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Induction of proteins involved in multidrug resistance (P-glycoprotein, MRP1, MRP2, LRP) and of CYP 3A4 by rifampicin in LLC-PK$_1$ cells

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Abstract

P-glycoprotein, multidrug resistance-related proteins (MRPs) and lung resistance-related protein (LRP) are involved in multidrug resistance in tumor cells but are also expressed in normal tissues. In the LLC-PK$_1$ tubular renal cell line, a 15-day treatment with 25 μM rifampicin significantly increased the mRNA levels of P-glycoprotein, MRP1, MRP2, LRP and cytochrome P450 3A4 (CYP 3A4). Western blot analysis confirmed a moderate increase in the expression of P-glycoprotein and MRP2, but not MRP1 also at the protein level. The intracellular uptake of doxorubicin was significantly lower in rifampicin pretreated cells. A pretreatment with 6-[82S,4R,6E)-4-methyl-2-(methylamino)-3-oxo-6-octenoic acid]cyclosporin D, valspodar (PSC 833), a specific inhibitor of P-glycoprotein, with (3-(3-(2-(7-chloro-2-quinidinyl)ethenyl-phenyl)((3-diimethyl amino-3oxo propyl)thio)methyl)thio)propanoic acid, sodium salt (MK-571), a specific inhibitor of MRP1, and with verapamil, that inhibits both proteins, significantly increased doxorubicin cell accumulation in rifampicin pretread cells. In rifampicin treated cells cultured on porous membranes, doxorubicin showed a polarized transport, that was reduced by a pretreatment with PSC 833. A chronic treatment with rifampicin induces the expression of transport proteins and of CYP 3A4 and could therefore alter the renal elimination kinetics of drugs that are their substrates.

Keywords: P-glycoprotein; MRP (Multidrug resistance-related protein); LRP (Lung resistance-related protein); Cytochrome CYP 3A4; LLC-PK$_1$ renal cell line; Rifampicin

1. Introduction

The most important mechanism responsible for resistance against certain anticancer agents is linked to decreased intracellular accumulation through enhanced cellular efflux of the antitumor compounds and usually involves the expression of P-glycoprotein, multidrug resistance-related proteins (MRPs) or lung resistance-related protein (LRP) (Loo and Clarke, 1999). P-glycoprotein is a 170 kDa ATP-dependent pump expressed constitutively in various normal cells and has been identified on apical membranes of cells with excretory or protective functions such as the enterocytes, the proximal renal tubular cells and the endothelial cells of brain capillaries (Thiebaut et al., 1987). MRPs are a family of drug transporters of 190 kDa involved in multidrug resistance but also expressed in normal tissues; MRP1 and MRP2 are expressed on the basolateral and apical membranes of cells, respectively (Borst et al., 1999). LRP is a 110 kDa major vault transporter protein, originally isolated from P-glycoprotein-negative multidrug resistant cells (Scheffer et al., 2000b) that appears to function as a bidirectional nucleo-cytoplasmic transporter of molecules, is widely expressed in normal human tissues and is important in intracellular drug sequestration (Sugawara et al., 1997). All these proteins seem to act as a defense mechanism against potential toxic substances, and are now recognised as the major determinants of the pharmacokinetics of various drugs. P-glycoprotein shows some similarities with cytochrome P450 3A4 (CYP 3A4), since both act to eliminate xenobiotics, are often expressed in the same cells, have common substrates and can be inhibited by the same molecules (Schuetz et al., 1996).

Many environmental factors can affect the expression of P-glycoprotein and MRPs. In vitro data have revealed that P-glycoprotein, the product of multidrug resistance (MDR1) gene in humans and of mdr1a and mdr1b genes in rodents, can be induced by a variety of drugs and hormones (Greiner

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et al., 1999; Seree et al., 1998), but the molecular mechanism of induction has not been completely elucidated (Geick et al., 2001). Recently it has been shown that P-glycoprotein is inducible in human gut by the prototypic CYP 3A4 inducer rifampicin; in this study Greiner et al. (1999) have suggested that P-glycoprotein induction may be restricted to some cell types, however, few data exist about the induction of P-glycoprotein in other tissues. An organ specific mdr1 inducibility has been previously shown in rodents: in fact, in mice, the P-glycoprotein inducer dexamethasone increased mdr1b expression in colon but not in kidney (Seree et al., 1998) and mdr1a and mdr1b were not inducible in rat liver in the presence of rifampicin or dexamethasone (Salphati and Benet, 1998). In humans, rifampicin does not induce lymphocyte P-glycoprotein expression (Becquemont et al., 2000). In addition, it has been recently shown that MRP2 can be induced by rifampicin in vivo in human duodenum (Fromm, 2000) and in monkey liver (Kauffmann et al., 1998).

In the normal kidney P-glycoprotein is constitutively expressed and plays a major role in the renal excretion of many substrates (Ernest and Bello-Reus, 1998); MRP1, MRP2 and LRP are also expressed in the normal kidney (Borst et al., 1999; Sugawara et al., 1997). An induction of renal P-glycoprotein has been demonstrated using substrates such as cyclosporin A and 6-[82S,4R,6E)-4-methyl-2-(methylamino)-3-oxo-6-octenoic acid]cyclosporin D, valspodar (PSC 833) (Jette et al., 1996) and cisplatin (Demeule et al., 1999). Furthermore the expression of renal MRP2 can be also induced by cisplatin (Demeule et al., 1999).

The LLC-PK_1 cell line is a proximal tubular renal cell line derived from pig kidney (Hull et al., 1976) which has structure and function similar to those of renal proximal tubular cells (Handler et al., 1980). These cells form an oriented monolayer with microvilli and tight junctions (Horio et al., 1990) and express low amounts of P-glycoprotein (Crivellato et al., 1999; Decorti et al., 1998) as well as of MRP1 and MRP2 (Decorti et al., 2001; Evers et al., 1996), and thus represent an useful in vitro model for studying drug transport pathways and the effect of drug induction in the proximal renal tubule.

The aim of this study was therefore to evaluate the effect of a treatment with rifampicin on the expression of P-glycoprotein, MRP1, MRP2, LRP and CYP 3A4 in LLC-PK_1 cells.

2. Materials and methods

2.1. Chemicals and reagents

Culture medium, fetal bovine serum, L-glutamine, Dulbecco's phosphate buffered saline (D-PBS), propidium iodide, RNAse solution, Nonidet P-40, rifampicin, verapamil, RedTaq™ DNA polymerase, alkaline phosphatase conjugated secondary antibodies and 100 bp DNA marker were purchased from Sigma, Milano, Italy. (3-(3-(2-(7-chloro-2-quinidinyl)ethenyl-phenyl)((3-diimethyl amino-3oxo propyl)thio)methyl) thio)propanoic acid, sodium salt (MK-571), C219 and M_2III6 monoclonal antibodies and A23 polyclonal antibody were obtained from Alexis Biochemicals, Vinci-Biochem, Vinci, Italy; SDZ-PSC 833 was a kind gift of Novartis Pharma, Basel, Switzerland. SV Total RNA Isolation System was purchased from Promega Italia, Milano, Italy; SuperScript™ II RNase H^- reverse transcriptase and synthetic oligonucleotide primers were from Invitrogen Life Technologies, San Giuliano Milanese, Italy. All other chemicals were of analytical grade.

2.2. Cell line and growth conditions

The pig kidney epithelial cell line LLC-PK_1, obtained from the American Type Culture Collection (Rockville, MD, ATCC-CRL-1392) was grown in plastic bottles in medium 199 supplemented with 3% fetal bovine serum without antibiotics under an atmosphere of 95% air and 5% CO_2 at 37 °C and subcultured twice weekly with 0.02% EDTA and 0.05% trypsin.

Preliminary experiments were performed to choose the rifampicin concentration to use in induction experiments. 75 cm^2 flasks were inoculated with 4×10^4 cells/ml in 15 ml of complete medium. The day after seeding, the medium was removed and 15 ml of complete medium containing rifampicin (25, 50 or 100 μM) were added, then cells were incubated for different experimental times and the doubling time was calculated for all concentrations. The 25 μM concentration was subsequently used in all experiments as with this concentration the doubling time was comparable with that of untreated controls.

2.3. Propidium iodide staining: morphologic and cell cycle analysis

Cells treated as above with 25 μM rifampicin for 3, 8 and 15 days, were detached and resuspended at the concentration of 1×10^6 cells/ml in D-PBS, centrifuged at 4 °C for 5 min at $150 \times g$, and resuspended in 1 ml of 70% ethanol to permeabilize the membrane. After two washings with D-PBS, cells were resuspended in 1 ml of a staining solution containing RNAse (1 mg/ml), propidium iodide (0.05 mg/ml) and 0.1% Nonidet P-40, then incubated at 37 °C for 30 min. Each sample was analyzed by a cytometer EPICS XL-MCL (Coulter Electronics, Miami, FL, USA), equipped with an argon ion laser excitation source emitting at 488 nm. Propidium iodide (λ EX max: 540 nm, λ EM max: 625 nm) fluorescence was collected with a 575 nm band pass. At least 10000 cells were analysed and gated on the basis of the forward (FS) and sideward (SS) scatter. Propidium iodide staining was registered in linear mode amplification and DNA content was related to the relative cell number. Cell cycle distribution was determined with the software Multicycle Multiple Options Cell cycle Fitting (Phoenix Flow

Systems, San Diego, CA, USA). Cell morphology was reported as dot density of combined signals FS and logSS to highlight differences in size.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Control cells and cells treated with 25 μM rifampicin for 15 days were detached and total RNA was extracted from about 2×10^6 cells using the SV Total RNA Isolation System kit. Five micrograms of total RNA were reverse transcribed using the SuperScript™ II RNase H⁻reverse transcriptase. Different aliquots (0.5–1 μl) of the cDNA were used in PCR reaction in a final volume of 10 μl. PCR reaction was performed using serial dilutions of cDNA according to Bates et al. (1999). Synthetic oligonucleotide primers were used to investigate the presence of MDR1, MRP1, MRP2, LRP and CYP 3A4 mRNA transcripts. MDR1 sense: 5′-CCCATCATTGCAATAGCAGG-3′, antisense: 5′-GTTCAAACTTCTGCTCCTGA-3′ (for primer designing human sequence Accession number M14758 was used, amplified fragment 157 bp long); MRP1 sense: 5′-TTCCCATTTCAACGAGACCTTGCT-3′, antisense: 5′-TCCGCCCCACGATGCCGACCT-3′ (human sequence Accession number NM004996, 533 bp fragment); MRP2 sense: 5′-TGGCTGAGATTGGAGAG-3′, antisense: 5′-TTTGTCCTTTCACTAGTTC-3′ (human sequence Accession number X96395, 596 bp fragment); LRP sense: 5′-TGTAGGTGATGCCTGCAAAG-3′, antisense: 5′-TTTCTCGGCTTCTGACTGGT-3′ (human sequence Accession number X79882, 405 bp fragment); CYP 3A4 sense: 5′-GACCTGATCCCAGGCTTTTCCACAGAA-3′, antisense: 5′-CAACACGGGCTGCCGACCATGAAA-3′ (Sus Scrofa sequence Accession number AF109068, 245 bp fragment); β-actin sense: 5′-CACCAAAGCTGAGAGG-GAAATCGTCGTGA-3′, antisense: 5′-AATTGCGGTG-CACGATGGAGGGGCCGGACT-3′ (human sequence Accession number M10277, 500 bp). PCR was carried out using RedTaq™ DNA polymerase. The reaction was performed using a DNA thermal cycler Gene Amp® 9700 (PE Applied Biosystems, Foster City, CA). After denaturing at 94 °C for 5 min, the amplification was obtained by 30 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min each. A final extension step at 72 °C for 10 min was employed. Only for MDR1 the amplification cycles were performed at 94 °C for 30 s, 55 °C for 1 min, 72 °C for 2 min, according to Noonan et al. (1990); for MRP2 amplification was obtained by 35 cycles of 94 °C for 5 min, 50 °C for 1 min, 72 °C for 1 min, according to Kauffmann et al. (1998). PCR products were subjected to electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining. The DNA marker was 100 bp ladder. cDNA from pig liver was used as positive control while a negative control containing water instead of cDNA was always included in each experiment. To normalize data, the ratio between the densitometric quantification obtained with specific primers and with β-actin primers was calculated (Bates et al., 1999). The percentage of increase was calculated versus the data obtained for the same day in control cells.

2.5. Western blot analysis

Control cells and cells treated with 25 μM rifampicin for 15 days were detached and dissolved in a lysis buffer containing NaCl 100 mM, EDTA 1 mM, Tris–HCl 10 mM pH 7.5, and added with protease inhibitors phenylmethylsulphonyl fluoride 1 mM, aprotinin 2 μg/ml, leupeptin 2 μg/ml, pepstatin A 1 μg/ml. After centrifugation at 4 °C for 10 min at $1600 \times g$, the supernatant was centrifuged at $28{,}000 \times g$ for 1 h at 4 °C. Membranes were kept at −20 °C till use. Samples were resuspended in loading buffer (Laemmli, 1970), boiled for 5 min and loaded on 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane by electroblotting for 2 h at 2 mA/cm^2 with the semi-dry protein blotter system (Pharmacia Biotech, Cologno Monzese, Italy).

The membrane was stained with red Ponceau to verify the homogeneity of samples loaded on gel, then blocked for 2 h in TTBS (Tris buffer saline: 150 mM NaCl, 50 mM Tris–HCl, pH 7.5, containing 0.05% v/v Tween-20) added with 3% skim milk, and incubated overnight at 4 °C with the primary antibodies diluted in blocking solution: C219 monoclonal antibody, specific for P-glycoprotein (Kartner et al., 1985) (dilution 1:200), M_2III-6 monoclonal antibody, specific for MRP2 (Scheffer et al., 2000a) (dilution 1:50) and A23 polyclonal antibody, which recognizes MRP1 (Fernetti et al., 2001) (dilution 1:500). Blots were washed three times with TTBS, and incubated with alkaline phosphatase conjugated secondary antibody (goat anti-mouse immunoglobulin G (IgG), dilution 1:30,000 for C219 and M_2III-6; goat anti-rabbit IgG, dilution 1:20,000 for A23) for 1 h at room temperature. The membrane was extensively washed and finally stained by addition of bromocloroindolyl phosphate and nitroblue tetrazolium.

2.6. Doxorubicin accumulation

After a 2-week treatment with 25 μM rifampicin the cells were detached and resuspended at a concentration of 1×10^6 cells/ml and incubated at 37 °C with different concentrations of doxorubicin (5, 25 and 50 μM) for 60 min or for different times (from 30 to 240 min) with 50 μM doxorubicin in culture medium at 37 °C. After incubation, ice cold D-PBS was added to the cells and the samples were centrifuged at 4 °C for 5 min at $150 \times g$. The supernatant was aspirated and the cell pellet was resuspended gently in 6 ml of ice cold D-PBS buffer and centrifuged again. To evaluate doxorubicin uptake, the final pellet was resuspended in 1 ml of 0.3 N HCl in 50% ethanol, mixed thoroughly in a vortex mixer, and centrifuged at $700 \times g$. Doxorubicin content in the supernatant fraction was deter-

mined fluorimetrically with the method of Bachur et al. (1970). Standard curves of doxorubicin dissolved in 0.3 N HCl/50% ethanol were used for computation of doxorubicin content. Protein concentration of samples was determined by the method of Lowry et al. (1951).

2.7. Pretreatment with inhibitors

In some experiments the cells were pretreated with different concentrations of P-glycoprotein and MRP inhibitors. The tested substances were PSC 833 (1, 10 and 30 μM), MK-571 (1, 10 and 50 μM) and verapamil (1, 10 and 100 μM). After a pretreatment of 60 min at 37 °C with the inhibitors, cells were incubated with 25 μM doxorubicin for 60 min at 37 °C, then treated as above for the determination of doxorubicin intracellular uptake.

2.8. Transepithelial transport and inhibition studies

LLC-PK_1 cells, treated for 2 weeks with rifampicin 25 μM, and control, untreated cells, were detached and plated on day 0 on microporous polycarbonate membrane filters (3 μm pore size, 24.5 mm diameter, Transwell(™), Corning B.V. Life Sciences, Celbio, Pero, Italy), at a density of 2×10^6 cells/well. About 2 ml of medium or medium containing rifampicin 25 μM were applied at both sides. On day 1, medium was changed and, on day 3, the experiment was performed. The quality of the cell monolayers was determined by measuring the transepithelial electrical resistance, using a Millicel-ERS Voltohmeter (Millipore, Vimodrone, Italy). Medium at both the apical and the basolateral side of the monolayer was changed with fresh medium or medium containing the appropriate concentration of the inhibitors, 30 μM PSC 833 or 50 μM MK-571. After 1 h doxorubicin (final concentration 25 μM) was added to the apical or basolateral side. After 1, 2, 3 and 4 h, 50 μl samples were taken from the opposite side with respect to the side of doxorubicin application, and the concentrations of the antineoplastic drug were measured as above. Data are presented as percentages of the initial amount of doxorubicin added to the donor side.

2.9. Data analysis

Averages ± S.E. of the means were calculated; statistical analysis of results was performed by Student's *t*-test for

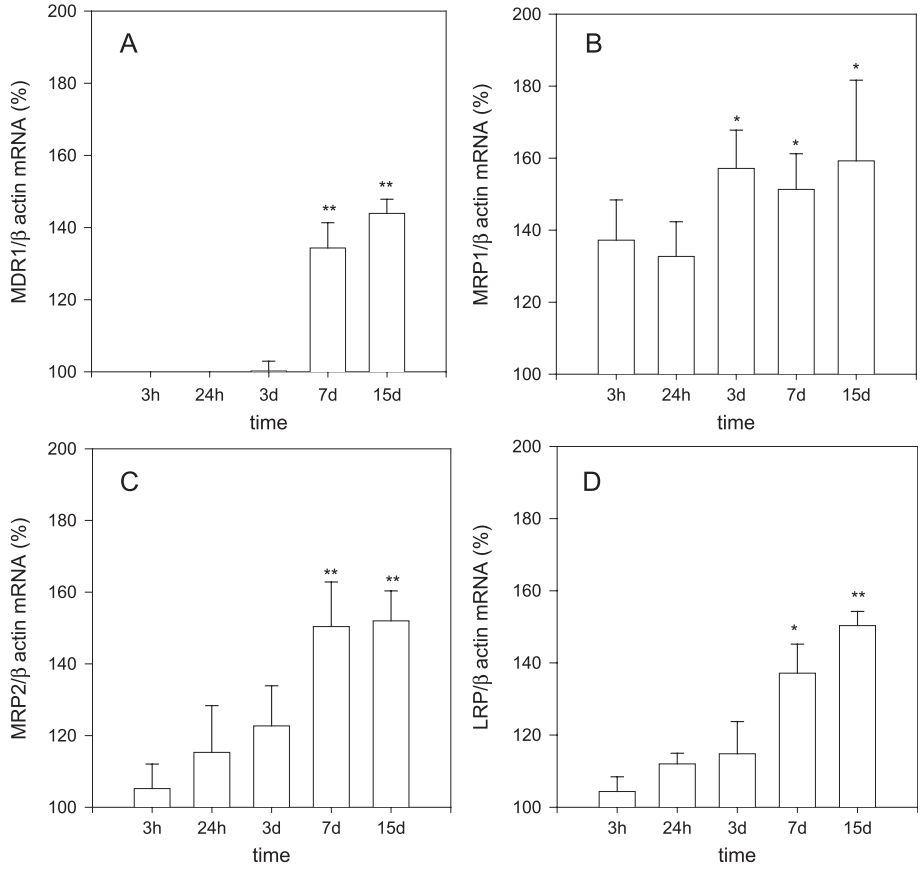

Fig. 1. RT-PCR analysis of MDR1 (A), MRP1 (B), MRP2 (C) and LRP (D) expression in LLC-PK_1 cells. The percentage of increase was calculated versus the data obtained for the same day in control untreated cells. Basal expression at different time points was set at 100% and the increase under rifampicin (25 μM) treatment was expressed as percentage of respective basal expression on the same day of culture. Each bar represents the mean ± S.E. of data from 6 to 12 determinations from a typical experiment. *$P < 0.05$, **$P < 0.01$, Student's *t*-test for independent data.

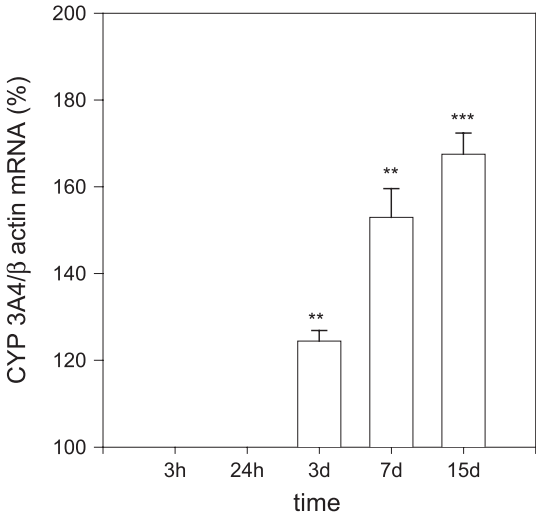

Fig. 2. RT-PCR analysis of CYP 3A4 expression in LLC-PK$_1$ cells. The percentage of increase was calculated versus the data obtained for the same day in control untreated cells. Basal expression at different time points was set at 100% and the increase under rifampicin (25 μM) treatment was expressed as percentage of respective basal expression on the same day of culture. Each bar represents the mean ± S.E. of data from 6 to 12 determinations from a typical experiment. *$P<0.05$, **$P<0.01$, ***$P<0.001$, Student's *t*-test for independent data.

independent samples. Values of $P<0.05$ were considered significant.

3. Results

Preliminary experiments revealed that 25 μM rifampicin did not modify the doubling time of LLC-PK$_1$ cells in culture; therefore this concentration was used for all following experiments. The cytofluorimetric analysis revealed that, in cells treated with this concentration of rifampicin for 3, 8 and 15 days, the cell cycle and morphology showed no difference compared to untreated controls (data not shown).

3.1. Induction of transport proteins by rifampicin

The expression of genes coding for proteins involved in multidrug resistance, MDR1, MRP1, MRP2 and LRP, was evaluated by RT-PCR with specific primers (Fig. 1). In addition the expression of CYP 3A4 gene was also evaluated (Fig. 2). Untreated parental LLC-PK$_1$ cells showed a significant expression of MRP1, MRP2 and LRP transcripts, a moderate expression of CYP 3A4 transcript and low levels of MDR1, and in these cells the expression level of all transporters did not change significantly with culture time. The 15-day treatment with rifampicin significantly induced the expression of all examined transcripts: besides a significant increase with respect to the control of CYP 3A4 expression (67%), there was a clear induction of MRP1 (59%), MRP2 (51%), MDR1 (43%) and LRP (50%) transcripts.

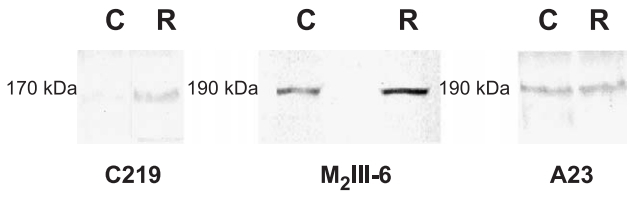

Fig. 3. Western blot analysis of proteins from LLC-PK$_1$ parental cells (C) and cells treated with rifampicin 25 μM for 15 days (R). Cell lysates were probed with C219, M$_2$III-6 or A23 antibodies. Molecular size standards are indicated in kDa.

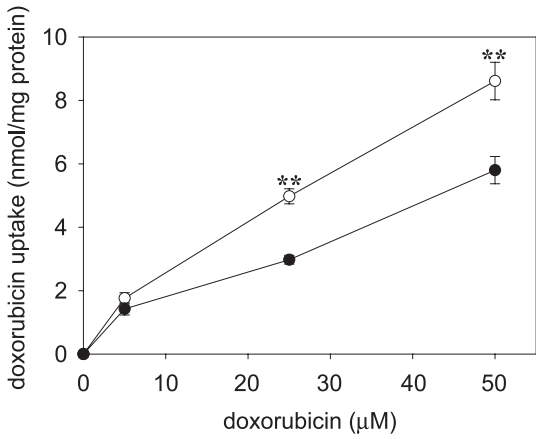

Fig. 4. Doxorubicin concentration-dependent uptake in LLC-PK$_1$ parental cells (○) and in cells pretreated with rifampicin 25 μM for 15 days (●). Cells were incubated with different concentrations of doxorubicin (5, 25 and 50 μM) in complete culture medium for 1 h. Each point represents the mean ± S.E. of data from three to six wells. **$P<0.01$, Student's *t*-test for independent data.

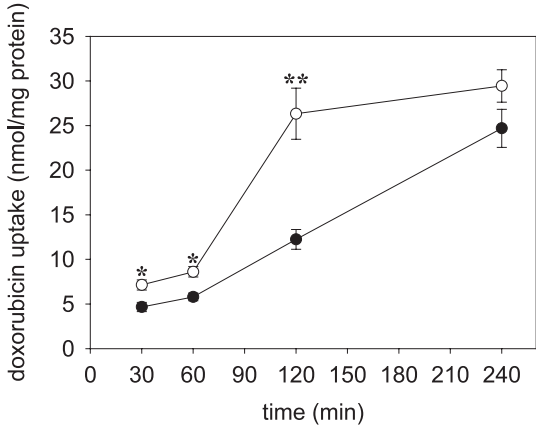

Fig. 5. Doxorubicin time-dependent uptake in LLC-PK$_1$ parental cells (○) and in cells pretreated with rifampicin 25 μM for 15 days (●). Cells were incubated with 50 μM doxorubicin in complete culture medium for up to 240 min. Each point represents the mean ± S.E. of data from three to six wells. *$P<0.05$, **$P<0.01$, Student's *t*-test for independent data.

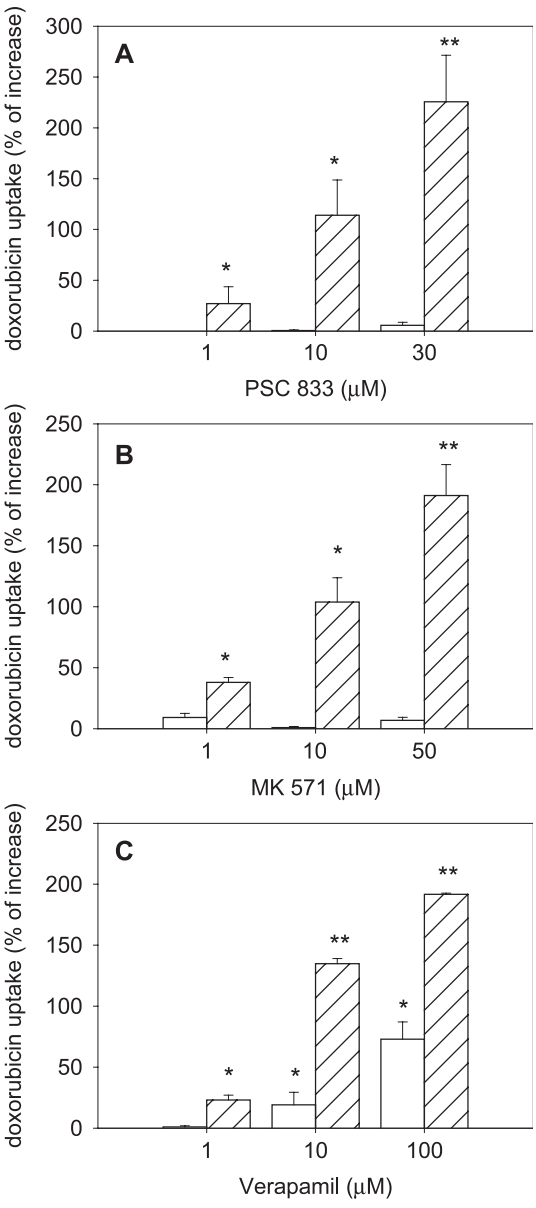

Fig. 6. Effect of 30 μM PSC833 (A), 50 μM MK571 (B) and 100 μM verapamil (C) on doxorubicin uptake in LLC-PK$_1$ parental cells (clear bars) and cells pretreated with rifampicin 25 μM for 15 days (hatched bars). The cells were preincubated in complete culture medium without or with inhibitors for 1 h and then doxorubicin (25 μM final concentration) was added and the incubation continued for 1 h. Each point represents the mean ± S.E. of data from three to six wells. *$P<0.05$, **$P<0.01$, Student's *t*-test for independent data.

The induction was time dependent and levels of significance were observed from the seventh day. Only for MRP1 and CYP 3A4 the induction was earlier and evident from the third day.

Western blot analysis with C219 monoclonal antibody showed a moderate increase in the levels of P-glycoprotein after 15 days of treatment with rifampicin 25 μM; similar results were obtained for MRP2 with M$_2$III-6 monoclonal antibody; on the contrary, no difference in the expression of MRP1, evaluated with A23 polyclonal antibody, was evident (Fig. 3).

3.2. Intracellular accumulation of doxorubicin

The intracellular uptake of doxorubicin, evaluated at different concentrations (Fig. 4) and at different experimental times (Fig. 5), was significantly lower in rifampicin pretreated cells. In this cells doxorubicin intracellular concentrations continued to increase until 4 h whereas in the parental cells the uptake reached the equilibrium within 2 h. The effect of substances that are known inhibitors of P-glycoprotein or MRP was also evaluated. We used a specific inhibitor of P-glycoprotein (PSC 833), a specific inhibitor of MRP (MK-571) and an inhibitor of both protein (verapamil), at different concentrations. For all inhibitors tested a significant increase in doxorubicin uptake was observed in rifampicin pretread cells. On the contrary no effect was evident in the parental cell line with the exception of the highest concentrations of verapamil (Fig. 6).

3.3. Transepithelial transport

In LLC-PK$_1$ cells the rate of doxorubicin transport (% per hour) was almost the same in either direction (apical to basal and basal to apical). In cells chronically treated with rifampicin the polarized flux was enhanced when the drug was loaded into the basal compartment (Fig. 7). Inhibition studies showed a significant reduction of doxorubicin transport from basal to apical side in monolayers of rifampicin treated cells after a pretreatment with the P-glycoprotein inhibitor PSC 833 (Fig. 8A). A slight, but significant reduction in the apical to basal flux was observed with the MRP inhibitor MK-571 (Fig. 8B). On the contrary, almost no effect of the two inhibitors was

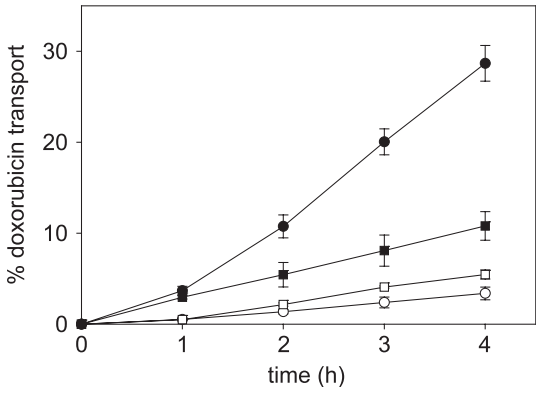

Fig. 7. Timecourse of trancellular transport of doxorubicin in LLC-PK$_1$ cells (open symbols: ○, □) and LLC-PK$_1$ cells chronically treated with rifampicin 25 μM (closed symbols: ●, ■). Circles (○, ●) show the basal to apical transport and squares (□, ■) indicate the apical to basal transport. Each point represents the mean ± S.E. of data from six wells.

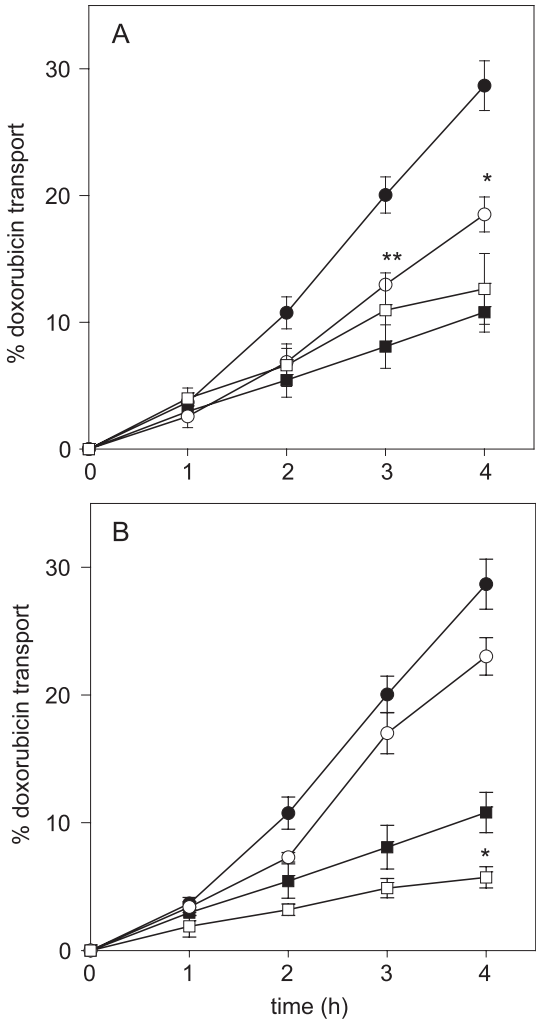

Fig. 8. Effect of PSC 833 30 μM (A) and MK-571 50 μM (B) on transcellular transport of doxorubicin in LLC-PK$_1$ cells chronically treated with rifampicin. Closed (●,■) and open (○, □) symbols show the transport in the absence and presence of the inhibitors, respectively. Circles (○, ●) show the basal to apical transport and squares (□, ■) indicate the apical to basal transport. Each point represents the mean ± S.E. of data from six wells. *$P<0.05$, **$P<0.01$, Student's t-test for independent data.

observed in untreated LLC-PK$_1$ cell monolayers (data not shown).

4. Discussion

Rifampicin is an antibiotic widely used in the therapy of tubercolosis; this drug is a powerful inducer of drug-metabolizing enzymes and various drug interactions occur when it is used in polytherapy. The main mechanism of these interactions has been ascribed to induction of CYP 3A4 in liver and small intestine (Fromm et al., 1996). However not all the interactions can be explained by induction of drug-metabolizing enzymes, for example an interaction between rifampicin and digoxin has been described but this compound is not metabolized by cytochromes (Greiner et al., 1999). Many of the substrates which are metabolized by CYP 3A4 are also substrates of P-glycoprotein (Schuetz et al., 1996). P-glycoprotein is an ATP-dependent transporter playing a role in drug clearance; other ATP-dependent transporters are MRPs. Initially discovered in cancer cells as responsible for the resistance against certain cytostatic drugs, P-glycoprotein and MRP1 were later demonstrated to be also expressed in different non malignant cells in various organs (Borst et al., 1999; Thiebaut et al., 1987). The level of their expression shows wide interindividual variability controlled by genetic (Hoffmeyer et al., 2000) and environmental factors (Licht et al., 1991). P-glycoprotein seems to be inducible at different levels in several tissues and its regulation may be different from one cell type to another. For this reason results obtained in vitro from human tumor cell lines should be interpreted cautiously and cannot be simply extrapolated to normal human cells (Becquemont et al., 2000).

In vitro experiments with human colon carcinoma cells have shown that rifampicin up-regulates the expression of P-glycoprotein coordinately with CYP 3A4 (Schuetz et al., 1996). Using intestinal biopsies, Greiner et al. (1999) have demonstrated that P-glycoprotein is inducible in human gut and a 3.5-fold increase in P-glycoprotein expression after the coadministration of rifampicin was observed, with a profound effect on the rate and extent of absorption of orally administered digoxin. Similar results have been obtained with administration of verapamil or talinolol, resulting in a lower absorption of these drugs (Hanafy et al., 2001; Sandstrom and Lennernas, 1999). On the contrary in primary cultures of human hepatocytes (Runge et al., 2000) and in lymphocytes (Becquemont et al., 2000) rifampicin did not induce MDR1 or MRP1, but only CYP 3A4. Rifampicin was also shown to induce MRP2: a marked induction of hepatic MRP2 transcript was observed in non-human primates and it was suggested that this may represent an adaptive response aimed at an enhanced biliary elimination of the inducing drugs and/or their metabolites (Kauffmann et al., 1998). In humans rifampicin induces MRP2 in the duodenum and this phenomenon could be responsible of an increased elimination of MRP2 substrates into the lumen of gastrointestinal tract (Fromm et al., 2000).

LLC-PK$_1$ is a cell line derived from pig kidney (Hull et al., 1976) similar to renal proximal tubular cells both in morphology and functions and represents a useful in vitro model for studying epithelial drug transport (Horio et al., 1990). Since LLC-PK$_1$ cells express low levels of P-glycoprotein, MRP1 and MRP2 (Crivellato et al., 1999; Decorti et al., 1998, 2001), the aim of this study was to investigate the possible induction of P-glycoprotein and MRPs by rifampicin in this cells. Furthermore the expression and the possible induction of LRP was also analyzed in these cells. The LLC-PK$_1$ cells were treated with the highest concentration that, in a period of treatment of 15 days, did not affect the doubling time, the cell morphology and cell cycle, as evaluated by cytofluorimetric analysis. The effect of this

treatment on the expression of MDR1, MRP1, MRP2 and LRP transcripts codifing for the respective multidrug resistance proteins and on the expression of CYP 3A4 transcript, was evaluated by RT-PCR using specific primers and compared with time-matched controls. Indeed in some cell lines the expression of transporters, among which P-glycoprotein, changes with culture time (Anderle et al., 1998; Behrens and Kissel, 2003), but this phenomenon was not observed in our study with LLC-PK_1 cells. In rifampicin treated cells an increase of mRNA, for all evaluated genes, indicated a transcriptional activation by rifampicin. These results were confirmed for P-glycoprotein and MRP2 at the protein level by Western blot analysis. To evaluate if the induction resulted in a reduced accumulation of drugs into the cell, functional studies have been performed using doxorubicin as substrate. This compound enters the cell by diffusion and is extruded by means of P-glycoprotein and MRPs with a mechanism of active transport; for all concentrations and times tested, a significant reduced uptake of doxorubicin in rifampicin treated cells was evident. To evaluate if this reduced uptake was due to an increased expression of MDR proteins, specific inhibitors were used: PSC 833, specific for P-glycoprotein (Twentyman and Bleehen, 1991), that does not interact with CYP 3A4, MK-571, specific for MRP (Gekeler et al., 1995), and verapamil, substrate for P-glycoprotein, MRP (Aszalos et al., 1999) as well as for CYP 3A4 (Tracy et al., 1999). All tested substances caused a significant increase of doxorubicin cell accumulation in rifampicin pretreated cells. Only verapamil, that is active on P-glycoprotein and MRPs, as well as on CYP 3A4, increased the uptake both in treated and parental cells.

Similar results were obtained in cells cultured on porous membranes, where LLC-PK_1 cells form a tight monolayer, with P-glycoprotein and MRP2 localized in the apical, and MRP1 in the basolateral plasma membrane. In bidirectional transport experiments, PSC 833, as expected, reduced the basal to apical transport, confirming an increased P-glycoprotein activity, whereas MK-571 only slightly reduced the apical to basal transport of the antineoplastic drug. However, it should be noted that MRP1 and MRP2 are both inhibited by MK-571, the substance probably acts at the apical side, where MRP2 is localized, as well as at the basolaterateral membrane, where MRP1 is expressed, hence its overall effect is not clear (Keppler et al., 1998). These results indicate a significant induction of P-glycoprotein, MRP1, MRP2, LRP and CYP 3A4 by rifampicin; doxorubicin accumulation in LLC-PK_1 cells appears to be P-glycoprotein and MRPs dependent and can't be explained only by an enhanced metabolism of the drug due to CYP 3A4 induction. Moreover these data confirm the results obtained by RT-PCR analysis which indicates an increased expression of the transcripts for these proteins involved in doxorubicin transport.

Induction of CYP 3A4 (Lehmann et al., 1998) and MDR1 (Geick et al., 2001) by rifampicin is thought to be mediated by the orphan nuclear receptor PXR. In humans, this receptor is predominantly expressed in the liver and intestine, while in the human kidney a low or absent expression has been described (Lehmann et al., 1998); on the contrary, in the rat, PXR gene has been detected also in the kidney (Zhang et al., 1999). The induction observed in our experimental model should therefore be ascribed to species differences, or to other, as yet unidentified, induction mechanisms.

In summary our work describes a drug–drug interaction based mainly on the induction of renal transport proteins. An important feature of induction is a marked species specificity, for example rifampicin is a potent inducer of CYP 3A4 in humans, moderate in mice, and almost completely ineffective in rats (Strolin Benedetti and Dostert, 1994). Hence, even though induction observed in a porcine cell line cannot be simply extrapolated to humans, this phenomenon should be considered if unusual effects are observed when the antibiotic rifampicin is associated with a therapy based on drugs mostly eliminated by the kidney.

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