Uptake of Doxorubicin by Cultured Kidney Epithelial Cells LLC-PK₁

Masafumi SASAYA,^a Ikuo WADA,^b Masahiko SHIDA,^a Michiko SATO,^a Yuko HATAKEYAMA,^a Hiroshi SAITOH,^a and Masahiko TAKADA^{*,a}

Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido,^a 1757 Kanazawa, Tobetsu-cho, Ishikari-gun, Hokkaido 061–0293, Japan and Department of Pharmacy, Sapporo National Hospital,^b 4–2 Kikusui, Shiroishi, Sapporo 003–0804, Japan. Received October 14, 1997; accepted January 16, 1998

The renal handling of doxorubicin (DXR) was investigated using a kidney epithelial cell line, LLC-PK₁. The uptake of DXR by LLC-PK₁ cells cultured on plastic dishes was shown to be temperature and concentration dependent. The initial uptake of DXR was slightly saturable. The K_m and V_{max} of the saturable component were calculated to be 20.2 μM , and 0.355 nmol/mg protein/10 min, respectively. The release of DXR from LLC-PK₁ cells was very slow at 37°C and almost negligible at 4°C, indicating that most of the DXR in the cells irreversibly binds to cellular constituents and that only a slight amount of unbound DXR participates in the efflux out of the cells. DXR uptake at 37°C was significantly decreased in the presence of 2,4-dinitrophenol. However, organic cations and aminoglycoside antibiotics, such as tetraethylammonium, *N*¹-methylnicotinamide, guanidine, gentamicin and neomycin, did not inhibit DXR uptake, suggesting that a process distinct from the organic cation transport system and absorptive endocytosis might be involved in the uptake of DXR by LLC-PK₁ cells.

Key words doxorubicin (DXR); LLC-PK₁ cell; uptake; organic cation transport system; absorptive endocytosis

Doxorubicin (adriamycin, DXR) is an anthracyclic antibiotic widely used in cancer chemotherapy in man. Accordingly, many studies have been undertaken to clarify the mechanism responsible for the membrane transport of DXR in various cultured cancer cells.^{1,2)} However, the rapid uptake and high binding property of DXR to cellular constituents sometimes complicates DXR transport studies.³⁾ DXR is cleared rapidly from plasma after intravenous administration, and is highly distributed to various tissues, including the kidney. However, details of the renal handling of DXR have not been fully characterized. Moreover, the relationship between DXR accumulation and toxicity in the kidney is rarely evaluated. Since DXR behaves as an organic cation in the body, various endogenous factors are capable of affecting DXR movement in the kidney. There may be an interaction between DXR and the renal organic cation transport system. Since anthracyclines are shown to be transported by P-glycoproteins,^{4,5)} the membrane protein expressed in the kidney is likely to influence DXR disposition. The endocytosis reported for some aminoglycosides may not be excluded.⁶⁾ However, little information is currently available on the secretion and reabsorption of DXR across renal tubular cells.

A pig kidney epithelial cell line, LLC-PK₁, which expresses known organic cation transport systems, often gives us useful information on the renal handling of various drugs.^{7,8)} Previously, we reported a specialized transport system for enoxacin (ENX) and ciprofloxacin (CPFX), new fluorinated quinolone derivatives, in LLC-PK₁ cells.^{9,10)} The basolateral-to-apical transport of ENX and CPFX was greater than apical-to-basolateral transport, and the transport was inhibited by guanidine, but not by tetraethylammonium (TEA). Therefore, it is considered that at least two organic cation transport systems exist on LLC-PK₁ cells. These results are consistent with a recent paper by Chun *et al.*¹¹⁾ describing the existence of two kinds of organic cation transporters on the brush-border membrane of human renal tubules; one prefers TEA to guanidine and the other prefers guanidine to TEA. However, there seems to be a considerable overlap in the substrate specificity of these two organic cation transporters for

organic cations having a larger molecular size than either TEA or guanidine. Even verapamil, a typical substrate of P-glycoprotein, interacts with both organic cation transport systems. This indicates that the substrate specificity of P-glycoprotein may in part overlap with those of the organic cation transport systems. This study was performed as a preliminary work to evaluate the uptake characteristics of DXR by LLC-PK₁ cells in connection with the renal accumulation of DXR and to examine whether organic cation transporters are involved in the renal handling of DXR.

MATERIALS AND METHODS

Materials DXR, daunorubicin (DNR) and *N*¹-methylnicotinamide (NMN) were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). TEA, guanidine, 2,4-dinitrophenol (DNP), gentamicin sulfate, neomycin sulfate, and bovine serum albumin (BSA) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Cimetidine was from Tokyo Kasei Hanbai Company (Tokyo, Japan). Medium 199 and fetal calf serum (FCS) were from Gibco Laboratories (Grand Island, NY, U.S.A.). All other chemicals used were of the highest purity commercially available.

Cell Culture LLC-PK₁ cells at passage 191 were purchased from the HSRRB cell bank (Osaka, Japan). The cells at passages 206–212 and 232–243 were seeded on 60-mm plastic culture dishes coated with rat tail collagen type I (Becton Dickinson, Bedford, MA, U.S.A.) at a density of about 4×10^5 cells in 5 ml culture medium. The culture medium used was Medium 199 supplemented with 5% FCS. The medium was replaced with fresh medium every 2–3 d after inoculation, and developed to confluence on day 6–7 of culture in an atmosphere of 5% CO₂ in air at 37°C. Subcultures were carried out every 5–6 d using 0.02% EDTA and 0.25% trypsin.

Uptake of DXR The uptake of DXR (10 μM) was measured at 37°C or 4°C, as described by Takano *et al.*¹²⁾ The uptake assays were performed in Dulbecco's phosphate-buffered saline (PBS, pH 7.4) [137 mM NaCl, 3 mM KCl,

* To whom correspondence should be addressed.

8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 1 mM CaCl_2 , 0.5 mM MgCl_2] containing 5 mM D-glucose. After the culture medium was removed, the cells were washed three times with 5 ml of PBS buffer and preincubated at 37 °C or 4 °C for 10 min. Then, 2 ml of PBS buffer containing DXR was added to each dish, and the cells were incubated for specified periods. After incubation, the medium was immediately aspirated, and the cells were washed three times with 5 ml of ice-cold PBS buffer. The cells were scraped from the dish with a cell scraper and transferred into 2 ml of ice-cold PBS buffer and then homogenized with polytron (Kinematica, Kriens-Luzern, Switzerland) at a setting of 7 for 1 min. DXR in the cell homogenate was determined by HPLC. The initial uptake of DXR into LLC-PK₁ cells was analyzed by a Damping Gauss-Newton iterative method, using a PC9801V personal computer and the curve fitting program MULTI as described by Yamaoka *et al.*¹³⁾

Analytical Methods A high performance liquid chromatograph LC-6A (Shimadzu, Kyoto, Japan) equipped with a fluorescence detector, RF-535 (Shimadzu), was used for the DXR assay. The HPLC conditions used were as follows: column, LiChrospher 100 CN (5 μm , 4 mm i.d. $\times$ 250 mm, E. Merck, Darmstadt, Germany); mobile phase, 0.05 M KH_2PO_4 -acetonitrile (65:35); flow rate, 1.0 ml/min; wave length, excitation 502 nm, emission 557 nm; temperature, 40 °C. In the measurement of the uptake sample, 1 ml of MeOH, 1 ml of 0.5 μM PIC A low reagent (Waters Assoc., Milford, MD, U.S.A.) and 10 μl of 50 μM DNR as an internal standard were added to 1 ml of cell homogenate. The mixture was applied onto a Sep-Pak C18 cartridge column (Waters Assoc., Milford, MD, U.S.A.) which was prewashed with 3 ml of MeOH and subsequently with 3 ml of 0.5 μM PIC A reagent-MeOH (3:1). DXR was eluted with 3 ml of 0.03 M phosphoric acid in MeOH. After adding 100 μl of 0.1 M KH_2PO_4 , the solution was concentrated to 0.2–0.3 ml under nitrogen stream at 40 °C, and 20 μl of the residue was applied to HPLC. Protein was determined by the method of Bradford¹⁴⁾ using a Bio-Rad Protein Assay Kit. BSA was used as a standard.

Enzyme Assays The LLC-PK₁ cells grown on 60-mm dishes were washed three times with 5 ml of ice-cold PBS buffer. The cells were scraped with a cell scraper and transferred into 2 ml of ice-cold PBS buffer, then homogenized with polytron at a setting of 7 for 1 min. All steps were carried out at 0–4 °C and aliquots were stored on ice until

Fig. 1. Time Course of DXR Uptake by LLC-PK₁ Cells

LLC-PK₁ cells were incubated in 2 ml of PBS buffer containing 10 μM DXR for 10, 30, 60, 90, 120, and 150 min at 37 °C (●) or 4 °C (○). Each point represents the mean $\pm$ S.E. of three to four determinations.

being assayed. The homogenates were assayed for alkaline phosphatase using Alkaliphospha K (Wako, Osaka, Japan). In another experiment, LLC-PK₁ cells were incubated with 10–50 μM of DXR for 1 h. The cells were treated and assayed for alkaline phosphatase, as mentioned above.

Quantification of Cell Numbers After incubation with 10–50 μM of DXR for 1 h, the viable cells adhering to the dish were counted with a microscope in the presence of trypan blue after trypsinization.

Data Analysis All data points were determined from 2 to 3 experiments performed in triplicate. The values represent means $\pm$ S.E. Statistical differences were analyzed by ANOVA and Student's *t*-test. The level of significance was $p < 0.05$.

RESULTS AND DISCUSSION

In order to confirm that DXR at the concentrations used in this study does not exhibit any cytotoxic effect on LLC-PK₁ cells, the activity of alkaline phosphatase, a marker enzyme specific for the apical membrane of LLC-PK₁ cells, was compared in the presence and absence of DXR treatment. There was no significant change in the enzyme activity between the control cells and the cells which were treated with DXR. Moreover, the addition of DXR did not affect the number of cells under the experimental conditions (data not shown). These results demonstrated that DXR does not exert any cytotoxicity under the experimental conditions used here.

The uptake of DXR by LLC-PK₁ cells cultured on plastic dishes was compared at 37 °C and 4 °C. As shown in Fig. 1, the uptake of DXR at 37 °C rapidly increased in the initial stage (up to 60 min) and then moderately reached a steady-state until 120 min. The uptake of DXR at 4 °C was remarkably retarded compared with that at 37 °C, suggesting that DXR uptake by LLC-PK₁ cells is highly temperature-dependent.

The time course of DXR release from LLC-PK₁ cells preincubated with 10 μM DXR for 120 min is shown in Fig. 2. Very little release of DXR was observed at 37 °C. In contrast, DXR release was almost negligible up to 60 min at 4 °C. Therefore, the process of DXR release from LLC-PK₁ cells was also regarded to be sensitive to incubation tempera-

Fig. 2. Efflux of DXR from LLC-PK₁ Cells

LLC-PK₁ cells were preincubated in 2 ml of PBS buffer containing 10 μM DXR for 120 min at 37 °C. After preincubation, the cells were rapidly washed with ice-cold PBS buffer. Then, 2 ml of drug free PBS buffer was added to each dish, and the cells were incubated for 10, 30, and 60 min at 37 °C (●) or 4 °C (○). At the stated times, the DXR remaining in the cells was determined. Each point represents the mean $\pm$ S.E. of four to five determinations. ** $p < 0.01$, Significantly different from control.

ture, possibly indicating the involvement of an energy-dependent process. Although it is reported that P-glycoprotein transports anthracyclines, such as DXR,^{4,5)} LLC-PK₁ cells express relatively low levels of P-glycoprotein.¹⁵⁾ Therefore, the involvement of P-glycoprotein in the DXR release from LLC-PK₁ cells seems to be small, if any. Therefore, it could be said that another type of transport system other than P-glycoprotein is involved in the DXR release from LLC-PK₁ cells at 37 °C. However, the results shown in Fig. 2 suggest more strongly that most of the DXR transported into LLC-PK₁ cells irreversibly bind to an intracellular compartment and that this binding is responsible for the slight release of DXR out of the LLC-PK₁ cells.

The rates of DXR uptake in the early stage were determined over the concentration range of 5 to 100 μM by incubating LLC-PK₁ cells for 10 min. As shown in Fig. 3, there was a non-linear relationship, and slight saturability, between the concentration and uptake rate. This indicates that DXR uptake by LLC-PK₁ cells consists of saturable and nonsaturable components. To obtain kinetic parameters for DXR uptake, the data were fit to the following Michaelis-Menten model: $V = V_{\text{max}}[S]/(K_m + [S]) + k_d[S]$, where V_{max} is the maximal uptake rate, K_m is the concentration needed to reach half of V_{max} , k_d is the coefficient for the nonsaturable component, and $[S]$ is the DXR concentration. K_m , V_{max} , and k_d were calculated to be 20.2 μM , 0.355 nmol/mg protein/10 min, and 0.0082 nmol/mg protein/10 min/ μM , respectively. It is demonstrated that the nonsaturable component accounts for the major part of the total uptake.

The effect of a metabolic inhibitor and various organic cations on DXR uptake was examined. The uptake of DXR (10 μM) for 30 min was significantly decreased, from 0.391 ± 0.025 to 0.264 ± 0.06 nmol/mg protein/30 min, in the presence of 0.2 mM DNP, a metabolic inhibitor. However, the same concentrations of TEA, NMN, guanidine, neomycin, and gentamicin did not exhibit any significant inhibitory effect on DXR uptake (data not shown). These results suggest that known organic cation transport systems are unrelated to the renal handling of DXR. There is a report which describes gentamicin as a substrate of an organic cation transport system in the renal brush-border membrane.¹⁶⁾ On the other hand, it has been reported that gentamicin is taken up by LLC-PK₁ cells via an absorptive endocytosis.⁶⁾ However, the uptake of DXR was not affected by gentamicin in this experiment. Therefore, it might be concluded that DXR uptake can be driven by a mechanism distinct from either known organic cation transport systems or absorptive endocytosis. Organic cations with amphiphilic structures are known to bind to biomembranes with saturability and competitiveness.¹⁷⁾ Therefore, it is possible that the saturable uptake of DXR by LLC-PK₁ cells (Fig. 3) merely reflects the binding of DXR to the surface of the apical membrane of the cells. Although further studies are required to precisely distinguish between binding to the membrane and transport into intracellular space, the fact that the uptake of 10 μM DXR was not altered in the presence of gentamicin and neomycin with a concentration of 0.2 mM might also give a negative point of view on the saturable binding of DXR to the LLC-PK₁ cell membrane. That's why these organic cations are expected to more or less bind to the apical membrane of LLC-PK₁ cells.

It is known that DXR has an enormous capacity to bind

Fig. 3. Concentration Dependence of DXR Uptake by LLC-PK₁ Cells

DXR uptake for 10 min at various concentrations over 5 to 100 μM was determined. LLC-PK₁ cells were incubated in 2 ml of PBS buffer containing DXR at 37 °C. Each point represents the mean $\pm$ S.E. of five to six determinations.

cellular constituents and that DXR might be accumulated in the lysosomes and nucleus.^{18,19)} It is also known that DXR administered intravenously are rapidly distributed to various tissues, restored in the tissues for an expanded period of time, and then slowly released.²⁰⁾ Although the mechanism underlying the small release of DXR from cells is not clear, the present results obtained by LLC-PK₁ cells suggest that the irreversible binding of DXR in renal proximal tubular cells might retard the release of DXR out of these cells.

REFERENCES

- Nagasawa K., Natazuka T., Nomiya M., Ohnishi N., Yokoyama T., *Biol. Pharm. Bull.*, **19**, 100—105 (1996).
- Marsh W., Sicheri D., Cebter S. M., *Cancer Res.*, **46**, 4053—4057 (1986).
- Scigfried J. M., Burke T. G., Tritton T. R., *Biochem. Pharmacol.*, **34**, 593—598 (1985).
- Borrel M.-N., Fiallo M., Priebe W., Garnier-Suillerot A., *FEBS Lett.*, **356**, 287—290 (1994).
- Tanaka K., Hirai M., Tanigawara Y., Yasuhara M., Hori R., Ueda K., Inui K., *Pharm. Res.*, **13**, 1073—1077 (1996).
- Saito H., Inui K., Hori R., *J. Pharmacol. Exp. Ther.*, **238**, 1071—1076 (1986).
- Saito H., Yamamoto M., Inui K., Hori R., *Am. J. Physiol.*, **262**, C59—C66 (1992).
- Takakura Y., Morita T., Fujikawa M., Hayashi M., Sezaki H., Hashida M., Borchardt R. T., *Pharm. Res.*, **12**, 1968—1972 (1995).
- Sasaya M., Shida M., Hirano T., Oda M., Endo T., Sakata M., Miyazaki S., Takada M., *Yakugaku Zasshi*, **116**, 497—503 (1996).
- Sasaya M., Oda M., Endo T., Saitoh H., Takada M., *Biol. Pharm. Bull.*, **20**, 887—891 (1997).
- Chun J. K., Zhang L., Piquette-Miller M., Lau E., Tong L.-Q., Giacomini K. M., *Pharm. Res.*, **14**, 936—941 (1997).
- Takano M., Kato M., Takayama A., Yasuhara M., Inui K., Hori R., *Biochim. Biophys. Acta*, **1108**, 133—139 (1992).
- Yamaoka K., Nakagawa T., Tanaka H., Yasuhara M., Okumura K., Hori R., *J. Pharmacobio-Dyn.*, **8**, 246—256 (1985).
- Bradford M. M., *Anal. Biochem.*, **72**, 248—254 (1976).
- Tanaka K., Hirai M., Tanigawara Y., Ueda K., Takano M., Hori R., Inui K., *Biochem. Pharmacol.*, **53**, 741—746 (1997).
- Sokol P. P., Huiatt K. R., Holohan P. D., Ross C. R., *J. Pharmacol. Exp. Ther.*, **251**, 937—942 (1989).
- Saitoh H., Kawai S., Iseki K., Miyazaki K., Arita T., *J. Pharm. Pharmacol.*, **40**, 776—780 (1988).
- Rutherford A. V., Willingham M. C., *J. Histochem. Cytochem.*, **41**, 1573—1577 (1992).
- Weaver J. L., Pine P. S., Aszalos A., Schoenlein P. V., Currier S. J., Dadmanabhan R., Gottesman M. M., *Exp. Cell Res.*, **196**, 323—329 (1991).
- Mross K., Maessen P., Vijgh W. J. M., Gall H., Boven E., Pinedo H. M., *J. Clin. Oncol.*, **6**, 517—526 (1988).