslips were inverted, mounted on glass slides and kept on ice until analysis. Cells were in addition incubated 1 h with 20 µM brefeldin A (Sigma, St.Louis, MD). Thereafter, cells were 10 min exposed to 5 µM CMFDA or 10 µM doxorubicin. Vesicle formation was studied after 20 min incubation. Fluorescence was analyzed with Leica TCS (Leica, Heidelberg, Germany) incorporating an inverted Leitz DM-IRB microscope with a 100x oil immersion lens. The 488-nm line from the argon/krypton laser was used for excitation and emission at 515 nm for CMFDA and doxorubicin. All experiments were performed three times.

Transmission electron microscopy
The GLC4 and GLC4/Adr cells were studied before and after drug exposure. A total of 0.5-1.10^6 cells/ml were 20 min incubated at 37°C with 50 µM MCB or 10 µM doxorubicin. Thereafter, cells were centrifuged with 180 x g at 4°C, washed with phosphate buffered saline (PBS, 0.14 M NaCl, 2.7 mM KCl, 6.4 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) and processed immediately or after an additional 30 or 60 min in culture medium at 37°C. Then, cells were centrifuged 10 min at 180 x g and fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (Na₂HPO₄·2H₂O, NaH₂PO₄, pH 7.4) for at least 1 h at 4°C. Blocks of 3x3 mm were prepared of the cell pellet and rinsed in phosphate sucrose buffer (6.8% sucrose in 0.1 M phosphate buffer). Post-fixation was performed in 1% OsO₄ for 2 h, after which the specimens were dehydrated with increasing alcohol concentrations followed by propylene oxide. Thereafter the specimens were infiltrated overnight with polypropylene oxide/Epon 812 1:1 (Serva, Heidelberg, Germany). The next day the specimens were, after 2 h rotating in Epon 812 and 2 h vacuum, embedded and polymerized overnight at 80°C. Ultrathin sections were stained with uranylacetate, dissolved in methanol and lead citrate. The analysis was performed with a Philips 201 transmission electron microscope (Philips, Eindhoven, The Netherlands). Experiments were performed three times.

Immuno-transmission electron microscopy
The GLC4 and GLC4/Adr cell lines were studied before and after MCB and CDNB exposure. A total of 0.5-1.10^6 cells/ml were incubated 20 min at 37°C with 50 µM MCB. Thereafter, cells were centrifuged at 180 x g at 4°C, washed and pelleted immediately or after an additional 30 or 60 min in culture medium at 37°C. The cells were fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) in 0.1 M phosphate buffer or with 2% glutaraldehyde for at least 1 h at 4°C. Then, cells were embedded in 2% agarose (Sigma) and placed at 4°C, followed by a short postfixation in 4% paraformaldehyde or glutaraldehyde. Small pieces of the agarose specimen were washed with 6.8% sucrose in 0.1 M phosphate buffer and with 2% glutaraldehyde for at least 1 h at 4°C. Then, cells were embedded in 2% agarose (Sigma) and placed at 4°C, followed by a short postfixation in 4% paraformaldehyde or glutaraldehyde. Small pieces of the agarose specimen were washed with 6.8% sucrose in 0.1 M phosphate buffer and then infiltrated with 2.3 M sucrose for at least 2 h at 4°C and mounted on copper pins. They were then frozen in liquid nitrogen. About 80 nm cryosections were made on LKB-Reichert-Jung ultra cryomicrotome (Leica, Rijswijk, The Netherlands) with a glass knife. Sections were picked up with a drop of 2.3 M sucrose, placed on Formvar coated nickel grids (Stork, Eerbeek, The Netherlands) and put, face down, on 2% gelatin plates on ice.19,20 Before labelling, plates were placed in an incubator at 38°C until the gelatin had become fluid.
Grids were transported to drops of PBS/gelatin (0.15%) in order to block free aldehyde groups. The cells were incubated for 1 h with MRPm6 (1:50) diluted in PBS/1% bovine serum albumin. After washing with PBS/glycine the antibody binding was detected with 10 nm gold particles conjugated with goat anti-mouse immunoglobulin (1:50) (Sanver Tech, Breda, The Netherlands). After washing with PBS and distilled water, sections were stained and covered with a methyl-cellulose (MC400cp, Fluka, Buchs, Switzerland) uranylacetate mixture (9:1) and air dried. Cells were also incubated 10 min with 50 µM CDNB and then the same procedure was performed, apart from the fact that CDNB was visualized with the guinea pig antibody (1:30.000) raised against CDNB. As second antibody a mouse anti guinea pig biotin label (1:250) was used and visualized with 5 nm antibiotin (1:50) gold particles (Sanver Tech). In case of double labelling with MRPm6 and CDNB antibody, 0.5% glutaraldehyde for 5 min was applied after the first labelling to prevent co-labeling. At the optimal antibody dilutions no background labelling over nuclei was seen. Evaluation was performed with the Philips 210 transmission electron microscope. Experiments were performed three times.

Cytotoxicity assay
Cytotoxicity for MCB and CDNB in GLC4 and GLC4/Adr were determined with the microwell tetrazolium assay as described before. Cells were incubated 20 min with the drug and after a washing procedure cultured for 4 days. For GLC4 and GLC4/Adr, respectively, 7.5 x 10^4 and 20 x 10^3 cells were used per well. Each experiment was performed three times in quadruplicate.

Results

Immunocytochemistry and Western blotting
MRP1 expression was not detectable in A2780 and A2780AD, slightly positive at the plasma membrane in GLC4 and strongly positive at the plasma membrane in more than 95% of GLC4/Adr cells. In GLC4/Adr cells there were also dense MRP positive spots close to the nucleus, most likely the Golgi area and in these cells there was an increased number of MRP positive spots in the cytoplasm after incubation with MCB or CDNB. Western blot of membrane fractions showed a low MRP expression in A2780, A2780AD, and in GLC4 while a strong signal, 25x higher than GLC4, was detectable in GLC4/Adr.

Fluorescence microscopy
For fluorescence microscopy studies the non-fluorescent substrate MCB was used. MCB conjugated intracellularly with glutathione, results in the fluorescent bimane glutathione. After 20 min MCB exposure, a strong homogenous fluorescence was observed in GLC4 while after 30 min incubation in drug free medium some fluorescent vesicles were observed. Fluorescence in GLC4/Adr was mainly observed in intracellular vesicles. The
intensity of this fluorescence decreased in time, as did the number of vesicles per cell. Most cells did no longer contain fluorescent vesicles 90 min after MCB exposure.

Figure 1. Confocal scanning laser microscopy scans after 10 min 5 μM CMFDA exposure in GLC4 (A), in GLC4/Adr (B) and in GLC4/Adr after 60 min 20 μM brefeldin A preincubation (C); and after 10 min 10 μM doxorubicin exposure in GLC4 (D), GLC4/Adr (E) and GLC4/Adr after 60 min preincubation 20 μM brefeldin A exposure (F). N = nucleus, bar = 5 μm.
Fluorescence microscopy allows separate detection of bimane glutathione and doxorubicin. Doxorubicin exposure for 20 min resulted in a homogenous fluorescence in the cytoplasm with strong nuclear staining in GLC\textsubscript{4}, while the fluorescence was concentrated in vesicles without nuclear staining in GLC\textsubscript{4}/Adr. When doxorubicin and vincristine were used as other substrates for MRP1, together with MCB, again vesicular glutathione bimane was observed in GLC\textsubscript{4}/Adr. However, vesicle size was increased and vesicle content was stained less intense compared to MCB incubation alone. The topoisomerase I inhibitor, topotecan did not influence vesicular transport of bimane glutathione in GLC\textsubscript{4} and GLC\textsubscript{4}/Adr.

After MCB incubation the A2780 and A2780AD cell lines showed strong overall homogeneous fluorescence. No vesicle transport could be detected in either cell line.

Confocal scanning laser microscopy
To evaluate more precisely vesicular transport, confocal scanning laser microscopy (CSLM) was performed. Because MCB and its conjugate are not suitable probes for CSLM, CMFDA was used. CMFDA is intracellularly converted by esterases and glutathione S-transferases into the fluorescent organic anion glutathione methylfluorescein. After CMFDA exposure, fluorescent vesicles were observed in GLC\textsubscript{4} and GLC\textsubscript{4}/Adr cells, but the number of vesicles was much higher in GLC\textsubscript{4}/Adr cells (Figure 1a, b).

After extending periods the vesicles disappeared possible due to insertion in the plasma membrane. Pre-incubation with brefeldin A, an inhibitor of vesicle formation from the Golgi apparatus,\textsuperscript{23} showed CMFDA fluorescence especially in one spot with tubular extensions in GLC\textsubscript{4}/Adr (Figure 1c) but not in GLC\textsubscript{4}. Doxorubicin exposure resulted in mainly nuclear fluorescence with some cytoplasmatic staining in the GLC\textsubscript{4} cells and no nuclear staining but vesicular staining in the cytoplasm of GLC\textsubscript{4}/Adr (Figure 1d, e). In GLC\textsubscript{4}/Adr cells, brefeldin A pre-incubation, followed by doxorubicin resulted in an intensive large fluorescence spot close to the nucleus (Figure 1f). From these data it can be concluded that glutathione methylfluorescein and doxorubicin are transported in GLC\textsubscript{4}/Adr, in vesicles originating from the Golgi complex.

Transmission electron microscopy
Morphological analysis at the ultrastructural level showed in GLC\textsubscript{4}/Adr compared to GLC\textsubscript{4} cells an extensive Golgi apparatus sometimes associated with small clustered vesicles (Figure 2). Exposure of the cells to MCB did not affect the quality of the ultrastructural morphology. After MCB exposure GLC\textsubscript{4} cells have a normal Golgi apparatus and small and sometimes clustered vesicles. The number of vesicles was decreased after 90 min in drug free medium. In GLC\textsubscript{4}/Adr cells MCB exposure resulted in a more pronounced Golgi apparatus and several cells contained very large vesicles and (clusters of) many small vesicles (Figure 3a). Furthermore, some multivesicular bodies were present. After 60 min in drug free medium the vesicle number was strongly decreased, the Golgi apparatus reduced to its original state and myelin figures, representing degenerated residual membranes, were formed (Figure 3b).

Doxorubicin exposure resulted in GLC\textsubscript{4} cells in some vesicles, as also observed after MCB exposure. Immediately after incubation of GLC\textsubscript{4}/Adr cells with doxorubicin, a large Golgi area with small vesicles and induction of larger (multi) vesicles were found.
Following 60 min in drug free medium the vesicles number was strongly reduced, the Golgi apparatus was reduced to its original state and in several cells swollen mitochondria were present.

**Figure 2.** Electron micrographs at magnifications 45,000 x of A: GLC1 cells with some rough endoplasmatic reticulum (RER, arrows) and mitochondria (M) and B: GLC1/Adr cells with the Golgi apparatus (Go), RER (arrow heads), M and myelin figures (my). Bar = 1 µm.

**Figure 3.** Electron micrographs of GLC1/Adr cells at magnification 70,000 x. A: after MCB exposure with Golgi apparatus (Go) with a well developed trans Golgi region, dilatation of the RER (arrow heads), many small and larger (multi) vesicles (large arrows). B: 60 min after MCB exposure Go is reduced to its original state (see 2B) and several large mitochondria (M), RER (arrow heads) and several myelin figures (my) are present. Bar = 1 µm.
Immuno-transmission electron microscopy

Immuno-transmission electron microscopy was performed to visualize MRP1 at the ultrastructural level. A clear labelling for MRP1 was observed on and just under the plasma membrane in GLC\textsubscript{4} cells, which was more pronounced in GLC\textsubscript{4}/Adr cells. After MCB exposure, GLC\textsubscript{4}/Adr cells showed vesicles with MRP1 in their membrane (Figure 4a, b) and the Golgi apparatus. CDNB exposed cells also showed MRP1 in the Golgi apparatus and extensively in the trans Golgi region (Figure 4d). No MRP1 was detected in the mitochondria (Figure 4b). Optimal fixation with 2\% glutaraldehyde was required to crosslink the glutathione-conjugate of CDNB, DNP-GS, inside the vesicles (Figure 4c). DNP-GS could be found immediately after CDNB exposure in the trans Golgi area and in (multi)vesicles (Figure 4b, c). Double labelling showed that these vesicles did contain MRP in their membrane (Figure 4e). CDNB was also detected in mitochondria, in which glutathione levels are high. It can be concluded that trans Golgi derived vesicles contain functionally active MRP1 which transports in an ATP dependent manner glutathione-conjugates inside these vesicles.

Cytotoxicity

To check whether differences between the GLC\textsubscript{4} and GLC\textsubscript{4}/Adr cells were due to large differences in handling of these compounds, cytotoxicity of MCB and CDNB was analyzed. There was only a very low cross resistance between the GLC\textsubscript{4} and GLC\textsubscript{4}/Adr cells for MCB and CDNB. The MCB concentration that inhibited the cell growth by 50\% (IC\textsubscript{50}) was (mean ± SD) 8.0 ± 1.3 µM for GLC\textsubscript{4} and 9.7 ± 2.3 µM for GLC\textsubscript{4}/Adr resulting in a resistance factor of 1.2. The IC\textsubscript{50} for CDNB was 56.7 ± 8.1 µM in GLC\textsubscript{4} and 72.2 ± 15.8 µM in GLC\textsubscript{4}/Adr resulting in a resistance factor of 1.3. These low cross resistance factors underscore the suitability of these compounds for above experiments.

Discussion

MDR is often due to the presence of active pumps, such as MDR1 and MRP1, in the plasma membrane of cells. These ABC-proteins have been shown to be involved in ATP-dependent efflux of natural product drugs from cells resulting in decreased intracellular levels of chemotherapeutic compounds. However, drug efflux did not always correlate with overexpression of the respective transporter gene. MDR cells with high levels of MRP1 have been shown to exhibit sometimes no or only low reduction of intracellular drug level.\textsuperscript{2} In the present article we show an explanation for this observation. We demonstrated that glutathione-conjugates of MCB- and CMFDA in the MRP1 overexpressing cell line GLC\textsubscript{4}/Adr were transported in intracellular vesicles, while in the sensitive cell line GLC\textsubscript{4}, with low MRP1 expression there were less vesicles. The vesicles were observed after exposure to MCB and CMFDA and disappeared from the cells within 60 min after exposure. Pre-incubation with brefeldin A, an inhibitor of vesicle formation from the Golgi apparatus,\textsuperscript{25} followed by CMFDA exposure resulted in an accumulation of fluorescent vesicles close to the nucleus. This suggest that glutathione-conjugate
Figure 4. Micrographs of immunogold labelled ultrathin cryosections of GLC\textsubscript{s}/Adr cells at magnification 70,000x. A, B: 20 min after MCB exposure, MRP1 staining in membrane of intracellular vesicles (large arrow) and in plasma membrane (arrow head), no MRP1 staining in mitochondria (M). C: Positive CDNB staining (gold 5 nm) inside a vesicle 10 min after CDNB exposure. D: Double labelling of CDNB (gold 5 nm, arrows) and MRP (gold 10 nm, large arrows) in Golgi apparatus 10 min after CDNB exposure. E: Micrographs at magnification 70,000x, 10 min after CDNB exposure, of a vesicle labeled for MRP (large arrows) and CDNB (arrows), located just under the plasma membrane (arrow heads). Bar = 100 nm.
transporting vesicles are blocked in their formation from the Golgi apparatus by brefeldin A. In addition, induction of some fluorescent tubular structures was observed in the cytoplasm, this is in agreement with reported formation of tubular structures from the Golgi apparatus by brefeldin A. Interestingly, the observed relation between glutathione-conjugates and the Golgi complex is in agreement with findings from Lutzky et al. In 1989 they analyzed the effect of MCB in the human myelogenous leukemia anthracycline-resistant HL60/AR cell line and its sensitive parent cell line HL60. As shown recently HL60/AR is a strongly MRP1 overexpressing cell line. After 10 min MCB incubation fluorescence was only observed in these cells in the Golgi apparatus. In their study co-localisation experiments with MCB and NBD-ceramide, a fluorescent probe specific for the Golgi apparatus, confirmed that the perinuclear localization of MCB corresponded to the Golgi apparatus. They observed no vesicles but that may due to the fact that effects were evaluated only after 10 min MCB incubation. There was no vesicular concentration after MCB observed in a MDR1 overexpressing doxorubicin resistant A2780AD cell line with very low MRP1 level (Figure 1).

With electron microscopy it was shown that the Golgi apparatus was more extensively present in GLC4/Adr than in GLC4. This indicates an increased activity of the biosynthetic pathway. MCB induced in GLC4/Adr a more pronounced Golgi apparatus and membrane surrounded vesicles partly related to the trans Golgi region which were almost disappeared 60 min after MCB exposure. Immuno-transmission electron microscopy to study MRP1 localisation has been used by Flens et al. They observed with a pre-immuno labelling technique, followed by plastic embedding, only MRP1 expression on the plasma membrane. This is in contrast to immunocytochemistry studies that localized the MRP1 in cytoplasm, the Golgi region and the cell membrane. In the present study with ultracryomicrotomy and immuno-transmission electron microscopy technique, MRP1 was observed on the plasma cell membrane, especially in the GLC4/Adr cells. However, MRP1 staining was also shown, although to a lesser extent, in the cytoplasm. After MCB exposure, MRP1 was present in membranes of the Golgi apparatus, the trans Golgi region and in vesicle membranes. It was previously shown that DNP-GS is a substrate for the glutathione-conjugate pump in the GLC4/Adr cell line. Double staining for MRP1 and DNP-GS, after exposure to CDNB showed MRP1 on the membranes of vesicles containing DNP-GS. The fixation procedure with glutaraldehyde results in crossing-linking of the highly water-soluble glutathione-conjugate of CDNB inside the MRP1 positive secretory vesicles. The results presented here show that MRP1 is not only active in the plasma cell membrane, but that it is also functionally active inside the cell. Moreover, the association of MRP1 with vesicles would make MRP1 another member, after e.g. the Transporter-associated with Antigen Processing (TAP), of the ATP-binding cassette superfamily to function not only as an entity exclusively at the plasma membrane but also inside the cell. This function can protect cells intracellularly from toxic compounds by compartmentalisation of its conjugates.

The fact that the glutathione-conjugate pump is present in many cell types in the human body may mean that the above described finding also applies to many non-tumour cells. It is still an unresolved problem whether MRP1 apart from glutathione-conjugates and other conjugates, also transports chemotherapeutic drugs. Various options can be considered e.g. MRP1 may catalyze co-transport of drug and glutathione. Alternatively, the drugs might indeed be transported as glutathione-conjugates even though for doxorubicin, etoposide and vincristine no glutathione-conjugates are known to occur. It
may, however, be that these conjugates do exist but elude detection because they are unstable. If the chemotherapeutic drugs will be transported by MRP1 in a GSH-dependent manner we would expect that doxorubicin, vincristine and etoposide are also transported, at least partly, in intracellular vesicles to the plasma membrane. The present study shows that vesicular transport does occur for doxorubicin in GLC4 and to a larger extent in GLC4/Adr cells. This has also been observed by others for other MRP1 overexpressing cell lines.4 Vesicles disappeared 60 min after doxorubicin incubation. In GLC4/Adr brefeldin A pre-incubation, just as for CFMDA, resulted, if also incubated with doxorubicin, in fluorescent spots in the Golgi region. Co-incubation of MCB with doxorubicin or vincristine in GLC4/Adr cells resulted in larger vesicles with a weaker bimane glutathione fluorescence. All these findings favour an identical pathway for doxorubicin- and glutathione-conjugate transport. Regretfully, double staining electron microscopy for MRP1 and doxorubicin or vincristine is as yet not possible because no antibodies against these chemotherapeutic drugs are available. Drug accumulation and efflux are commonly measured with techniques that assume identical cellular distribution of drug in resistant and sensitive cells with respect to localization and exchangeability. The fact that a part of the chemotherapeutic drug can be inside the cell in an extracellular compartment explains why in the GLC4/Adr sublines with increasing doxorubicin resistance an increasing MRP1 expression the difference in doxorubicin accumulation showed no linear relation with MRP1 expression.18 The results presented here as well as the data of others also suggesting vesicular transport in MRP positive cell lines forms a likely explanation for this confusion. This probably also explains why Cole et al. observed in their doxorubicin MRP1 overexpressing cell line no cellular difference for doxorubicin compared to the sensitive cell line.2

In the present article we showed that in the MRP1 overexpressing doxorubicin resistant GLC4/Adr cell line, glutathione-conjugates are rapidly transported intracellularly in vesicles which contain MRP1 in their membranes. Doxorubicin was transported in the same pathway. The vesicles originate from the well developed Golgi complex in GLC4/Adr.

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