

MDR3 P-glycoprotein, a phosphatidylcholine translocase, transports several cytotoxic drugs and directly interacts with drugs as judged by interference with nucleotide trapping

Alexander J. Smith^{1,6}, Ardy van Helvoort^{1,5}, Gerrit van Meer^{2,5}, Katalin Szabó^{3,4}, Ervin Welker^{3,4}, Gergely Szakács³, András Váradi⁴, Balázs Sarkadi³ and Piet Borst¹

¹ Division of Molecular Biology and Center for Biomedical Genetics, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands. ² Laboratory of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands. ³ National Institute of Haematology and Immunology, Membrane Research Group of the Hungarian Academy of Sciences, H-1113 Budapest, Hungary. ⁴ Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, H-1113 Budapest, Hungary. ⁵ Part of this work was carried out at the Department of Cell Biology, Faculty of Medicine and Institute of Biomembranes, Universiteit Utrecht, Utrecht, The Netherlands. ⁶Present address: Dept of Molecular Genetics, Institute of Ophthalmology, London, UK.

Running title: Contribution of MDR3 P-glycoprotein to transport of cytotoxic drugs

Corresponding author:

Prof. Dr P. Borst

Division of Molecular Biology

The Netherlands Cancer Institute

Plesmanlaan 121

1066 CX Amsterdam

The Netherlands

tel: +31-20 512 2880

fax: +31-20 669 1383

E-mail: pborst@nki.nl.

Summary

The human *MDR3* gene is a member of the multidrug resistance (MDR)¹ gene family. The MDR3 P-glycoprotein is a transmembrane protein that translocates phosphatidylcholine. The product of the other member of the gene family, MDR1 P-glycoprotein, transports cytotoxic drugs. Its overexpression can make cells resistant to a variety of drugs. Attempts to show that MDR3 P-glycoprotein can cause MDR have been unsuccessful thus far.

Here, we report an increased directional transport of several MDR1 P-glycoprotein substrates, such as digoxin, paclitaxel and vinblastine, through polarized monolayers of *MDR3* transfected cells compared to the parent cell line. Transport of other good MDR1 P-glycoprotein substrates, including cyclosporin A and dexamethasone, was not detectably increased. MDR3 P-glycoprotein dependent transport of a short-chain phosphatidylcholine analog and drugs was inhibited by several MDR reversal agents and other drugs, indicating an interaction between these compounds and MDR3 P-gp. This interaction was further studied in insect cell membranes from Sf9 cells overexpressing *MDR3*. These membranes showed specific MgATP binding and a vanadate-dependent, NEM sensitive nucleotide trapping activity, which could be visualized by covalent binding with [α -³²P]-8-azido-ATP. Trapping was not significantly stimulated by the addition of possible substrates of MDR3 P-glycoprotein, e.g. phospholipids or drugs. Nucleotide trapping was (nearly) abolished by paclitaxel, vinblastine and by the MDR reversal agents verapamil, cyclosporin A and PSC833. We conclude that MDR3 P-glycoprotein can bind and transport a sub-set of MDR1 P-glycoprotein substrates. The rate of MDR3 P-glycoprotein mediated transport is

low for most drugs, explaining why this protein is not detectably involved in multidrug resistance. It remains possible, however, that drug binding to MDR3 P-glycoprotein could adversely affect phospholipid or toxin secretion under conditions of stress, *e.g.* in pregnant heterozygotes with one *MDR3* null allele.

Keywords: multidrug resistance, reversal agents, phosphatidyl choline, chemotherapy, nucleotide trapping, baculovirus

Introduction

P-glycoproteins (P-gps) are 170 kD glycosylated membrane proteins that actively transport their substrates out of the cell. Two P-gp genes have been identified in humans. The human *MDR1* gene encodes a drug transporting P-gp that can actively extrude a range of cytotoxic anticancer drugs from the cytoplasm. Overexpression of this P-gp gene results in a decreased intracellular accumulation of these drugs and renders the cell multidrug resistant (MDR) (1-3). The second human P-gp gene is *MDR3* (also known as *MDR2*) (4,5). Attempts to obtain resistance against cytotoxic drugs by transfecting drug-sensitive cell lines with the *MDR3* cDNA, or its mouse homolog *Mdr2*, yielded only negative results (4,6-9). Amplification or activation of the *MDR3* gene, independent of the closely linked *MDR1*, has never been found in multidrug resistant cell lines (10-12). Nevertheless, two studies suggest a role for MDR3 P-gp in drug transport: (i) analysis of B-cell leukemias showed a correlation between *MDR3* overexpression and daunorubicin transport (13,14) and (ii) Kino *et al.* found low-level resistance against the antifungal agent aureobasidin A in yeast transformed with the human *MDR3* cDNA (15).

The function of the murine homolog of human MDR3 P-gp, Mdr2 P-gp, became clear when Smit *et al.* (16,17) generated mice, homozygous for a disruption of the *Mdr2* gene (*Mdr2* (-/-) mice). These mice were unable to excrete phospholipids (PL) into their bile, indicating that Mdr2 P-gp is a PL translocator or flippase, translocating PL from the cytosolic leaflet to the luminal leaflet of the canalicular membrane. The excretion of phosphatidylcholine (PC; the

major PL in the bile) into the bile can be restored in these mice by expression of the human *MDR3* gene in the hepatocytes (18,19), demonstrating that the closely related Mdr2 and MDR3 P-gps fulfill the same function. Further evidence for the PC translocator function of the MDR3/Mdr2 P-gps came from studies in yeast and cultured mammalian cells. Gros and coworkers showed that secretory vesicles from yeast transformed with *Mdr2* cDNA can accumulate fluorescent short chain PC (20,21). We demonstrated increased translocation of PC through the plasma membrane of cells overproducing the MDR3 P-gp (22,23) and defined the substrate specificity of the translocator for PL (23). Whereas the MDR1 P-gp was found to translocate a variety of PL analogs, the MDR3 P-gp proved highly selective, only translocating PL with a choline headgroup, and a diacyl glycerol backbone. Even a PC with two short acyl chains (C₈-C₈-PC) was not transported (23). This selectivity seemed an adequate explanation for the inability of the MDR3/Mdr2 type of P-gp to contribute to MDR. It remains puzzling, however, that transport of a PC analogue by the MDR3 P-gp could be inhibited by verapamil, a known substrate and inhibitor of MDR1 P-gp (20,23). To resolve this paradox, we re-investigated drug transport by the MDR3 P-gp, using monolayers of polarized kidney cells transfected with a *MDR3* mini-gene (23), in which the transporter localizes in the apical membrane. We found that the transfected cells transport digoxin, a substrate of the MDR1 P-gp (24,25) and several other cytotoxic drugs, and that transport is inhibited by several inhibitors (reversal agents) of MDR1 P-gp. Moreover these drugs appear to interact directly with the MDR3 P-gp, as they are able to inhibit vanadate-dependent nucleotide trapping by MDR3 P-gp produced in insect cells. Here we present a detailed

analysis of these unexpected results. Part of these results was included in the thesis of Smith (1998).

Experimental procedures

Materials

[12 α -³H(N)]digoxin (15.0 Ci/mmol), [³H(G)]daunorubicin (4.3 Ci/mmol) and [N-methyl-³H]morphine (86.5 Ci/mmol) were obtained from DuPont NEN, Wilmington, DE. [MeBMT- β -³H]CsA (6.6 Ci/mmol), [1,2,4,6,7-³H]dexamethasone (87 Ci/mmol) and inulin[¹⁴C]carboxylic acid (MW approximately 5200) (5.95 mCi/mmol) were from Amersham, Little Chalfont, England. [³H]paclitaxel (11.6 Ci/mmol) was from Moravek Biochemical Inc, La Brea, CA. [¹⁴C]SDZ PSC 833 was a gift from Sandoz Pharma Ltd. (now part of Novartis Pharma Inc.), Basel, Switzerland. [22,23-³H]ivermectin (51.9 mCi/mmol) was kindly provided by Merck Research Laboratories. 1-hexadecanoyl-2-(C₆-(7-nitro-2,1,3-benzoxadiazol-4-yl))-*sn*-glycero-3-phosphocholine (C₆-NBD-PC) and C₆-NBD-

phosphatidic acid (C₆-NBD-PA) were from Avanti (Alabaster, AL). C₆-NBD-ceramide was from Molecular Probes (Eugene, OR). [α -³²P]-8-azido-ATP (666 GBq/mmol) and [α -³²P]-ATP (111 TBq/mmol) were obtained from ICN Biomedicals (Costa Mesa, CA) All tissue culture materials were from GIBCO BRL, Paisley, Scotland. Other chemicals were from Sigma Chemical Co., St. Louis, MO.

Tissue culture

The LLC-PK1 pig kidney epithelial cells were obtained from the American Type Culture Collection and cultured in M199 medium supplemented with 50 units penicillin and 50 μ g streptomycin per ml and 10% fetal bovine serum at 37°C in 5% CO₂. The cells were trypsinized and subcultured every 3 to 4 days. The *MDR1*, *MDR3* and *MRP* transfected clones of LLC-PK1 were obtained by calcium phosphate coprecipitation of the corresponding cDNAs as described (23,25,26). The cells were maintained routinely in the absence of drugs and tested regularly for P-gp content by immunoblotting with the monoclonal antibody C219, that recognizes all mammalian P-gps tested (27). This was followed by the enhanced chemiluminescence procedure (Amersham, 's-Hertogenbosch, The Netherlands), as described (25,28).

Digoxin accumulation experiments

Digoxin accumulation in the various cell lines was measured by seeding 1 x 10⁶ cells in a

single well of a 6-wells plate, followed by incubation of the cells for 3 days. The medium was replaced by 1.5 ml medium containing 10 μM [^3H]digoxin (0.25 $\mu\text{Ci/ml}$). After a 2h incubation period the medium was removed and the cells were washed twice with 1 ml ice cold phosphate buffered saline. The cells were scraped and removed from the wells in 0.5 ml phosphate buffered saline. Radioactivity was determined by liquid scintillation counting. Accumulation was measured in triplicate in the presence and absence of 5 μM PSC 833.

Drug transport assays

Drug transport assays were performed as described (25,29) with minor modifications. Cells were seeded on microporous polycarbonate membrane filters (pore size 3.0 μm , diameter 24.5 mm, TranswellTM, Costar^R) at a density of 1.5 - 2 x 10⁶ cells per filter. The cells were grown in complete medium for three days with a medium replacement the day after seeding. One hour before the start of the experiment, medium at both sides of the monolayer was replaced. The experiment was started by replacing the medium at either the apical or the basolateral side by medium containing [^3H]-labeled substrate drugs (0.25 $\mu\text{Ci/ml}$) and inulin[^{14}C]carboxylic acid (0.025 $\mu\text{Ci/ml}$, 4.2 μM). The cells were incubated at 37°C in 5% CO₂ and 50 μl aliquots were taken from both compartments at t=1, 2, 3 and 4h. The appearance of radioactive drug in the opposite compartment was measured and presented as the fraction of total radioactivity added at the beginning of the experiment. Directional transport was measured in duplicate. The paracellular flow was monitored by the appearance

of [^{14}C]inulin in the opposite compartment and was always less than 1.5% of total radioactivity per hour.

Inhibition of transport was measured similarly. One hour before the start of the experiment the medium at both sides of the monolayer was replaced by complete medium containing the appropriate concentration of reversal agent. At $t=0$ medium in one of the compartments was replaced by medium with radioactive drug and the indicated amount of reversal agent.

Lipid transport assays

Transport of $\text{C}_6\text{-NBD-PC}$ and $\text{C}_6\text{-NBD-glucosylceramide}$ ($\text{C}_6\text{-NBD-GlcCer}$) was measured as described (23,30). LLC-PK1 derived cell lines were seeded on microporous membrane filters and grown for 4 days. Lipid precursors, $\text{C}_6\text{-NBD-PA}$ or $\text{C}_6\text{-NBD-ceramide}$, were complexed to 1% (w/v) bovine serum albumin (BSA) in HEPES buffered Hanks' balanced salt solution (pH 7.35) (HBSS') to the following concentrations: 25 μM $\text{C}_6\text{-NBD-PA}$ and 5 μM $\text{C}_6\text{-NBD-ceramide}$. To discriminate surface synthesized $\text{C}_6\text{-NBD-PC}$ (30) from transport of intracellularly synthesized $\text{C}_6\text{-NBD-PC}$, cells were preincubated with 10 μCi [^3H]choline/ml HBSS' for 1h at 37°C, after a 1h choline depletion in HBSS' at 37°C. Incubation with the lipid precursors in HBSS' + BSA followed for 3h at 15°C in the presence of 10 μCi [^3H]choline. For inhibition studies, the appropriate concentration of reversal agents was present 1h before and during the 3h incubation at 15°C. During the incubations short-chain lipid products appearing on the cell surface were depleted into the medium by the BSA.

After 3h, the apical and basolateral medium were collected and the cells were washed in HBSS' + BSA for 30' on ice. The apical and basolateral washes were pooled with the respective incubation media, the filters were cut from the wells and the lipids from media and cells were analyzed.

Lipid analysis

Lipids were extracted from the cells and media by a modified Bligh and Dyer extraction (31). The upper phase contained 20 mM acetic acid. The organic (lower) phase was dried under N₂ and the lipids were applied to TLC plates. Lipid products were separated in two dimensions by borate-TLC as described (32), but over 20 cm in the first (alkaline) dimension. In the case of C₆-NBD-[³H]PC in cellular lipid extracts, the fluorescent spot was circled and the plate was exposed to a film to allow accurate separation of C₆-NBD-[³H]PC and long-chain [³H]PC. Fluorescent spots were quantitatively analyzed in a fluorimeter, radiolabeled spots were detected by fluorography and the radioactivity was quantified by liquid scintillation counting as described (33).

MDR3 expression and membrane preparation

Sf9 (*Spodoptera frugiperda*) cells were cultured and infected with baculovirus vectors as described in (34). Baculo transfer virus for *MDR3* was constructed as follows: The plasmid constructs harbouring human *MDR3* cDNA were pJ3Ω-MDR3 and pJ3Ω-MDR3-NotI. As

the *MDR3* cDNA was found to be unstable in several baculovirus transfer vectors in *E. coli* an alternative approach has been developed, which does not involve bacterial propagation of the recombinant vector. *MDR3* cDNA and pieces of the appropriate transfer vector containing essential segments for homologous recombination with the viral DNA were ligated and this ligation mix was used for cotransfection of insect cells. The 5' region of the *MDR3* cDNA (-33 to 1621) was removed from pJ3Ω-MDR3-NotI by NotI and ApaI digestion, and the 3' region (1621 to 4002) was removed from the pJ3Ω-MDR3 by ApaI and XbaI digestion.

Two segments of the pVL 1392 baculovirus transfer plasmid (Invitrogen) were isolated: the first one extends from 4154 to 8361 (obtained by XbaI and BglII digestion) and the second one covers region 9479 to 4138 (obtained by BglII and NotI digestion).

The two *MDR3* fragments were ligated with the two pVL1392 fragments, and this “ligation mix” was used for generating recombinant viruses with the Baculogold Transfection Kit (Pharmingen, San Diego, CA).

Sf9 insect cells were infected and cultured as described previously (34). The virus-infected Sf9 cells were suspended in a low ionic strength medium (50 mM Tris-HCl pH 7.0, 50 mM mannitol, 2 mM EGTA, 10 µg/ml leupeptin, 8 µg/ml aprotinin, 0.5 mM PMSF, 2 mM DTT) and disrupted using a glass-teflon homogenizer. Membrane fractions were isolated by repeated centrifugations and homogenizations, and the membrane protein concentrations determined as described in (35). After cloning the baculoviruses producing high-level *MDR3* expression, the sequences of the cDNA inserts were checked by PCR amplification.

Electrophoresis and immunoblotting

Membranes were suspended in a disaggregation buffer (35). Samples (20 μ l) were run on 6% Laemmli-type gels and electroblotted onto PVDF membranes. Quantitative estimation of the expression of human MDR3 and MDR1 P-gps was performed using the monoclonal antibody C219 (27), which recognizes both proteins. Additionally, a monoclonal anti-human MDR3 antibody (P3II26) (36), and polyclonal anti-MDR1 antisera 4077 and 4007 were applied. Anti-mouse IgG and anti-rabbit IgG, peroxidase conjugated IgGs (10,000 x diluted, Jackson Immunoresearch), were used for secondary antibodies as described (37). HRP-dependent luminescence (ECL, Amersham) was determined by luminography and quantitated by the Bio-Rad Phosphoimager system.

ATP binding to MDR3 P-gp

Binding of ATP to MDR3 P-gp was measured by incubation of isolated Sf9 cell membranes (100 μ g protein) for 5 min at 4°C in a reaction buffer containing 50 mM Tris-HCl (pH7.0), 0.1 mM EGTA, 2 mM MgCl₂, in a final volume of 50 μ l, in the presence of 5 μ M Mg-8-azido-ATP, containing 0.2 MBq of [α -³²P]-8-azido-ATP. The solution was irradiated for 10 min with an UV lamp (λ_{max} about 250 nm) at a distance of 3 cm, the membranes were washed 3 times with 500 μ l ice-cold Tris-EGTA-MgATP buffer (50 mM Tris-HCl (pH7.0), 0.1 mM EGTA, 10 mM MgATP), collected in 40 μ l of the electrophoresis buffer, and the samples were run and electroblotted as described above. The blots were subjected to autoradiography in a Phosphoimager, and the identity of the ³²P-azido-nucleotide labeled

bands was verified by immunostaining of the same blot.

Vanadate-dependent nucleotide trapping

Isolated Sf9 cell membranes (100 μ g protein) were incubated for 30 sec to 10 min at 37°C in reaction buffer (50 mM Tris-HCl (pH7.0), 0.1 mM EGTA, 2 mM MgCl₂, 200 μ M Na-orthovanadate) in a final volume of 50 μ l, in the presence of 5 to 200 μ M Mg-8-azido-ATP, containing 0.2 MBq of [α -³²P]-8-azido-ATP. The reaction was stopped with 500 μ l ice-cold Tris-EGTA-MgATP buffer (50 mM Tris-HCl (pH7.0), 0.1 mM EGTA, 10 mM MgATP, 200 μ M Na-orthovanadate), the membranes were washed twice in this buffer and the pellet was resuspended in 20 μ l Tris-EGTA. The membranes were irradiated, and collected in 40 μ l of electrophoresis buffer. The samples were run on SDS-PAGE, electroblotted and the blot was processed further as described above. The effects of various cytotoxic agents on vanadate-dependent nucleotide trapping was measured by addition of the drugs to the reaction mixture. The effect of phospholipids was assessed by the addition of sonicated liposomes.

Statistical analysis

In the transport experiments, two replicate slopes were measured per experiment. Statistical analysis of the experiments was performed by a one-way analysis of variance approach with the individual slopes as experimental units. The slope of the line through the four time points of each well was determined, resulting in two estimations of the slope per experiment. Because we assumed that the random variation of the slope was equal in all experiments, we

used the differences between the two independent estimations of all experiments to determine this random variation. All P values are two-sided. Differences are considered significant if $P < .05$.

Results

Transport of drugs by MDR3 transfected cells

Fig. 1 shows the transport of several MDR1 P-gp substrate drugs through monolayers of polarized pig kidney epithelial cell lines. LLC-PK1 is the untransfected parent cell line, LLC-M3.4.44 (23) is a subclone of the *MDR3* transfectant LLC-M3.4 and LLC-1.1 is the *MDR1* transfected LLC-PK1 clone (25). Directional transport of the cardiac glycoside digoxin, of the cytostatic drugs paclitaxel, daunorubicin and vinblastine (VBL) and of the antihelmintic drug ivermectin to the apical compartment is significantly increased in the *MDR3* transfected cells compared to the parental cell line (two sided $P < .00001$ for all drugs using one-way analysis of variance). With the exception of paclitaxel, the net transport rate (flow from basolateral to apical minus the flow from apical to basolateral) of these drugs is lower in the *MDR3* transfected cells than in the *MDR1* transfectant (two-sided $P < .00001$ for daunorubicin, vinblastin and ivermectin; two-sided $P = .001$ for digoxin), whereas the P-gp expression levels are comparable (see fig. 2). The transport rate of paclitaxel is extremely high in both cell lines and this makes it difficult to compare the activities of the transporters. The transport experiments are performed twice for ivermectin and three times or more for the other drugs with various batches of the cell lines. The statistically significant differences were comparable in the various experiments showing that the increased transport of drugs in the *MDR3* transfected cell line is reproducible (results not shown). It should be noted that the expression of *MDR3* is rather heterogeneous (23), in contrast to the expression of *MDR1*

(25); this might influence the transport efficiency through the monolayers. These results were reproduced in an independent *MDR3* transfectant clone LLC-M3.1 (see e.g. fig. 2A). The transport rate of digoxin in several subclones of LLC-M3.4 with various *MDR3* expression levels, correlated with the amount of MDR3 P-gp determined on immunoblot (fig. 2). Transport rates of 0.1 μM PSC 833 and 2 μM cyclosporin A (CsA), dexamethasone, ondansetron and morphine did not differ significantly between *MDR3* transfectants and parental cells (not shown). Directional transport of these drugs was readily detectable in the *MDR1* expressing cells at these concentrations (not shown; *c.f.* refs (25,29,38)).

Inhibition of drug transport by reversal agents

The active transport of drugs by MDR1 P-gp through the monolayers of LLC-PK1 derived cell lines can be inhibited by addition of P-gp substrates and inhibitors, also called reversal agents. We have tested the effect of several of these inhibitors on MDR3 P-gp mediated digoxin transport. Fig. 3 shows that the increased transport of 2 μM digoxin through the LLC-M3.4.44 monolayers is abolished by 10 μM cyclosporin A, 5 μM PSC 833, 20 μM verapamil, 10 μM vinblastine. We also found inhibition by 10 μM paclitaxel (not shown). The low level of digoxin transport through the parental cells, due to the presence of endogenous porcine transporters (25,29), is inhibited only partially by verapamil, whereas this drug completely inhibits the increased MDR3 P-gp mediated transport. In the *MDR1* transfectant inhibition of digoxin transport by PSC 833 is complete, the other reversal agents are not able to inhibit directional transport of digoxin by MDR1 P-gp completely at the

concentrations used here. Fig. 4 shows that the *MDR3* transfectant accumulated far less digoxin than the parental cells as compared to conditions where active digoxin transport was inhibited by 5 μM PSC 833. This suggests that the MDR3 P-gp in the transfected cells extrudes digoxin or prevents its influx by removal of the drug from the inner leaflet of the plasma membrane. However, indirect effects of MDR3 P-gp on drug transport, e.g. by influencing the activity of another transporter, are not excluded by these experiments.

Inhibition of lipid translocation by MDR3 P-gp

An established physiological function of MDR3 P-gp is the translocation of PC (16,22). We therefore tested whether the substrates transported by *MDR3* transfectants and the inhibitors blocking this process had an effect on the translocation of PC to the extracellular leaflet of the apical membrane. Instead of PC, we used C₆-NBD-PC, a fluorescent short chain analog of PC, as the translocation substrate (23). In order to measure specifically the translocation across the plasma membrane, the intracellular pool of newly synthesized C₆-NBD-PC was double-labeled with [³H]choline and vesicular transport mechanisms were inhibited by performing the experiment for 3 h at 15°C.

Fig. 5A shows that reversal agents that inhibit the increased drug transport in the LLC-M3.4.44 cells, also decrease the rate of C₆-NBD-PC translocation by MDR3 P-gp in these cells. Lipid translocation is also reduced in the presence of 10 μM of the putative substrate drugs paclitaxel and vinblastine, suggesting that a direct interaction exists between MDR3 P-gp and the compounds tested. To exclude that the inhibition of lipid translocation was due to

a non-specific toxic effect, *e.g.* on the cytoskeleton, rather than a direct interaction with the transporter the effect of paclitaxel was measured on the lipid translocation by the multidrug-resistance associated protein MRP1 (26). We have shown that MRP1 translocates C₆-NBD-GlcCer in *MRP1* transfected LLC-PK1 cells (39), whereas MRP1 is known to be a poor transporter of paclitaxel (40,41). We found that 10 μM paclitaxel did not inhibit the translocation of C₆-NBD-GlcCer to the outer leaflet of the membrane, whereas 2 mM sulfinpyrazone, a known inhibitor of MRP1 (26) completely abolished transport of the lipid (fig. 5B). The results in figures 5A and B indicate that paclitaxel has an inhibitory effect on MDR3 P-gp itself rather than on lipid metabolism or lipid trafficking in general.

Attempts to inhibit MDR3 P-gp lipid translocation activity with 10 μM digoxin were unsuccessful (fig. 5A). However, translocation of C₆-NBD-GlcCer and C₆-NBD-PC by MDR1 P-gp was not inhibited by 10 μM digoxin either (fig. 5C), showing that digoxin is unable to inhibit P-gps in this assay system. This is probably due to the inability of digoxin to penetrate the cells at 15°C. To study translocation of PL from the inner to the outer leaflet of the plasma membrane, all assays have to be performed at 15°C to minimize the contribution of vesicular transport to the phospholipid reaching the outer leaflet of the plasma membrane. At 15°C, however, we find no diffusion of digoxin through the monolayer, suggesting that digoxin is unable to pass the lipid bilayer and reach P-gp at this temperature.

Expression of MDR3 in Sf9 cells

To verify that the MDR3 P-gp can interact directly with drugs, we analyzed whether the

drugs transported by this P-gp also affect enzymatic reactions associated with P-glycoprotein, such as ATPase activity and vanadate-dependent nucleotide trapping. *MDR3* expression levels in mammalian cells were not high enough for studying these partial reactions and we therefore cloned *MDR3* cDNA into baculovirus and expressed the protein in insect (Sf9) cells. Since baculovirus vectors containing this cDNA could not be amplified in any bacterial strain, we had to clone it directly into baculovirus by co-transfection of Sf9 cells (see methods). As shown in Fig. 6A infection of the Sf9 cells with the recombinant *MDR3* baculovirus resulted in a high-level expression of the *MDR3* gene. Antibody C219 recognizes both MDR3 (lane 2) and MDR1 (lane 3) and does not immunostain control Sf9 cell membranes (lane 1). The polyclonal antiserum 4077, generated against the N-terminal portion of MDR1, only stains MDR1, while the polyclonal antiserum 4007, generated against the C-terminal part of MDR1 (37) reacts both with MDR1 and MDR3 (not shown). In Sf9 cells MDR1 and MDR3 P-glycoproteins are underglycosylated relative to their mammalian counterparts and appear at 140 kDa on the immunoblots following SDS-PAGE. The two proteins are expressed at about equal level judged from the immunoblots.

ATP binding to MDR3 P-gp

ATP binding to MDR3 was estimated by using the radiolabeled photoaffinity ATP analog [α -³²P]-8-azido-ATP. Isolated membranes were incubated with this reagent at 4°C, the samples UV-irradiated, the labeled proteins separated on SDS-PAGE and examined by a phospho-imager, as described in the Experimental Procedures. As documented in Fig. 6B,

MDR3 P-gp in isolated Sf9 cell membranes showed a specific binding of [α - 32 P]-8-azido-ATP (lane 3). This binding, measured at 5 μ M 8-azido-ATP, required the presence of free Mg^{2+} , as it was eliminated by 1 mM EDTA (lane 4). ATP binding to MDR3 P-gp (similarly to that seen for MDR1 P-gp – see lane 2, (35)) was eliminated by the addition of 1 mM ATP (lane 5), but not by 1 mM AMP, and strongly inhibited by 500 μ M NEM (not shown). ATP binding was not influenced by CsA, verapamil, digoxin or paclitaxel (not shown). In control Sf9 cell membranes there was no ATP binding observed in the region of MDR1/MDR3 P-gp (lane 1)

Nucleotide trapping in MDR3 P-gp

Low concentrations of vanadate can inhibit ATP-hydrolysis by MDR1 P-gp, probably by replacing inorganic phosphate bound to the protein. This results in the formation of a complex between ADP and the protein that cannot be dissolved by washing with high $MgATP$ concentrations (42,43). Vanadate-dependent nucleotide trapping by MDR3 P-gp was studied in isolated Sf9 cells membranes using [α - 32 P]-8-azido-ATP. Labeling was performed in the presence of Na-orthovanadate for 2 min at 37°C, and UV-irradiation was performed after thorough washing of the membranes in high- $MgATP$ -containing media. Fig. 7 shows labeled nucleotide trapping in MDR3 and MDR1 (lanes 1 and 6) at the expected molecular mass, but not present in control Sf9 cell membranes (lane 8). Nucleotide trapping by MDR3 P-gp required the presence of Mg^{2+} (lane 2); it was inhibited by 500 μ M NEM (lane 3), and decreased by 50 μ M verapamil (lane 4) and even more effectively by 5 μ M CsA

(lane 5). For comparison, Fig. 7 also demonstrates nucleotide trapping by MDR1 P-gp. This labeling is also NEM-sensitive (not shown), but greatly stimulated by verapamil (lane 7, (44)). No nucleotide trapping was observed in either P-gp if no vanadate was added to the incubation media. Equal amounts of P-gp were present in each lane as verified in each experiment by immunoblotting of the same membrane filters (Fig 7B).

In experiments not documented here, we studied the time course and ATP dependence of nucleotide trapping in MDR3 P-gp. Labeling increased up to 20 min incubation time at 37°C, and up to 8-azido-ATP concentrations of 50 μ M. Since modulating effects of substrates or inhibitors are expected to be more pronounced at relatively lower levels of nucleotide trapping (44), we have performed such experiments at conditions similar to those in Fig. 7.

Table I summarizes the effects of several compounds on the nucleotide trapping by MDR3 P-gp in the presence of 5 μ M ATP during 2 min incubations at 37°C. A strong inhibition of nucleotide trapping in MDR3 P-gp was observed after addition of several compounds that inhibit drug transport by this protein. Phospholipids (phosphatidylcholine, phosphatidylserine or phosphatidylinositol) added as sonicated liposomes up to 50 μ g/ml, did not modulate nucleotide trapping of MDR3 P-gp or MDR1 P-gp (not shown). This may be due to saturation of P-gp with endogenous lipids or to a low spontaneous exchange of PL between the liposomes and the membrane vesicles.

Discussion

Previous attempts to demonstrate drug transport by the human MDR3 P-gp and its murine homolog Mdr2 P-gp have met with little success (4,6-9). It came therefore as a surprise that expression of *MDR3* in kidney cell monolayers results in the directional transport of digoxin, paclitaxel, daunorubicin, vinblastine and ivermectin. The transport of these drugs is specific as the transport of several other drugs and reversal agents is not increased in the *MDR3* transfected cell lines. The rate of digoxin transport correlates with the amount of MDR3 P-gp in various subclones of *MDR3* transfectant LLC-M3.4 (Fig. 2). Transport is inhibited by the MDR1 P-gp reversal agents, CsA, PSC 833 and verapamil, agents that also inhibit the translocation of short-chain PC analogs by the MDR3 P-gp. Conversely, C₆-NBD-PC translocation is also inhibited by the MDR3 P-gp substrates, paclitaxel and vinblastine. PSC 833 causes an increase in the accumulation of digoxin in the LLC-M3.4.44 cells, suggesting that in the absence of PSC 833 digoxin is extruded from these cells against a concentration gradient.

Two earlier studies have suggested that the MDR3 P-gp may be able to transport some drugs. Nooter *et al.* and Herweijer *et al.*, (13,14) found overexpression of *MDR3* (and not of *MDR1*) in certain drug resistant leukemias and an increase in daunorubicin accumulation after treatment of the cells with cyclosporin A (CsA). This is in line with our results on daunorubicin transport by MDR3 P-gp and its inhibition by CsA. Like our assays, their accumulation experiments had a sensitive read-out that allowed the detection of small

changes in transport efficiency.

Drug resistance caused by MDR3 P-gp has been shown by Kino and coworkers in an *MDR3* transformed yeast strain (15). In their experiments resistance to aureobasidin A, a fungicide, correlated with the expression of *MDR3* and was inhibited by high concentrations of CsA, verapamil and vinblastine. The transport of aureobasidin A by MDR1 P-gp was more efficient than by MDR3 P-gp, comparable to what we find for the drugs tested in our study.

To exclude the possibility that the drug transport found in *MDR3* transfected cells is only an indirect effect of the presence of MDR3 P-gp – e.g. because it activates an endogenous transporter – we sought direct evidence of binding of drug to MDR3 P-gp. Experiments with the MDR1 P-gp have shown that this transporter catalyzes two reactions that can be used to study protein-drug interaction, hydrolysis of ATP (ATPase activity) and trapping of nucleotide in the ATP binding site in the presence of vanadate (nucleotide trapping). We have studied both reactions for MDR3 P-gp, expressed to high levels in Sf9 cells. We were unable to find any ATPase activity of MDR3 P-gp above the background present in uninfected Sf9 cells with or without a range of phospholipids or drugs, under conditions that allow ready detection of the ATPase activity of MDR1 P-gp (35) and the Multidrug Resistance Protein 1 (results not shown). This negative result could be due to a low turnover number of MDR3 P-gp, but other explanations cannot be excluded at this stage.

We were able to demonstrate vanadate-dependent nucleotide trapping by MDR3 P-gp, however, and substantial drug effects on this process. Whereas most drugs at moderate concentrations increase the rate of nucleotide trapping by MDR1 P-gp and only decrease this

rate at much higher concentrations, we only observed inhibitory effects on MDR3 P-gp. The drug concentrations required for inhibition of trapping (Table I) were similar to those needed to inhibit transport of NBD-PC (Fig. 5A) or drugs (Fig. 3) by MDR3 (and MDR1 P-gp). We conclude that the MDR3 P-gp can bind a range of cytotoxic drugs and P-gp inhibitors with relatively high affinity.

If drug is bound with rather high affinity to MDR3 P-gp, why is this P-gp such a poor drug transporter that it is unable to cause MDR in transfected cells? We consider three explanations for this paradox:

1. PC is the preferred substrate of MDR3 P-gp; as membranes are full of PC, drugs cannot compete with PC. This explanation is in line with our inability to block PC transport with PSC833 in mice *in vivo*. Mayer et al (45) have shown that PSC833 has no effect at all on secretion of PC into bile at drug concentrations that strongly inhibit the secretion of digoxin or paclitaxel from the liver into bile. As the MDR3 P-gp concentration has been shown to be a controlling step for PC secretion into bile (18,46), it is unlikely that a partial inhibition by PSC833 would have been missed in these experiments. The fact that we can readily inhibit C₆-NBD-PC transport with reversal agents *in vitro* (Fig. 5), could be due to a lower affinity of MDR3 P-gp for short chain PC than for natural PC; *in vitro* inhibition of natural PC transport by reversal agents has not been tested.
2. PC is not a preferred substrate for MDR3 P-gp and the protein transports drugs at least as well (or better) than PC. Transport of drugs does not lead to drug resistance, because the catalytic efficiency is lower than of MDR1 P-gp. Our inability to detect ATPase activity

associated with MDR3 P-gp is in line with this explanation. It can also account for the PSC833 sensitive biliary excretion of digoxin and paclitaxel in mice that lack both the Mdr1a and the Mdr1b P-gp in the bile canalicular membrane (45,47). Mayer et al (45) conclude that another digoxin transporter must be present in the canalicular membrane, in addition to the Mdr1a/b P-gps. As human MDR3 P-gp can transport both digoxin and paclitaxel and is sensitive to inhibition by PSC 833, the unidentified drug transporter could be the Mdr2 P-gp.

3. A compromise between explanations 1 and 2 is that PC is the preferred substrate, but that the transport of natural PC by MDR3 P-gp might be so efficient that the inner leaflet of the membrane around the transporter runs out of substrate. If the (bile-type) PC molecules in the proximity of the transporter are transported to the outer leaflet of the membrane fast, this will leave only (non-bile-type PC molecules,) aminophospholipids and drugs. For the lack of better substrates, the latter may then be transported as well. This hypothesis implies that the transport of PC by MDR3 P-gp is faster than the lateral diffusion of PC molecules in the inner leaflet of the membrane or backflipping from the outer leaflet. The very high concentration of MDR3 P-gp in the canalicular membrane (18) would therefore create the conditions in which this P-gp would be able to contribute to drug transport.

It should be obvious that none of these explanations is satisfactory. Explanations 1 and 3 do not explain high-affinity binding of drugs under conditions where PC transport is low, *i.e.* the nucleotide-trapping experiments at low ATP concentration. Explanation 2 cannot account

for lack of inhibition of PC secretion by PSC833 *in vivo*. Clearly it is important to test the effect of PSC833 and other inhibitors on the transport of PC by MDR3 P-gp in cell lines. Unfortunately, this is technically difficult.

The *MDR3* and *MDR1* genes are very similar. They both contain 27 introns, inserted at identical positions in the coding sequence (48). The proteins encoded by these genes have virtually identical hydropathy plots, they are 77% identical and 82% similar in amino acid sequence (5,49). It does not require a bold leap of the imagination to infer that the PC translocators encoded by the *MDR3* and *Mdr2* genes evolved from drug transporting P-gps by gene duplication during vertebrate evolution when the need arose to package the aggressive bile salts required for fat digestion in mixed micelles with phospholipids. It is unlikely, though still untested, that MDR1-type P-gps transport natural membrane phospholipids. This would interfere with their physiological function, protection against toxins and could lead to futile flipping of *e.g.* phosphatidylethanolamine, resulting in useless ATP hydrolysis.

Since bile appeared in evolution more than a hundred million years ago, it is remarkable that the MDR3 P-gp is still able to transport/bind a range of drugs, if its only function were PC transport. We briefly consider three explanations for this paradox: Firstly, it is possible that drug transport by MDR3 P-gp is an accidental side-product of evolution. There is evidence for gene conversion between the *MDR1* and *MDR3* genes (4) and such gene conversion may have counteracted the genetic drift accompanying the evolutionary optimization of the protein as a PC transporter. Secondly, optimal transport of PC may require a protein that transports

some drugs as an unavoidable consequence. We find that unlikely. ABC-transporters have been adapted in evolution to a variety of highly specialized tasks and, in fact, the substrate specificity of the MDR3 P-gp towards membrane PLs is exquisite (23). Thirdly, MDR3 P-gp might be a dual function protein, available to transport PC, but also some toxins that are especially threatening to the liver, the only organ where the protein is present in high concentrations.

At present, both explanations 1 and 3 are compatible with the experimental evidence. More work on the effect of inhibitors on the transport of long-chain PC and on binding of drugs to MDR3 P-gp reconstituted in PC-free membranes is required to fully define the physiological functions of this interesting transporter. The importance of a better definition is reinforced by the discovery that the complete absence of the MDR3 P-gp in humans causes a severe liver disease requiring liver transplantation (50) and that women heterozygous for a *MDR3* null allele are at risk for developing intrahepatic cholestasis of pregnancy (51). It is therefore conceivable that the type of drug interactions with MDR3 P-gp described here could result in disease of heterozygotes under conditions of stress requiring maximal phospholipid secretion into bile.

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Abbreviations:

BSA, bovine serum albumin; CsA, cyclosporin A; HBSS', HEPES buffered Hanks Balanced Salt Solution; MDR, multidrug resistance; NEM, N-ethyl-maleimide PC, phosphatidylcholine; P-gp, P-glycoprotein; PL, phospholipid; PSC, PSC 833; VCR, vincristine; vrp, verapamil

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Table I

Effects of various agents on nucleotide trapping by MDR3 P-gp. Nucleotide trapping was quantified by phosphoimaging. Mean values from two independent experiments.

Agent added	Concentration	MDR3 nucleotide trapping (% of control)
verapamil	50/200 μM	70/20
CsA	2/5 μM	10/<5
PSC833	2/5 μM	10/<5
paclitaxel	5/50 μM	10/<5
verapamil	5/50 μM	30/10
ouabain	200/800 μM	90/40

Legends

Figure 1. Flow of radioactive drugs through monolayers of LLC-PK1 derived cell lines. LLC-M3.4.44 is an *MDR3* transfected clone of LLC-PK1, LLC-1.1 is an *MDR1* transfectant. The flow from the apical to the basolateral compartment (n) and *vice versa* (") are measured separately and plotted in a single graph. The horizontal bars indicate the values of two independent measurements. Note that the Y-axis is not identical for all drugs. The net transport (flow from basolateral to apical minus the flow from apical to basolateral) is significantly higher in the *MDR3* transfected cells than in the parental cell line (two sided $P < .00001$ for all drugs using one-way analysis of variance).

Figure 2. Net transport of 2 μ M digoxin through monolayers of LLC-PK1 derived cells correlates with the amount of MDR3 P-gp in the cells. (A) Left: directional transport of digoxin through monolayers of various subclones of *MDR3* transfectant LLC-M3.4 (3.4.1; 3.4.11; 3.4.44; 3.4.45). Right: directional transport of digoxin through monolayers of two independent *MDR3* transfectants, LLC-M3.1 and LLC-M3.4.44, and one *MDR1* transfectant, LLC-1.1. Depicted is the difference in the absolute amounts of drug transported from the basolateral to the apical compartment and *vice versa* in 4h (mean \pm range in two independent experiments). Each compartment contained 2ml tissue culture medium (B) Protein blot analysis of total cell lysates of the corresponding subclones. Detection of P-gp content with the monoclonal antibody C219, that recognizes all mammalian P-gps tested (27).

Figure 3. Inhibition of digoxin transport through monolayers of *MDR3* and *MDR1* transfected cells by reversal agents. The flow from the apical to the basolateral compartment (n) and *vice versa* (") are measured separately and plotted in a single graph. The horizontal bars indicate the values of two independent experiments.

Figure 4. Accumulation of digoxin in the cell lines transfected with P-gp genes in the absence (filled bars) and presence (open bars) of 5 μ M PSC 833. Cells on plastic dishes are incubated for 2h with 10 μ M [3 H]digoxin, washed and scraped. The radioactivity was measured by scintillation counting. The drug accumulation in the presence of the reversal agent is arbitrarily set at 1 for each cell line to allow for variation in the number of cells present in the wells. In this experiment the amount of digoxin accumulated in the presence of PSC 833 varied from 88 ± 6 pmol for LLC-M3.4.44 to 128 ± 5 pmol for LLC-1.1. Shown is the mean \pm SD of three measurements.

Figure 5. Inhibition by reversal agents of C₆-NBD-[3 H]PC and C₆-NBD-GlcCer translocation by MDR3 and MDR1 P-gp. (A) Relative amount of C₆-NBD-[3 H]PC translocated in the *MDR3* transfected cell line LLC-M3.4.44 during 3 h at 15°C. Translocation across the apical (filled bars) and the basolateral membrane (open bars) was measured in the presence of 10 μ M CsA, 5 μ M PSC 833 (PSC), 20 μ M verapamil (vrp) and 10 μ M VBL, paclitaxel (pacl.) or digoxin, as indicated in the bottom line. Incubation of the

cells with these drugs did not alter the efficiency of C₆-NBD-[³H]PC synthesis. (B) Relative amount of C₆-NBD-GlcCer translocated in the *MRP* transfected cell line LLC-MRP during 3 h at 15°C. Translocation across the apical (filled bars) and the basolateral membrane (empty bars) was measured in the presence of 10 μM paclitaxel and 2 mM sulfinpyrazone, as indicated in the bottom line. (C) Relative amount of C₆-NBD-GlcCer (left panel) and C₆-NBD-[³H]PC (right panel) that was translocated in the *MDR1* transfected cell line LLC-1.1. Translocation across the apical (filled bars) and the basolateral membrane (empty bars) was measured in the presence of 20 μM verapamil (vrp) and 10 μM digoxin, as indicated in the bottom line. The control values shown for cell line LLC-PK1 were obtained in an earlier experiment (23).

Figure 6. Expression and ATP-binding of *MDR3* in Sf9 cells. Panel A: Isolated Sf9 cell membranes, containing β-galactosidase (lane 1) recombinant MDR1 (lane 2) or MDR3 (lane 3) were subjected to SDS-PAGE and immunoblotting with the C219 monoclonal antibody. Panel B: Binding of azido-ATP to MDR3 P-gp and MDR1 P-gp. Isolated Sf9 cell membranes, containing β-galactosidase (lane 1) recombinant MDR1 (lane 2) or MDR3 (lane 3-5) were labeled with 5 μM of [α-³²P]-8-azido-ATP, the labeled proteins were separated on SDS-PAGE and subjected to autoradiography. The effect of the addition of 2 mM EDTA (lane 4) or 1 mM ATP (lane 5) is shown.

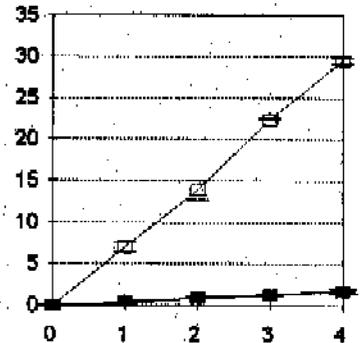
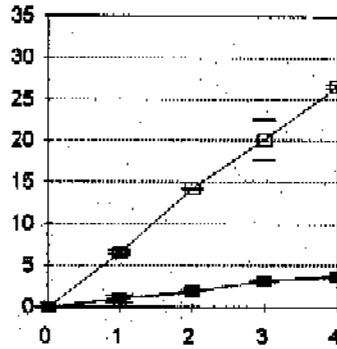
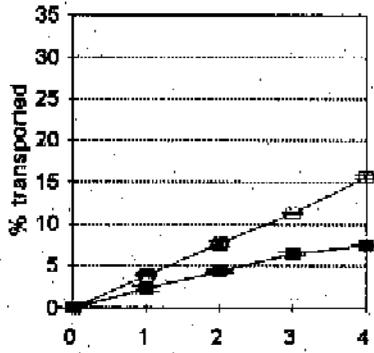
Figure 7. Trapping of [α - 32 P]-8-azido-ATP in MDR1 and MDR3 P-gp. Panel A: Labeling was performed by the incubation of isolated Sf9 cell membranes, expressing *MDR3* (lanes 1-5), *MDR1* (lanes 6 and 7) or β -galactosidase (lane 8), at 37°C for 2 min in the presence of 5 μ M Mg-[α - 32 P]-8-azido-ATP and 200 μ M Na-orthovanadate. The incubation media contained in lane 2: 1 mM EDTA, lane 3: 500 μ M NEM, lanes 4, 7: 50 μ M verapamil, lane 5: 5 μ M CsA. Nucleotide trapping, covalent photo-affinity labeling, SDS-PAGE and autoradiography were performed as described in the Methods section. Panel B: detection of P-glycoproteins in the samples corresponding to panel A by immunoblot analysis using monoclonal antibody C219.

LLC-PK1

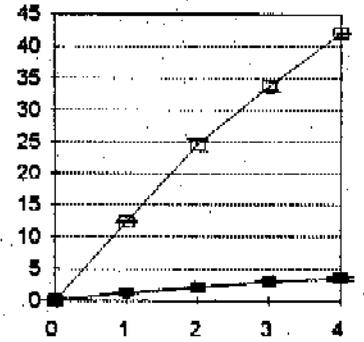
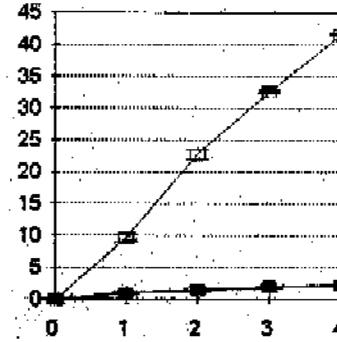
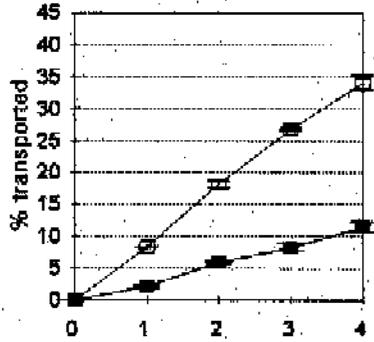
LLC-M3.4.44
MDR3

LLC-1.1
MDR1

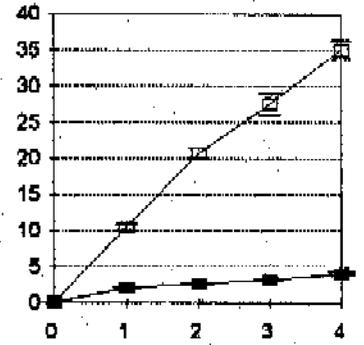
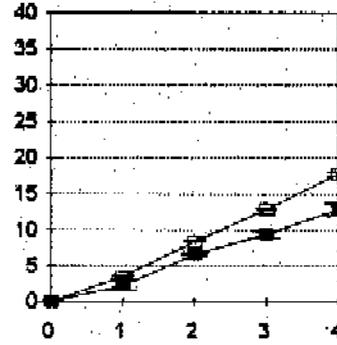
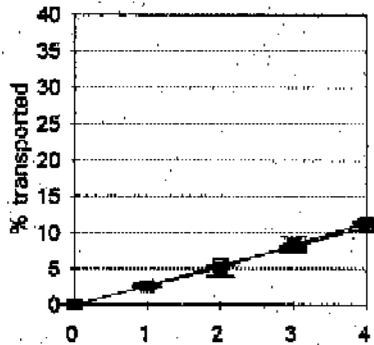
2 μ M
digoxin



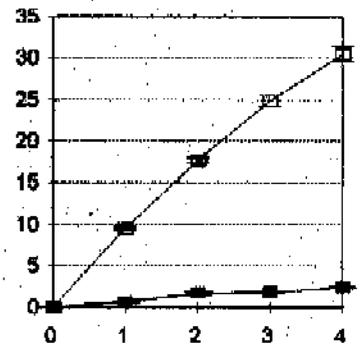
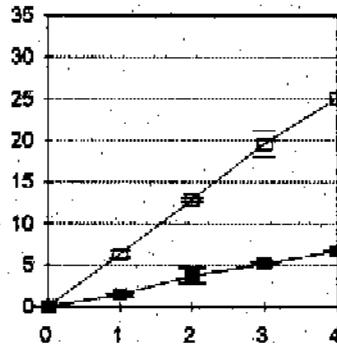
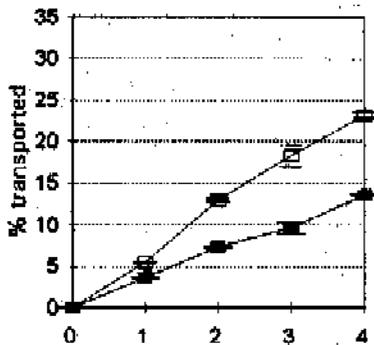
20 μ M
paclitaxel



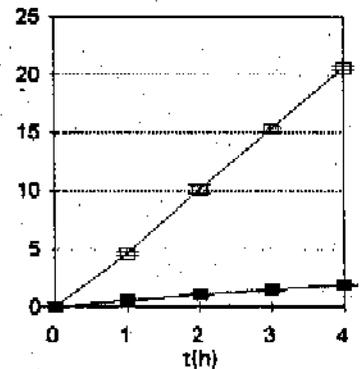
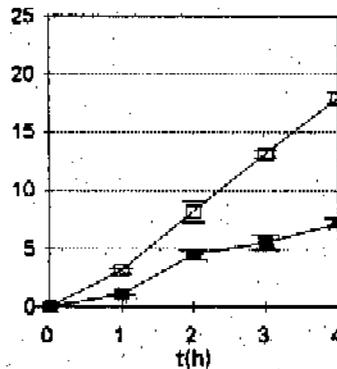
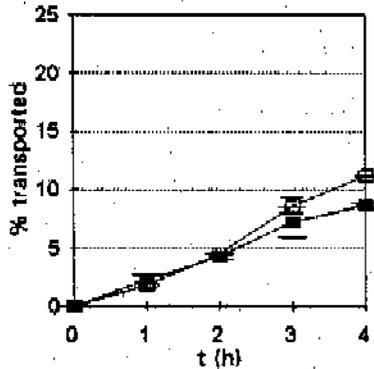
2 μ M
daunorubicin

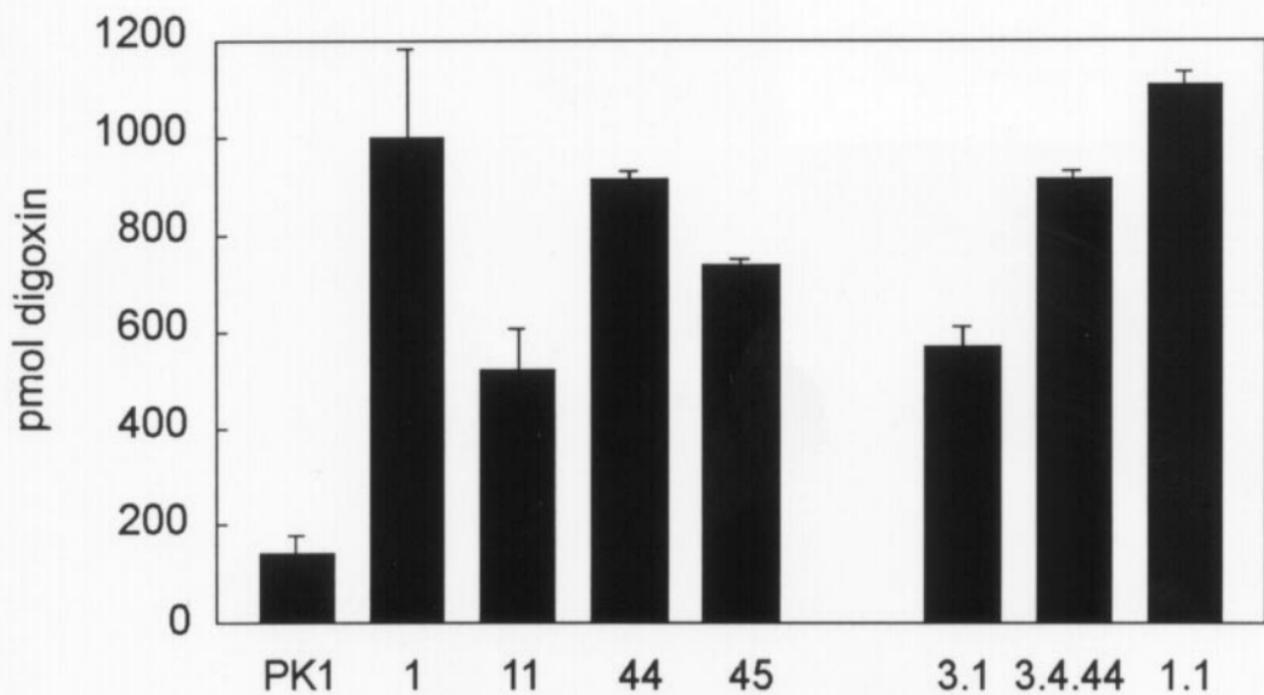
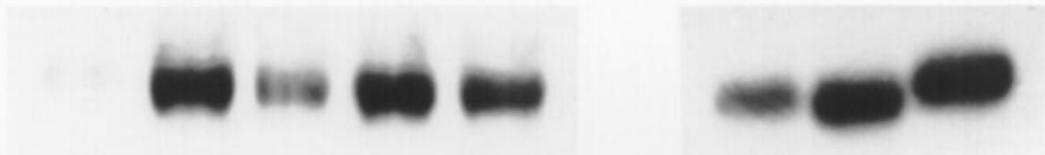


2 μ M
vinblastine



50 nM
ivermectin



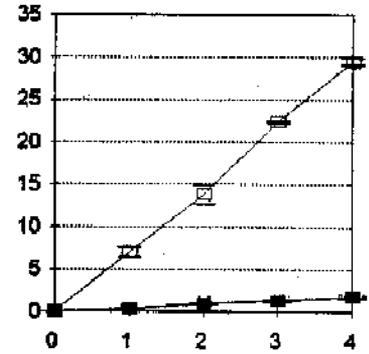
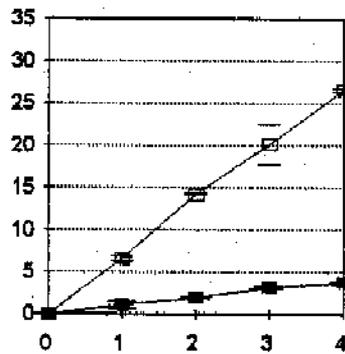
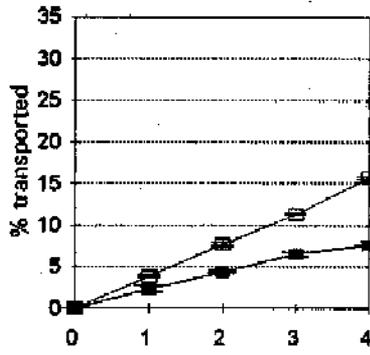
A**B**

LLC-PK1

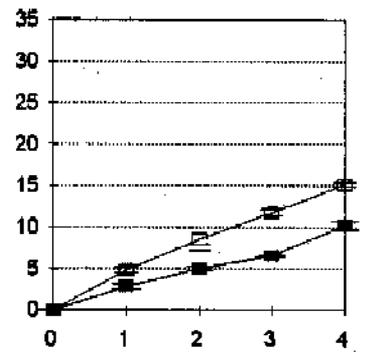
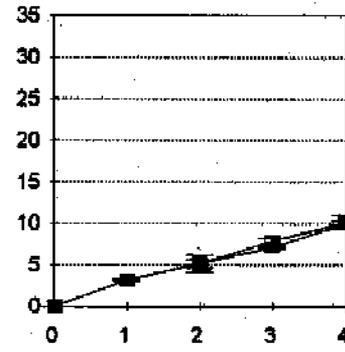
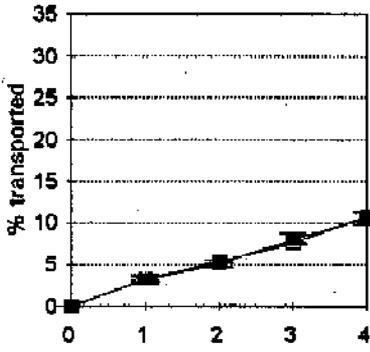
LLC-M3.4.44
MDR3

LLC-1.1
MDR1

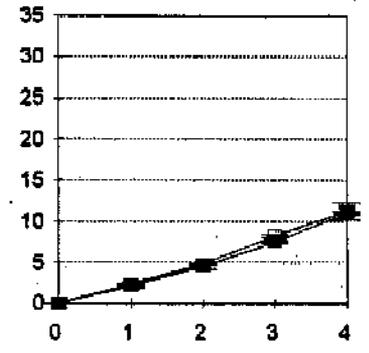
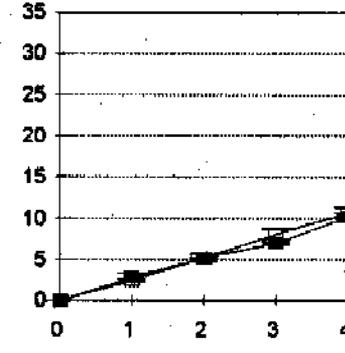
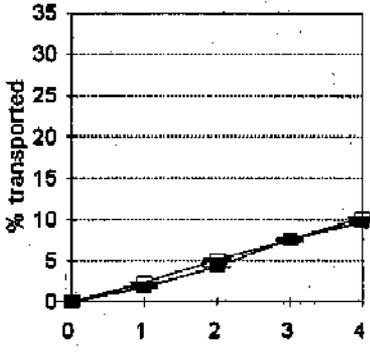
no inhibitor



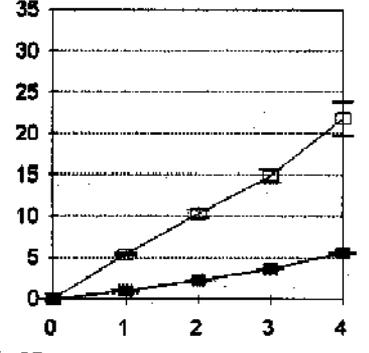
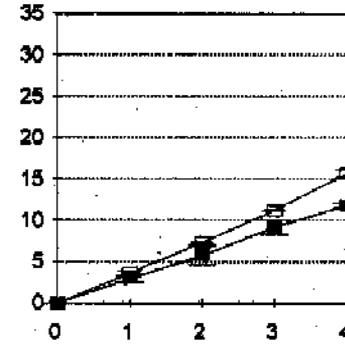
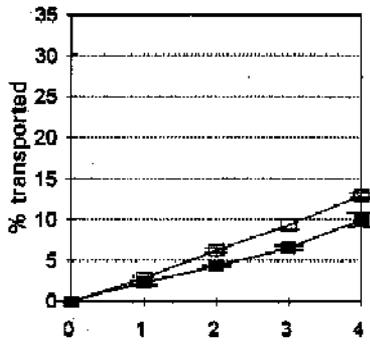
10 μ M
cyclosporin A



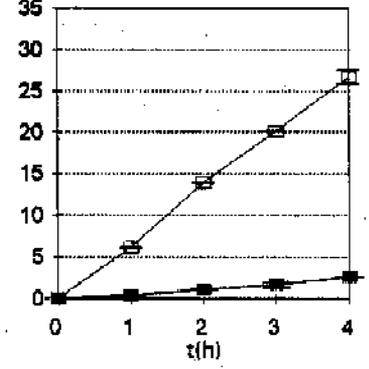
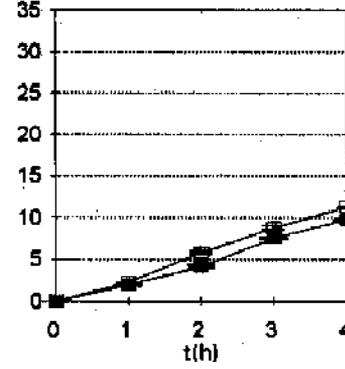
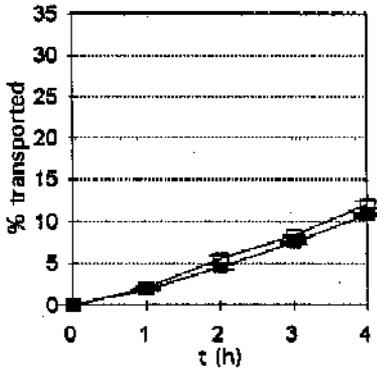
5 μ M
PSC 833

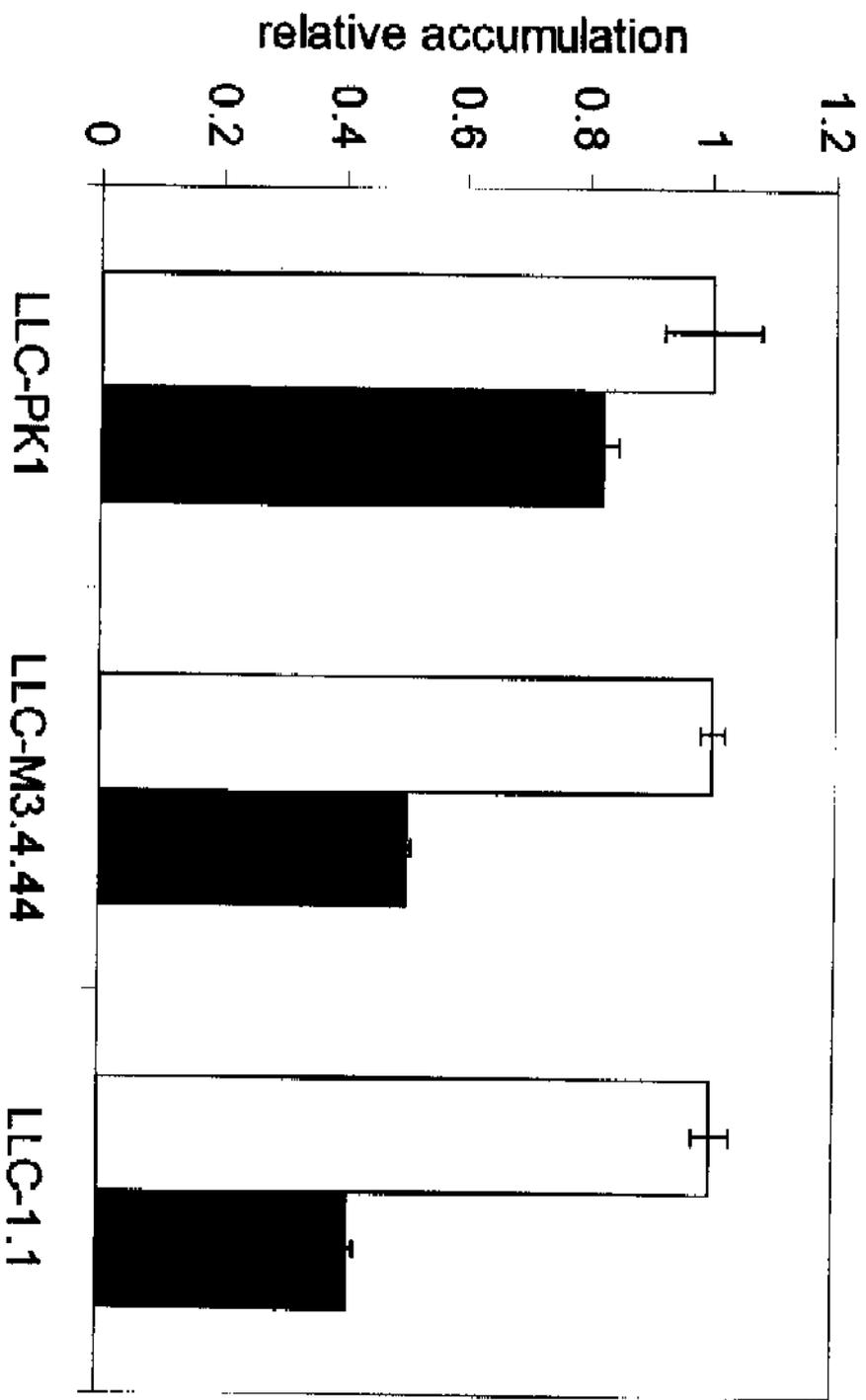


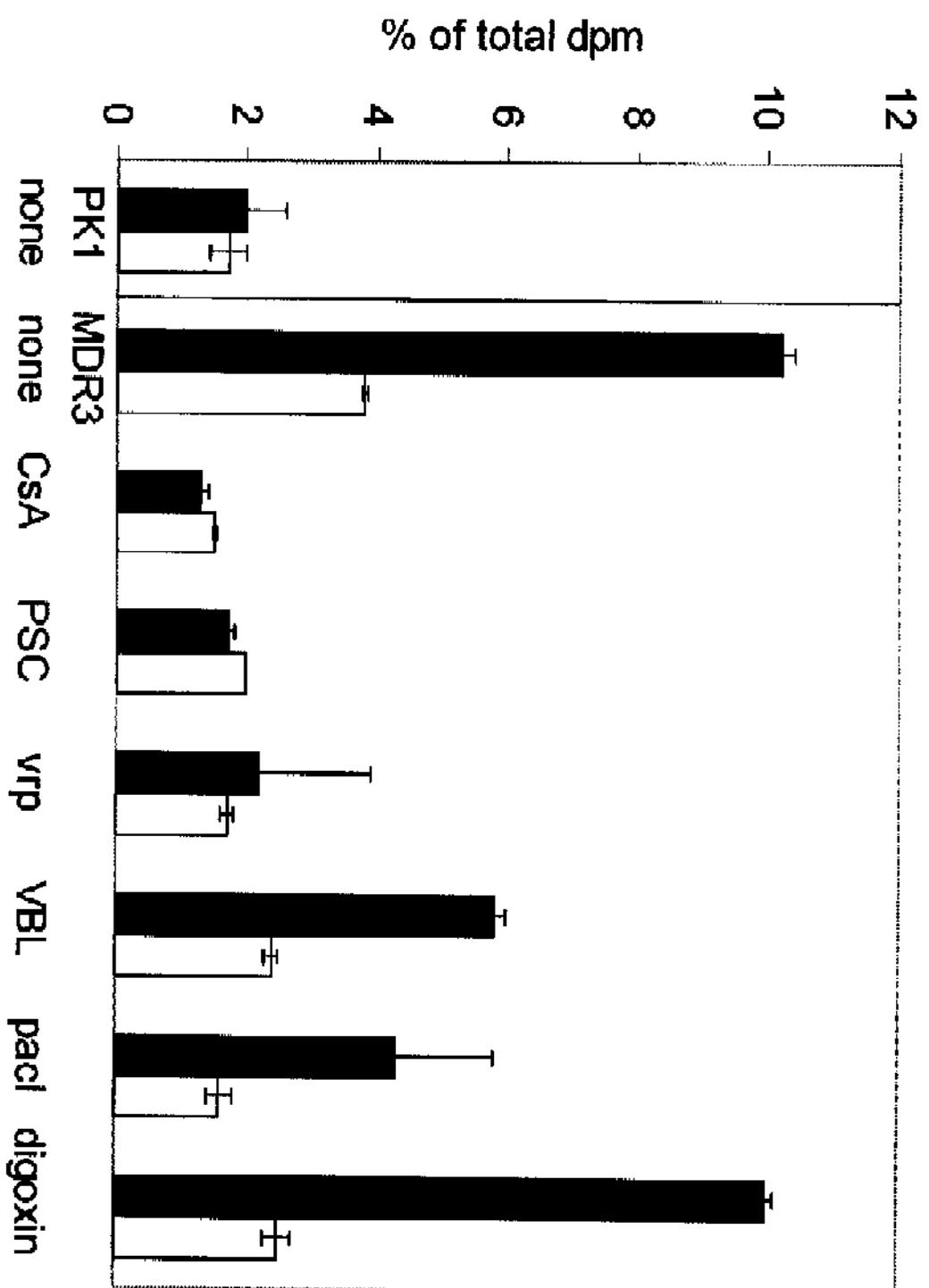
20 μ M
verapamil

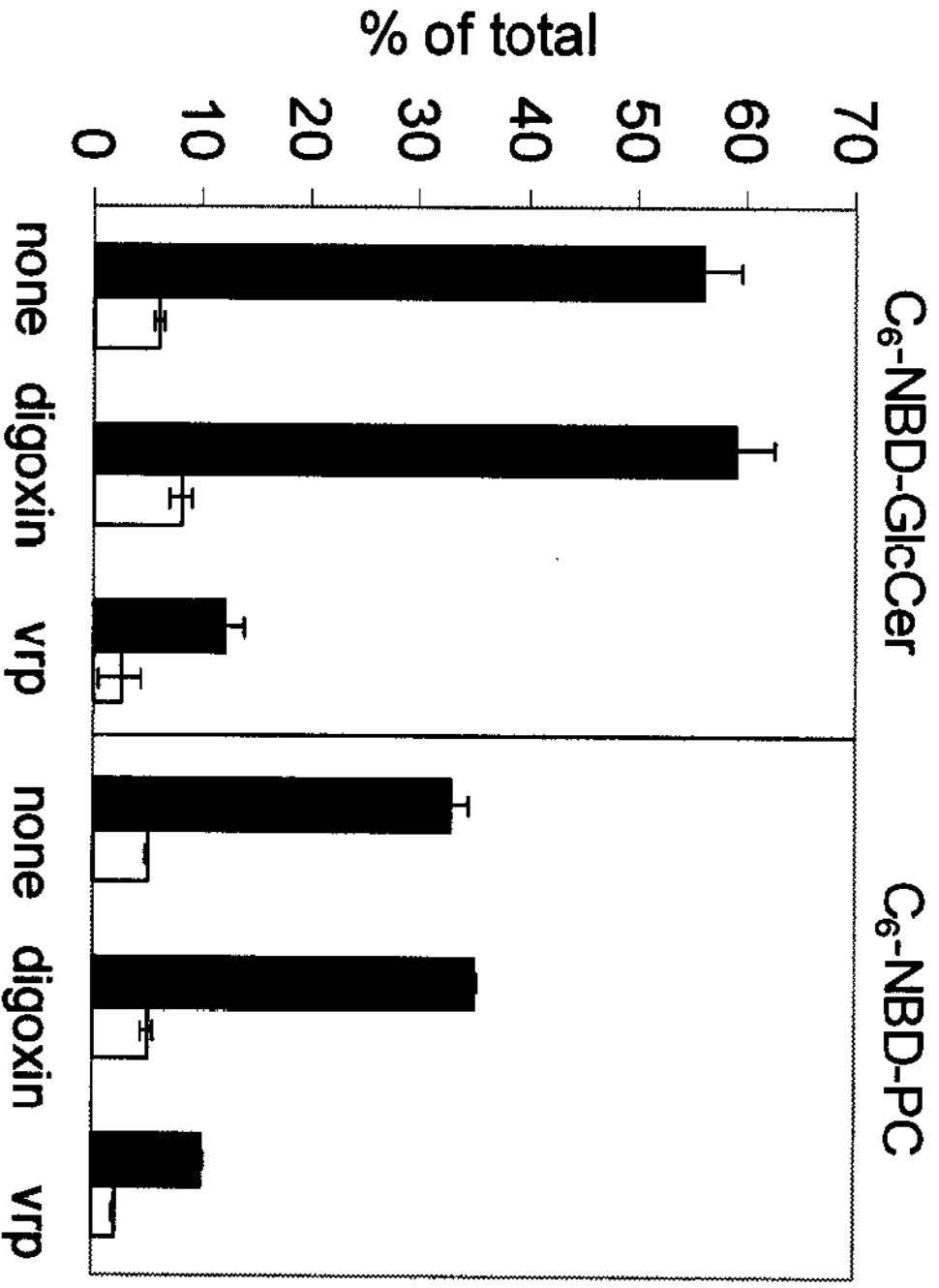


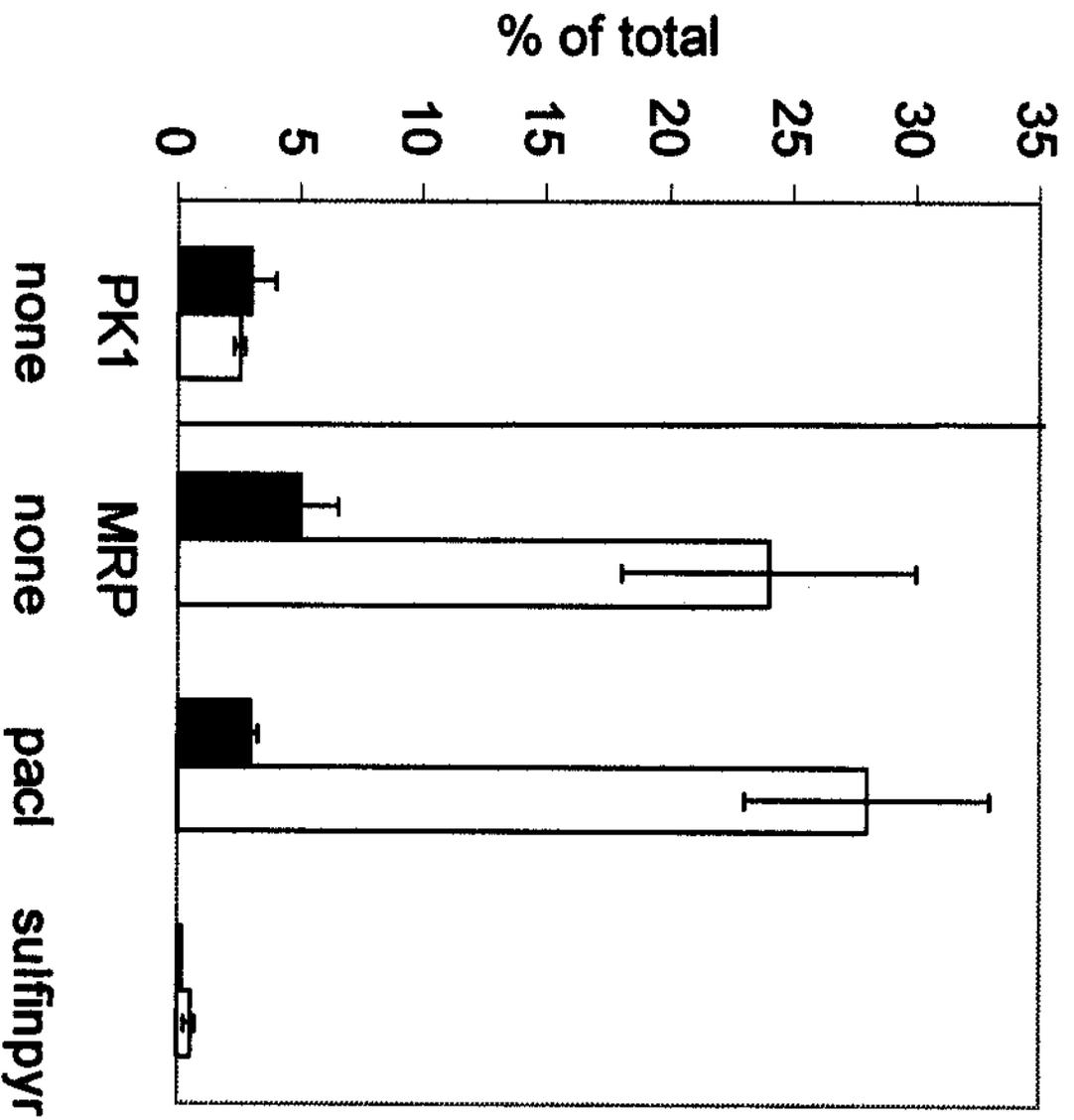
10 μ M
vinblastine











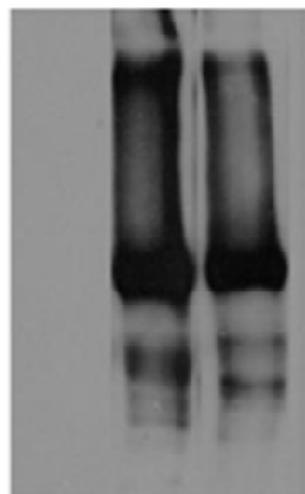
A (immunoblot)

1 2 3

200—

97—

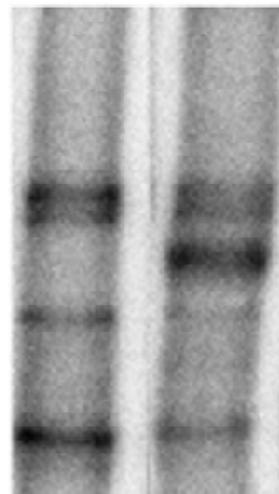
69—



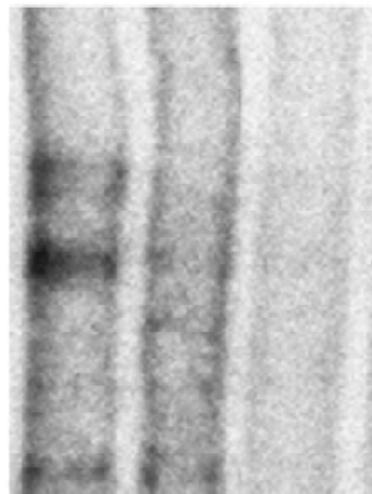
β -gal MDR1 MDR3

B (32 P)

1 2 3 4 5



β -gal MDR1



MDR3 MDR3 MDR3
+EDTA +ATP

^{32}P -incorporation

MDR3

MDR1

β -gal

1

2

3

4

5

6

7

8

200 —

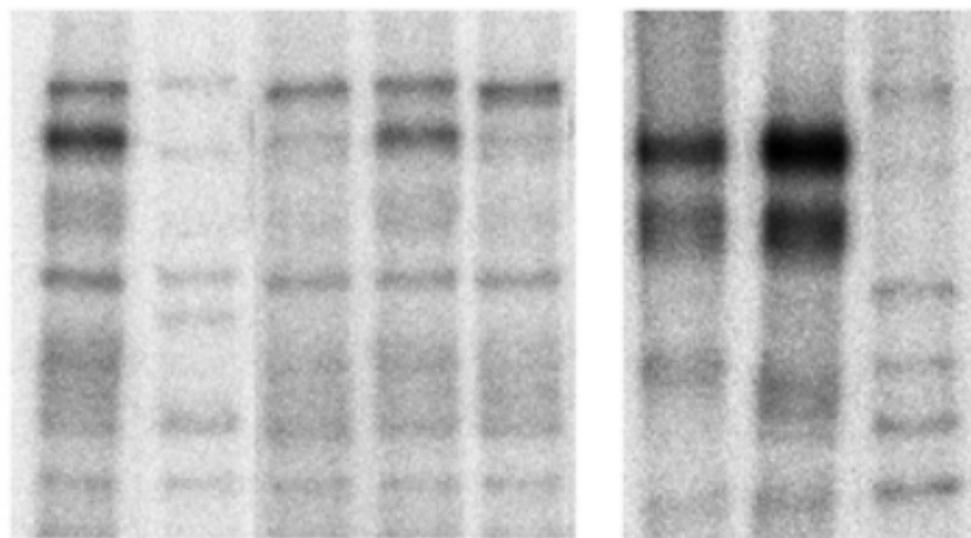
97 —

69 —

← MDR3
MDR1

EDTA NEM Ver CSA

Ver



IMMUNOBLOT

MDR3

MDR1

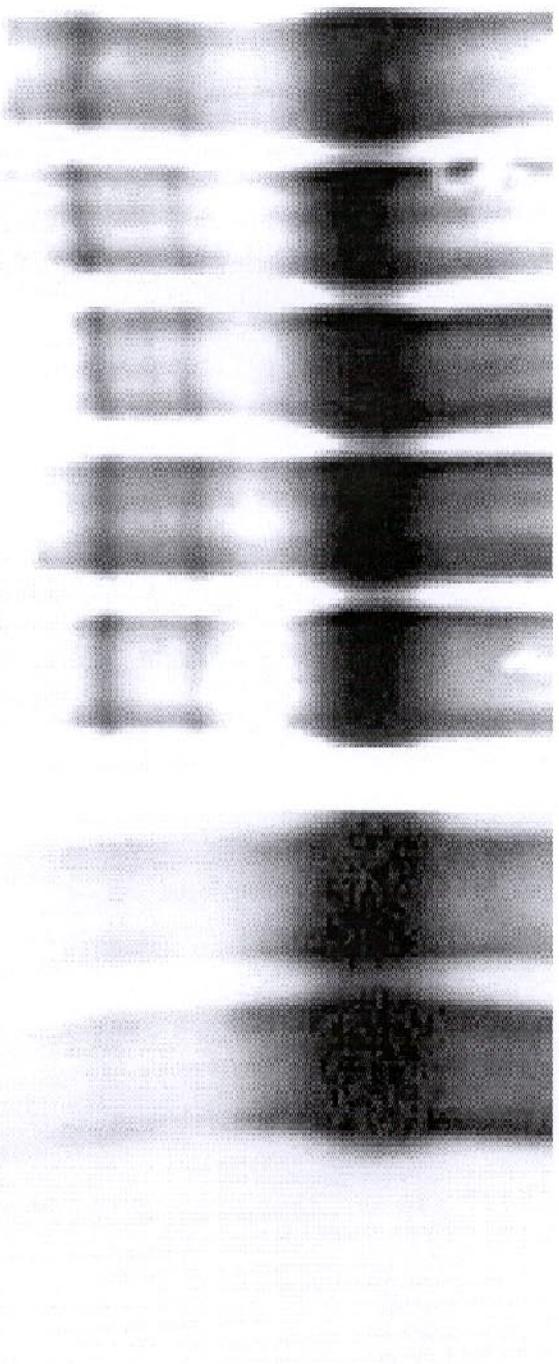
β -gal

1 2 3 4 5 6 7 8

200—

97—

69—



↑
MDR3
MDR1

EDTA NEM Ver CSA Ver