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# Handling of Doxorubicin by the LLC-$PK_1$ Kidney Epithelial Cell Line[1]

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## ABSTRACT

The characteristics of doxorubicin handling have been studied in the cultured kidney epithelial cell line LLC-$PK_1$, which has structure and function similar to those of renal tubular cells and expresses P-glycoprotein. The uptake of doxorubicin by LLC-$PK_1$ cells was time dependent, reaching a steady state at about 4 hr, and reduced at low temperature; the initial uptake was saturable. The efflux of doxorubicin from LLC-$PK_1$ cells was also temperature dependent but, even at 37°C, a significant percentage of the drug remained associated with the cells after 180 min, which suggests a strong cellular binding, and the fluorescence microscopy revealed that the drug was concentrated in intracellular organelles. Substances that are substrates for P-glycoprotein, such as verapamil, vinblastine, vincristine and quinidine, significantly increased doxorubicin concentrations in LLC-$PK_1$ cells. Similar results were obtained with the metabolic inhibitors sodium metavanadate and 2,4-dinitrophenol. On the other hand, the uptake was not affected by the classic organic cation transport drugs cimetidine, decynium 22 or decynium 24, nor by the organic anion drug probenecid. These results indicate that, in LLC-$PK_1$ cells, doxorubicin enters by passive diffusion, is trapped in intracellular organelles and then is extruded from cells by a mechanism that probably involves P-glycoprotein. On the contrary, substances that interfere with the renal organic cation or anion secretory system have no effect on doxorubicin net accumulation in these cells.

The multidrug transporter P-glycoprotein has been identified in many resistant tumor cells. This 170- to 180-kdalton membrane glycoprotein is part of the ABC family of transporters and mediates the extrusion of chemotherapeutic agents, decreasing their accumulation within cells and consequently conferring MDR to cancer cells (Kane, 1996). The protein also transports peptides (Saeki *et al.*, 1993a), steroids (Ueda *et al.*, 1992) and a variety of structurally unrelated, hydrophobic substances including calcium channel blockers and immunosuppressive agents (Saeki *et al*., 1993a; Tsuruo, 1991)

P-glycoprotein also has been identified in several normal human tissues and is most abundant on the luminal surface of polarized transporting epithelia of kidney proximal tubule, small intestine and colon, liver biliary hepatocytes and the secretory gland of the pregnant mouse endometrium, as well as in the adrenals and capillary endothelial cells such as those of the brain and testis (Thiebaut *et al.*, 1987, 1989; Sugawara *et al.*, 1988; Cordon-Cardo *et al.*, 1990). The location of P-glycoprotein expression suggests that one of the physiological roles of this protein in normal tissues may be the excretion of xenobiotics (Gottesman and Pastan, 1993); in particular, in the kidney it is unclear whether P-glycoprotein is involved in the transepithelial drug transport pathway or whether it simply has a protective role for those cells lining the nephron.

The anthracycline antibiotic doxorubicin is a well-known substrate for P-glycoprotein in MDR cells. Although many reports have studied the transport of anthracyclines in various tumor cells extensively, only few studies have examined the transport mechanism of these anticancer agents in normal cells and in particular in the renal proximal tubular cells, which are particularly rich in P-glycoprotein. Charuk *et al.* (1994) studied the interactions of anthracyclines with renal P-glycoprotein and observed that both doxorubicin and daunomycin interact with this protein, even if their relative affinities (half-maximal inhibition constant, 10 $\mu$M) were lower than those of several other drugs tested.

Renal elimination is not a hallmark of anthracycline phar-

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**ABBREVIATIONS:** ABC, ATP binding cassette; ATP. adenosine 5′-triphosphate; ATPase, adenosine 5′-triphosphatase; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; MDR, multidrug resistance; decynium 22, 1,1′-diethyl-2,2′-cyanine; decynium 24, 1,1′-diethyl-2,4′-cyanine; cDNA, complementary deoxyribonucleic acid; L-DOPA: L-3 dihydroxyphenylalanine.



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macology; indeed, dose modification is not required in patients with compromised renal function (Pratt and Ruddon, 1979) and renal clearance approximates that of creatinine (Krarup-Hansen *et al.*, 1988). However, urinary excretion of daunomycin has been observed in mice and rats (Finkel *et al.*, 1969); the drug is accumulated in the kidney after intravenous administration (Bachur *et al.*, 1970) and is metabolized by several tissues primarily to daunorubicinol (Bachur and Gee, 1971), which also is excreted in the urine of humans (Bachur, 1971). In the rabbit, in which the pharmacokinetics of doxorubicin have been found to be closest to those in humans (Koren *et al.*, 1992), the highest tissue levels of doxorubicin and doxorubicinol were found in the kidneys (Bachur, 1975).

The studies presented here were therefore designed to clarify the transport mechanism of doxorubicin in the proximal tubular cells and in particular to examine the effects of a series of compounds that are known to interact with P-glycoprotein or to interfere with the transporters of organic anions and cations in renal tubules. We have used the LLC-$PK_1$ 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). The LLC-$PK_1$ cells form an oriented monolayer with microvilli and tight junctions and have been shown to express P-glycoprotein (Horio *et al.*, 1990).

## Materials and Methods

**Cell cultures.** LLC-$PK_1$ cells obtained from the American Type Culture Collection (Rockville, MD) (ATCC-CRL-1392) were grown in medium 199 containing 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. The cells were used in the passages 199 to 230. For experiments, 35-mm dishes were inoculated with $2 \times 10^4$ cells/ml in 2 ml of complete culture medium. Unless otherwise stated, the uptake of doxorubicin was measured on confluent cells on the 5th day after inoculation.

After removal of the culture medium, each dish was washed twice with 4 ml of PBS at 4°C; then 2 ml of complete medium containing doxorubicin and other tested substances were added to each dish and the cells were incubated for a specified period. At the end of the incubation period, the medium was removed by suction and the dish was rinsed three times with 4 ml of ice-cold PBS buffer. The cells were scraped with a rubber policeman into 2 ml of ice-cold saline. The dishes were then rinsed again with 4 ml of ice-cold saline to improve the recovery of cells. The cells were centrifuged at 4°C for 5 min at $150 \times g$. The supernatants were aspirated and the cell pellet was resuspended gently in 6 ml of ice-cold 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 determined 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 on the acid-insoluble residue by the method of Lowry *et al.* (1951).

**Microscopic studies.** LLC-$PK_1$ cells were seeded in Leighton tubes at a cell density of $2 \times 10^4$ cells/ml in 1 ml of complete culture medium, and the uptake of doxorubicin was observed on confluent cells, on the 5th day after inoculation. Cells on glass slides were examined under a Zeiss Axioskop microscope; the instrument contained two illumination sources, a tungsten bulb for bright-field observation and a mercury lamp (Osram HBO 100W, Germany) for epifluorescence examination. The fluorescence setting was equipped with fluorescein/rhodamin optics. Cells were studied using a ×100 Plan-Neofluar N.A. 1.30 objective at oil immersion, by alternative bright field and fluorescence observations.

## Results

First, doxorubicin accumulation in LLC-$PK_1$ cells was examined as a function of growth in culture. As shown in figure 1, on day 3, when the cells were subconfluent, doxorubicin uptake was relatively low, and a marked increase in the uptake was observed on day 4 when the cells reached a confluent monolayer. After the development of confluence, the uptake was steady until day 6 and then started to decrease. Based on the above findings, all subsequent uptake studies were carried out with the LLC-$PK_1$ cells on day 5.

Figure 2 shows the time course of doxorubicin uptake at 37 and 4°C for up to 4 hr by renal tubular cells. At 37°C, doxorubicin uptake reached the equilibrium within about 4 hr. At 4°C, the uptake increased with time but was drastically lower than that at 37°C. Thus, temperature is a very important determinant for doxorubicin uptake.

Figure 3 shows the relationship between drug concentration and the initial doxorubicin uptake (15 min). At 4°C the relation was linear; when the experiments were carried out at 37°C, the uptake of doxorubicin was greater and showed a trend for saturation. According to other reports (Gachot *et al.*, 1991; Takano *et al.*, 1992), linear accumulation at 4°C and saturable accumulation at 37°C appear to correspond with uptakes by simple diffusion and by the sum of simple diffusion and carrier-mediated transport, respectively; thus the subtraction of doxorubicin uptake at 4°C from that at 37°C (dotted line in fig. 3) suggested a saturable process in doxorubicin uptake. The curve obtained from subtraction of doxorubicin uptake at 4°C from that at 37°C was analyzed further by the Lineweaver-Burk plot (fig. 4), and a linear relationship was clearly observed.

We next studied the kinetics of doxorubicin efflux from LLC-$PK_1$ cells cultured for 2 hr with 50 $\mu$M doxorubicin and, after washing to remove free doxorubicin, incubated for various times in drug-free medium. As shown in figure 5, doxorubicin was released slowly, and 75.7 ± 1.7% remained associated with the cells after 180 min, which suggests a strong

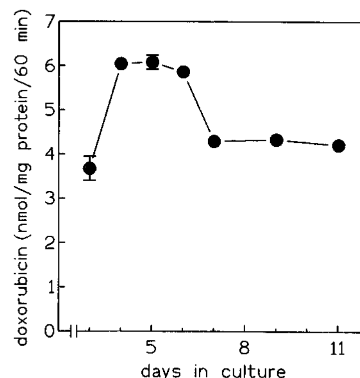


**Fig. 1.** Effect of culture time on doxorubicin uptake. Cells were plated in 35-mm wells at a density of $2 \cdot 10^4$ cells/ml in 2 ml of complete culture medium and were cultured for up to 11 days. On the day indicated in the graph, cells were incubated with 50 $\mu$M doxorubicin in complete culture medium for 1 hr. Each point represents mean ± S.E. of data from three to six wells.

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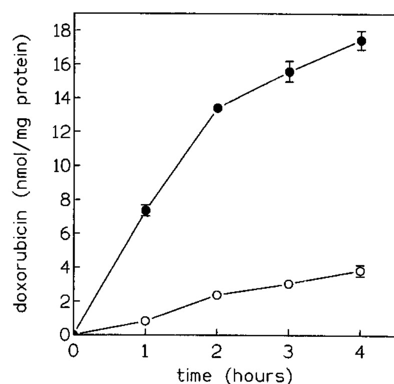


**Fig. 2.** Effect of temperature on doxorubicin uptake. Cells were incubated with 50 $\mu$M doxorubicin in complete culture medium for up to 4 hr at 37°C (●) or 4°C (○). Each point represents mean ± S.E. of data from three to six wells.

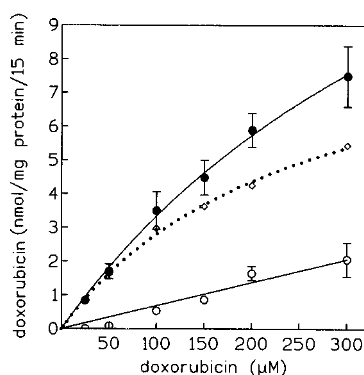


**Fig. 3.** Concentration dependence of doxorubicin uptake. Cells were incubated with different concentrations of doxorubicin in complete culture medium at 37°C (●) or 4°C (○) for 15 min. The dotted line indicates the difference of doxorubicin uptake at 37°C and 4°C. Each point represents mean ± S.E. of data from three to six wells.

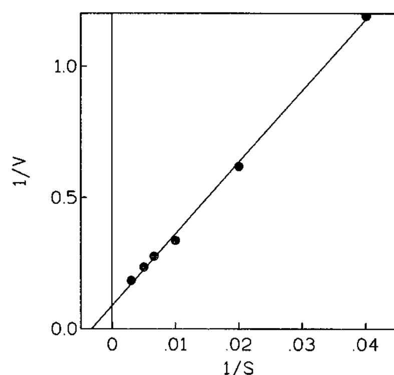


**Fig. 4.** The saturable uptake shown in figure 3 was plotted by the Lineweaver-Burk plot. The parameters ($K_m$, $V_{max}$) were calculated. The correlation coefficient was 0.999.

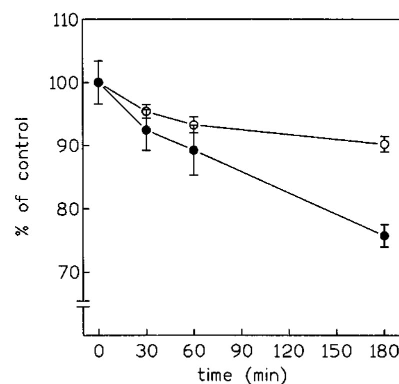


**Fig. 5.** Kinetics of doxorubicin release from LLC-$PK_1$ cells incubated in complete medium containing 50 $\mu$M doxorubicin. After 2 hr of incubation the cells were washed with ice-cold PBS to remove free doxorubicin (time 0, total uptake) and incubated at 37°C (●) or 4°C (○) in fresh PBS buffer. Doxorubicin remaining in the cells was measured at determined times. Each point represents mean ± S.E. of data from three to six determinations from a typical experiment.

cellular binding or trapping. The fluorescence microscopy observations revealed that doxorubicin was trapped and concentrated in intracellular organelles; in particular the fluorescent drug was confined to cytoplasmic perinuclear localization, with a faint fluorescence in the nucleus (fig. 6).

Doxorubicin is a well-known substrate for P-glycoprotein; hence, the effect of several MDR reversing agents on the concentration of the drug was studied. When cells were preincubated with 500 $\mu$M verapamil, doxorubicin concentration increased significantly to approximately 3-fold; other P-glycoprotein substrates, vinblastine (100 $\mu$M), vincristine (100 $\mu$M) and quinidine (200 $\mu$M), also significantly increased doxorubicin concentration (fig. 7).

Transport mediated by the MDR transporter depends on cellular metabolism and thus should be sensitive to metabolic inhibitors. Figure 8 shows the effect of 2,4-dinitrophenol (4 mM) and sodium metavanadate (10 $\mu$M) on doxorubicin uptake by LLC-$PK_1$ cells. The substances significantly increased the concentrations of doxorubicin in the cells.

The effect of classic organic cation transport drugs, such as cimetidine (200 $\mu$M) and the cyanine derivatives decynium 22 (5 $\mu$M) and decynium 24 (5 $\mu$M), and of the organic anion drug probenecid (50 $\mu$M) also was tested, but none of the substances modified doxorubicin concentrations in LLC-$PK_1$ cells (fig. 9).

## Discussion

P-glycoprotein is expressed in renal tissue and in particular in the proximal tubular epithelium. Charuk *et al.* (1994) showed that the anthracyclines doxorubicin and daunorubicin block $^3$H-azidopine photolabeling of renal P-glycoprotein. The relative affinities (half-maximal inhibition constant, 10 $\mu$M) of the anthracyclines were quite low, but high tissue, plasma and urinary levels attained during chemotherapy probably allow some interactions of these drugs with renal P-glycoprotein. Therefore we studied the uptake characteristics of doxorubicin in the LLC-$PK_1$ cell line, a cell line derived from the pig kidney (Hull *et al.*, 1976) that possesses structure and function similar to those of renal proximal tubules (Handler *et al.*, 1980) and expresses the P-glycoprotein (Horio *et al.*, 1990). A transepithelial drug transport pathway involving P-glycoprotein was demonstrated in several kidney-derived cell lines (Horio *et al.*, 1989, 1990, 1991). In particular, several authors showed that, in a canine kidney cell line (MDCK) carrying the human MDR-1 gene, P-glycoprotein is expressed in a polarized manner similar to the kidney (Pastan *et al.*, 1988) and plays a role in the transepithelial transport of anticancer agents such as vinblastine, vincristine, daunorubicin, actinomycin D and verapamil (Horio *et al.*, 1989). Several other studies were conducted in renal cell cultures transfected with the MDR-1 cDNA (Ito *et*

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**Fig. 6.** Fluorescence photomicrograph showing doxorubicin distribution in LLC-$PK_1$ cells exposed to 50 $\mu$M drug for 2 hr. Calibration bar, 5 $\mu$m.

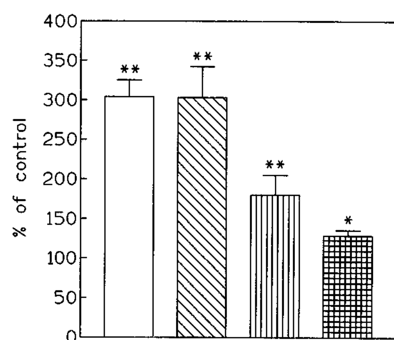


**Fig. 7.** Effect of 500 $\mu$M verapamil (□), 100 $\mu$M vinblastine (▧), 100 $\mu$M vincristine (▥) and 200 $\mu$M quinidine (▦) on doxorubicin uptake in LLC-$PK_1$ cells. The cells were preincubated in medium containing the inhibitors at 37°C for 30 min, then in medium containing 50 $\mu$M doxorubicin and the inhibitors for 30 additional min. Each point represents mean ± S.E. of data from three to six determinations from a typical experiment. *$P < .05$; **$P < .01$; Student's *t* test for independent data.

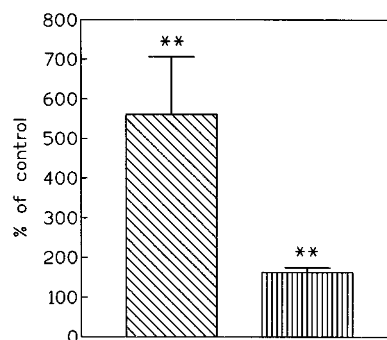


**Fig. 8.** Effect of the metabolic inhibitors 10 $\mu$M sodium metavanadate (▧) and 4 mM 2,4-dinitrophenol (▥) on doxorubicin uptake in LLC-$PK_1$ cells. The cells were preincubated in PBS containing the inhibitors at 37°C for 30 min, then in PBS containing 50 $\mu$M doxorubicin and the inhibitors for 30 additional min. Each point represents mean ± S.E. of data from three to six determinations from a typical experiment. **$P < .01$; Student's *t* test for independent data.

*al.*, 1993; Pan *et al.*, 1994; Saeki *et al.*, 1993a, b; Tanaka *et al.*, 1997). These transfectant cells have overexpressed human P-glycoprotein on the apical membrane. However, in these cells the levels of the protein are extremely high, whereas to evaluate the possible clinical effects of modulators, cells with a physiological level of expression of P-glycoprotein should be used. In our study we used the wild-type of LLC-$PK_1$ cells, that, similarly to normal mammalian proximal renal cells, express endogenous P-glycoprotein in low amounts.

When doxorubicin uptake was studied as a function of growth in culture, it was shown that the uptake strictly depends on cell growth and was maximal when cells reached a confluent monolayer, which suggests that the increased doxorubicin uptake corresponds to attainment of a confluent cell density and to the development of functional properties for apical membranes of the LLC-$PK_1$ cells.

The transport of doxorubicin in various neoplastic cell types has been studied; however, how the drug enters epithelial cells such as the renal proximal tubule cells is not yet completely clear. In cancer cells, the main route of entry is passive diffusion across the plasma membrane of the uncharged form of the drug, which is a weak base with a pKa of 8.3 (Frezard and Garnier-Suillerot, 1991; Harrigan *et al.*, 1993; Madden and Redelmeier, 1994); however, differences in the specific parameters of membrane transport systems between tumor and normal cells are likely and are suspected to be associated closely with the determinants of cytotoxicity. In our study with the LLC-$PK_1$ cells, the subtraction of doxorubicin uptake at 4°C from that at 37°C suggested a saturable process and so was analyzed by the Lineweaver-Burk plot. A linear relationship was observed, which suggests a carrier-

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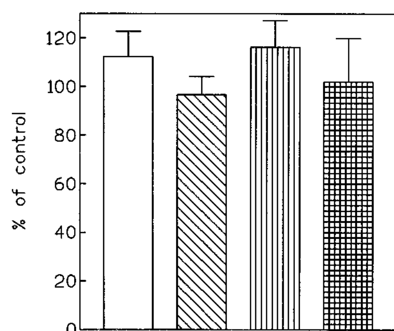


**Fig. 9.** Effect of the organic anion 50 $\mu$M drug probenecid (□) and of the organic cation drugs 200 $\mu$M cimetidine (▧), 5 $\mu$M decynium 22 (▥), and 5 $\mu$M decynium 24 (▦) on doxorubicin uptake in LLC-PK$_1$ cells. The cells were preincubated in medium containing the inhibitors at 37°C for 30 min, then in medium containing 50 $\mu$M doxorubicin and the inhibitors for 30 additional min. Each point represents mean ± S.E. of data from three to six determinations from a typical experiment.

mediated transport. The values of Michaelis constant ($K_m$) and maximum uptake rate ($V_{max}$) were estimated with the aid of a computer to be 0.779 nmol doxorubicin/mg protein/min and 319.96 $\mu$M respectively. In addition, doxorubicin was accumulated against the concentration gradient, and, assuming the intracellular volume of LLC-PK$_1$ cells as 7 $\mu$l/mg of protein (Saito *et al.*, 1986), the cellular concentration of doxorubicin at the equilibrium was approximately 50-fold compared with external concentrations.

The concentration-dependent uptake, along with the temperature dependent transport is evocative of the presence of a specific mechanism, such as facilitated diffusion; however, nonspecific factors also can be invoked as alternative explanations. Indeed, the relative increase in the rate of drug uptake as the temperature is raised by 10°C (the $Q_{10}$), in LLC-PK$_1$ cells is $<2$; this value is in the range expected for passive diffusion, whereas it is $>6$ for facilitated diffusion or active transport (Dalmark and Storm, 1981). On the other hand, doxorubicin could enter cells by passive diffusion and the concentration dependence can, for example, originate from the saturation of nonspecific binding sites on the cell membrane, or from ion trapping in acidic compartments of the cell. The fluorescence microscopy observations agree with this latter hypothesis. The fluorescence is indeed primarily punctate, an observation that is indicative of doxorubicin localization to intracellular organelles, primarily Golgi and lysosomes. The fluorescent drug is confined to cytoplasmic perinuclear localization, whereas the nucleus presents a fainter fluorescence. This picture is very similar to that observed by various authors in doxorubicin-resistant cancer cell lines (Coley *et al.*, 1993; de Lange *et al.*, 1992; Rutherford and Willingham, 1993; Simon and Schindler, 1994), and in these cells it has been suggested that trapping of doxorubicin in intracellular organelles decreases drug concentration in the cytoplasm and in the nucleus, and facilitates cell survival. Nephrotoxicity is not a major side effect of anthracyclines; doxorubicin usually induces a glomerular damage characterized by proteinuria, ipoproteinemia and peripheral edemas in animals (Burke, 1977; Hayashi *et al.*, 1984). It has been suggested that the drug also can induce a tubular toxicity (Landwehr *et al.*, 1977); however, the effects on tubular cells are usually not very important. In addition, *in vitro* studies have shown that fragments of proximal tubule are less sensitive to the cytotoxic effects of doxorubicin than glomerular tissue (Kastner *et al.*, 1990, 1991).

When the efflux of doxorubicin after incubation for 2 hr with 50 $\mu$g/ml was studied, more than 75% remained associated with the cells after 180 min, which confirms the very strong cellular binding or trapping in intracellular cell compartments.

To elucidate the role of P-glycoprotein, which is expressed in LLC-PK$_1$ cells, in the handling of doxorubicin, cells were treated with high concentrations of agents that interfere with the transport by this protein. The tested substances verapamil, vinblastine, vincristine and quinidine significantly increased doxorubicin concentrations, which suggests therefore that in this cell line, P-glycoprotein could play a role in the extrusion of the antineoplastic drug.

Because P-glycoprotein actively pumps out drugs in an ATP-dependent manner, higher uptake could be expected in LLC-PK$_1$ cells in the presence of metabolic inhibitors, and this was indeed the case. The effect was particularly evident with sodium metavanadate; as a matter of fact, P-glycoprotein belongs to a family of ATPases that are particularly sensitive to this substance.

The localization of P-glycoprotein in the brush-border membranes of proximal tubular cells and the observation that many P-glycoprotein substrates are organic cations, led to the hypothesis of a physiological role for the kidney P-glycoprotein in the energy-dependent renal secretion of organic cations (Nelson, 1988). Indeed, the mammalian kidney, like P-glycoprotein, has pleiotropic drug transport capacity, best characterized by the organic cation and anion secretory systems. These systems are considered distinct because probenecid inhibits the anion carrier, and cimetidine inhibits the cation carrier. Therefore it was decided to test the effect of probenecid, an organic anion transport inhibitor, and the effect of cimetidine and of two cyanine derivatives, decynium 22 and decynium 24, which recently have been shown to exert a potent inhibitory effect on the renal transport of organic cations (Schömig *et al.*, 1993) and were extremely effective in reducing the tubular uptake of L-DOPA (Pinto-do-Ó and Soares-da-Silva, 1996), but none of the tested substances interfered with the uptake of doxorubicin.

Our studies were conducted on cells reaching a confluent monolayer and hence it is very probable that the entrance *via* simple diffusion occurs across the luminal membrane; similarly, the efflux also probably is limited to the luminal site where the P-glycoprotein is located. Luminal entrance of doxorubicin in the readsorptive direction after glomerular filtration was demonstrated in dogs (Daoud and Huang, 1988), and the authors suggested that both readsorption and secretion of the drug occurred in the proximal tubular section. Hence, it seems that P-glycoprotein could have an important protecting role for cells lining the nephron.

In conclusion, doxorubicin enters LLC-PK$_1$ probably by passive diffusion and is then trapped and accumulated into intracellular organelles. P-glycoprotein seems to be very important in extruding doxorubicin from these cells, even if the levels of the protein expressed in wild-type LLC-PK$_1$ as well as in mammalian proximal tubules are quite low. On the contrary, the renal organic anion and cation secretory system is not involved in doxorubicin renal handling. It remains to be elucidated if interactions between doxorubicin and P-gly-

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coprotein substrates also could occur in clinical practice, hence increasing the risk of drug-induced nephrotoxicity.

**References**


Bachur NR, Moore AL, Bernstein JG and Liu A (1970) Tissue distribution and disposition of daunomycin (NSC-82151) in mice: Fluorometric and isotopic methods. *Cancer Chemother Rep* **54:**89–94.

Bachur NR (1971) Daunorubicinol, a major metabolite of daunorubicin: Isolation from human urine and enzymatic reactions. *J Pharmacol Exp Ther* **177:**573–581.

Bachur NR and Gee M (1971) Daunorubicin metabolism by rat tissue preparations. *J Pharmacol Exp Ther* **177:**567–572.

Bachur NR (1975) Adriamycin (NSC-123127) pharmacology. *Cancer Treat Rep* **6:**153–158.

Burke JF (1977) Doxorubicin-hydrochloride-associated renal failure. *Arch Intern Med* **137:**385–388.

Charuk JHM, Loo TW, Clarke DM and Reithmeier RAF (1994) Interaction of rat kidney P-glycoprotein with a urinary component and various drugs including cyclosporin A. *Am J Physiol* **266:**F66–F75.

Coley HM, Amos WB, Twentyman PR and Workman P (1993) Examination by laser scanning confocal fluorescence imaging microscopy of the subcellular localisation of anthracyclines in parent and multidrug resistant cell lines. *Br J Cancer* **67:** 1316–1323.

Cordon-Cardo C, O'Brien JP, Boccia J, Casals D, Bertino JR and Melamed MR (1990) Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. *J Histochem Cytochem* **38:**1277–1287.

Dalmark M and Storm HH (1981) A fickian diffusion transport process with features of transport catalysis: Doxorubicin transport in human red blood cells. *J Gen Physiol* **78:**349–364.

Daoud SS and Huang KC (1988) Renal handling of adriamycin. *Prog Clin Biol Res* **258:**161–171.

de Lange JH, Schipper NW, Schuurhuis GJ, ten Kate TK, van Heijningen TH, Pinedo HM, Lankelma J and Baak JP (1992) Quantification by laser scan microscopy of intracellular doxorubicin distribution. *Cytometry* **13:**571–576.

Finkel FM, Knapp KT and Mulligan LT (1969) Fluorometric determination of serum levels and urinary excretion of daunomycin (NSC-82151) in mice and rats. *Cancer Chemother Rep* **53:**159–164.

Frezard F and Garnier-Suillerot A (1991) Comparison of the membrane transport of anthracycline derivatives in drug-resistant and drug-sensitive K562 cells. *Eur J Biochem* **196:**483–491.

Gachot B, Tauc M, Morat L and Poujeol P (1991) Zinc uptake by proximal cells isolated from rabbit kidney: Effects of cysteine and histidine. *Pflugers Arch Eur J Physiol* **419:**583–587.

Gottesman MM and Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62:**385–427.

Handler JS, Perkins FM and Johnson JP (1980) Studies of renal cell function using cell culture techniques. *Am J Physiol* **238:**F1–F9.

Harrigan PR, Wong KF, Redelmeier TE, Wheeler JJ and Cullis PR (1993) Accumulation of doxorubicin and other lipophilic amines into large unilamellar vesicles in response to transmembrane pH gradients. *Biochim Biophys Acta* **1149:**329–338.

Hayashi Y, Imaqawa T, Kokubo T, Kurokawa Y and Takahashi M (1984) Nuclear alterations of myocardial cells and glomerular epithelial cells in rats after a single administration of adriamycin. *Toxicol Lett* **20:**105–110.

Horio M, Chin K-V, Currier SJ, Goldenberg S, Williams C, Pastan I, Gottesman MM and Handler J (1989) Transepithelial transport of drugs by the multidrug transporter in cultured Madin-Darby canine kidney cell epithelia. *J Biol Chem* **264:** 14880–14884.

Horio M, Pastan I, Gottesman MM and Handler JS (1990) Transepithelial transport of vinblastine by kidney-derived cell lines. Application of a new kinetic model to estimate the in situ Km of the pump. *Biochim Biophys Acta* **1027:**116–122.

Horio MM, Kakihara H, Nakahama Y, Fukuhara Y, Ueda N, Orita Y, Kamada T, Pastan I and Handler J (1991) Kidney-derived cells show multidrug secretory transport, in *Cellular and Molecular Biology of the Kidney* (Koide H, Endou H and Kurokawa K, eds) pp 143–148, Karger, Basel.

Hull RN, Cherry WR and Weaver GW (1976) The origin and characteristics of a pig kidney cell strain, LLC-PK1. *In Vitro* **12:**670–677.

Ito S, Woodland C, Harper PA and Koren G (1993) The mechanism of the verapamil-digoxin interaction in renal tubular cells (LLC-PK1). *Life Sci* **53:**PL399–PL403.

Kane SE (1996) Multidrug resistance of cancer cells, in *Advances in Drug Research* (Testa B and Meyer UA, eds) vol 28, pp 181–252, Academic Press Ltd., London.

Kastner S, Wilks MF, Gwinner W, Soose M, Bach PH and Stolte H (1990) Metabolic heterogeneity of isolated cortical and juxtamedullary glomeruli in adriamycin nephropathy. *Renal Physiol Biochem* **14:**48–54.

Kastner S, Wilks MF, Soose M, Bach PH and Stolte H (1991) Metabolic studies on isolated glomeruli: a valuable tool to investigate glomerular damage, in *Nephrotoxicity: Mechanisms, Early Diagnosis and Therapeutic Management* (Bach PH, Gregg NJ, Wilks MW and Delacruz L, eds) pp 467–474, Marcel Dekker, New York.

Koren G, Beatty K, Seto A, Einarson TR and Lishner M (1992) The effects of impaired liver function on the elimination of antineoplastic agents. *Ann Pharmacother* **26:**363–371.

Krarup-Hansen A, Wassermann K, Rasmussen SN and Dalmark M (1988) Pharmacokinetics of doxorubicin in man with induced acid or alkaline urine. *Acta Oncol* **27:**25–30.

Landwehr DM, Carvalho JS and Oken DE (1977) Micropuncture studies of the filtration and absorption of albumin by nephrotic rats. *Kidney Int* **11:**9–17.

Lowry OH, Rosebrough NJ, Farr AL and Randell RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:**265–275.

Madden TD and Redelmeier TE (1994) Transmembrane distribution of lipophilic cations in response to an electrochemical potential in reconstituted cytochrome c oxidase vesicles and in vesicles exhibiting a potassium ion diffusion potential. *J Bioenerg Biomembrane* **26:**221–230.

Nelson JA (1988) A physiological function for multidrug-resistant membrane glycoproteins: a hypothesis regarding the renal organic cation secretory system. *Cancer Chemother Pharmacol* **22:**92–93.

Pan BF, Dutt A and Nelson JA (1994) Enhanced transepithelial flux of cimetidine by Madin-Darby canine kidney cells overexpressing human P-glycoprotein. *J Pharmacol Exp Ther* **270:**1–7.

Pastan I, Gottesman MM, Ueda K, Lovelace E, Rutherford AV and Willingham MC (1988) A retrovirus carrying a MDR1 cDNA confers multidrug resistance and polarized expression of P-glycoprotein in MDCK cells. *Proc Natl Acad Sci USA* **85:**4486–4490.

Pinto-do-Ó PC and Soares-da-Silva P (1996) Studies on the pharmacology of the inward transport of L-DOPA in rat renal tubules. *Br J Pharmacol* **118:**741–747.

Pratt WB and Ruddon RW (1979) *The Anticancer Drugs*. Oxford University Press, Inc., New York.

Rutherford AV and Willingham MC (1993) Ultrastructural localization of daunomycin in multidrug-resistant cultured cells with modulation of the multidrug transporter. *J Histochem Cytochem* **41:**1573–1577.

Saeki T, Ueda K, Tanigawara Y, Hori R and Komano T (1993a) Human P-glycoprotein transports cyclosporin A and FK506. *J Biol Chem* **268:**6077–6080.

Saeki T, Ueda K, Tanigawara Y, Hori R and Komano T (1993b) P-glycoprotein-mediated transcellular transport of MDR-reversing agents. *FEBS Lett* **324:**99–102.

Saito H, Inui K-I and Hori R (1986) Mechanisms of gentamicin transport in kidney epithelial cell line (LLC-PK1). *J Pharmacol Exp Ther* **238:**1071–1076.

Schömig E, Babin-Ebell J and Russ H (1993) 1,1′-diethyl-2,2′cyanine (Decynium 22) potently inhibits the renal transport of organic cations. *Naunyn-Schmiedebergs Arch Pharmacol* **347:**379–383.

Simon SM and Schindler M (1994) Cell biological mechanisms of multidrug resistance in tumors. *Proc Natl Acad Sci USA* **91:**3497–3504.

Sugawara I, Kataoka I, Morishita T, Hamada H, Tsuruo T, Itoyama S and Mori S (1988) Tissue distribution of P-glycoprotein encoded by a multidrug-resistant gene as revealed by a monoclonal antibody, MRK 16. *Cancer Res* **48:**1926–1929.

Takano M, Kato M, Takayama A, Yasuhara M, Inui K and Hori R (1992) Transport of procainamide in a kidney epithelial cell line LLC-PK1. *Biochim Biophys Acta* **1108:**133–139.

Tanaka K, Hirai M, Tanigawara Y, Ueda K, Takano M, Hori R and Inui K (1997) Relationship between expression level of P-glycoprotein and daunorubicin transport in LLC-PK1 cells transfected with human MDR1 gene. *Biochem Pharmacol* **53:**741–746.

Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I and Willingham MC (1987) Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc Natl Acad Sci USA* **84:**7735–7738.

Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I and Willingham MC (1989) Immunohistochemical localization in normal tissues of different epitopes in the multidrug transport protein P170: evidence for localization in brain capillaries and crossreactivity of one antibody with a muscle protein. *J Histochem Cytochem* **37:**159–164.

Tsuruo T (1991) Reversal of multidrug resistance by calcium channel blockers and other agents, in *Molecular and Cellular Biology of Multidrug Resistance In Tumor Cells* (Roninson IB, ed) pp 349–372, Plenum, New York.

Ueda K, Okamura N, Hirai M, Tanigawara Y, Saeki T, Kioka N, Komano T and Hori R (1992) Human P-glycoprotein transports cortisol, aldosterone and dexamethasone, but not progesterone. *J Biol Chem* **267:**24248–24252.


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