Transport Mechanism of Anthracycline Derivatives in Rat Polymorphonuclear Leukocytes: Uptake of Pirarubicin, Daunorubicin and Doxorubicin

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We performed experiments on the cis-inhibition and trans-stimulation effect on pirarubicin uptake in order to clarify the involvement of a carrier in the pirarubicin, daunorubicin and/or doxorubicin transport systems in rat polymorphonuclear leukocytes. The uptake of daunorubicin and doxorubicin was a saturable concentration-dependent process. Since the apparent kinetic constants, Michaelis constant (K_m) and inhibition constant (K_i), were almost comparable, these drugs presented mutually competitive inhibition. Furthermore, the pirarubicin uptake by polymorphonuclear leukocytes was significantly elevated by increasing the preloaded amount of doxorubicin, indicating that there was a trans-stimulation effect on the pirarubicin transport in the leukocytes. These results suggest that carrier-mediated transport might be involved in the uptake of anthracycline derivatives, pirarubicin, daunorubicin and doxorubicin, by rat polymorphonuclear leukocytes.

Keywords anthracycline derivative; polymorphonuclear leukocyte; carrier-mediated transport

Anthracycline derivatives are very effective antineoplastic agents widely used clinically. However, because of their many side effects (e.g. cardiotoxicity and alopecia), clinical use has been limited.²⁾ In order to minimize their side effects in cancer chemotherapy, antitumor agents must be selectively transported into tumor cells. The findings obtained from basic studies on cellular transport systems were very useful for the development of clinically successful and safe chemotherapy. Although many reports which deal with the culture cell lines and the primary culture cells isolated from various organs containing tumors have been published,^{3–15)} there are only a few reports which have examined the transport mechanisms of antitumor agents in normal cells.^{16,17)}

In our previous paper,¹⁸⁾ we reported on the transport mechanism of pirarubicin (THP), an anthracycline antitumor antibiotic, in rat polymorphonuclear leukocytes (PMN cells). As a result, it was revealed that the uptake of THP by PMN cells was time-, temperature- and concentration-dependent, it was affected by medium pH and osmolarity, and was inhibited by metabolic inhibitors such as rotenone, 2,4-dinitrophenol, *etc.* These results suggested that a specific mechanism was involved in the transport of THP in PMN cells. However, we could not elucidate whether the transporter for THP is present in the membrane of PMN cells. For this reason, in this report we performed further experiments to clarify the participation of a carrier in the transport of several anthracycline derivatives, THP, daunorubicin (DNR) and/or doxorubicin (ADR), in PMN cells.

MATERIALS AND METHODS

Chemicals Pure THP, tetrahydropyranyldoxorubicinol (THP-OH) and DNR were obtained from Meiji Seika Kaisha, Ltd. (Tokyo, Japan) and ADR was provided by Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). All

other reagents were obtained commercially and were of an analytical grade requiring no further purification.

Animals Male Wistar strain rats (Japan SLC, Inc., Hamamatsu, Japan), weighing 200–300 g, were used in the experiments.

Preparation of PMN Cells 0.1% gel-Hanks solution (gel-HBSS) (pH 7.4) was used in this study as the incubation medium.

PMN cells were prepared from rats using the same procedure described previously.¹⁸⁾ The cells obtained were subjected to total and differential counts using an improved Neubauer counting chamber (Clay Adams). The PMN cells were stained with gentiana violet stain and counted as polymorphonuclear cells under a light microscope. If 98% or more of the cells showed normal function in the Trypan blue exclusion test to determine the cell viability,¹⁹⁾ the cells were used in experiments.

Uptake Experiments In the uptake experiments for THP, DNR and ADR, preincubation of the reaction suspension (cell density; 5×10^6 cells/ml) was performed for 30 min at 37 °C with shaking (160 strokes/min). The reaction was initiated by the addition of the drug solution dissolved in distilled water to the incubated medium, and terminated by centrifugation of the reaction suspension at appropriate time intervals as reported previously.¹⁸⁾

In the cis-inhibition experiments, the drugs (1 or 5 μ M) as either substrate or inhibitor (0.3, 1, 5, 10 or 20 μ M) were added simultaneously to the reaction suspension, and the uptake of the substrate by PMN cells was determined 1 min after incubation. In the trans-stimulation experiments, after the reaction suspension of PMN cells was preloaded by various concentrations of ADR (1, 3, 5 or 10 μ M) for 15 min, the cells were washed three times with a sufficient volume of ice-cold Ca^{2+} -free medium. Thereafter, the PMN cells were resuspended in the appropriate volume of the gel-HBSS, warmed at 37 °C, containing 0.3 μ M of THP, and they were then incubated for 1 min.

Fig. 1. Chemical Structures of Anthracycline Derivatives Used

Thereafter, the samples were treated in the same manner as the above experiments. In all experiments, the PMN cells after washing were resuspended in 1 ml of distilled water, and stored at -80°C until the time of assay.

Assay Procedure The THP, DNR and ADR concentrations in PMN cells were determined by HPLC as reported previously.²⁰⁾

Statistical Analysis The data are expressed as the mean $\pm$ S.E., and the statistical significance was evaluated by the Student's *t*-test or ANOVA.

RESULTS AND DISCUSSION

Recently, studies on the cellular transport mechanisms of drugs have been carried out in great detail, especially those mechanisms in the intestine,³⁻⁵⁾ kidney,⁶⁻⁹⁾ liver^{10,11)} and blood-brain barrier.¹²⁻¹⁴⁾ The findings derived from these studies have been very useful for the development of chemotherapy, and have contributed immensely to drug design. Accordingly, by adapting the characteristics of a drug transport system in the cells, it was thought that antitumor agents should be able to reach tumor cells selectively. On the other hand, we previously indicated that unknown factors, other than white or red blood cell count, were related to interindividual variations in THP blood cell distribution.²¹⁾ However, it is presumed that in cancer chemotherapy, organisms are exposed to low drug concentrations for an extended period of time due to the drug's distribution to blood cells, especially leukocytes. For these reasons, the uptake of THP by leukocytes is considered to be closely associated with the incidence of clinical toxicity. Therefore, we have been investigating the transport mechanism of THP in PMN cells,¹⁸⁾ and in this report, further experiments were performed in order to elucidate the involvement of a carrier in the THP, DNR and/or ADR transport systems in PMN cells.

Since THP, DNR and ADR are anthracycline derivatives and have similar structures (Fig. 1), it was speculated that these drugs were taken up by PMN cells *via* a common transport system. We first examined the basic characteristics of the uptake of THP, DNR and ADR in PMN cells.

In our previous paper,¹⁸⁾ THP uptake by PMN cells was extremely rapid, and reached equilibrium within about 10 min (Fig. 2). The uptake of DNR and ADR by PMN cells was also rapid, and reached equilibrium within

Fig. 2. Time Courses of THP, DNR and ADR Uptake by PMN Cells

Each medium was incubated with $0.3\ \mu\text{M}$ of THP (circle), DNR (triangle) or ADR (square) for the indicated time at 37°C . Each point represents the mean $\pm$ S.E. of three experiments.

Fig. 3. Concentration Dependency of DNR Uptake by PMN Cells

Each medium was incubated with the indicated concentration of DNR for 1 min at 37°C . The inset shows the Lineweaver-Burk plot. Each point represents the mean $\pm$ S.E. of three experiments.

about 15 and 5 min, respectively; these uptakes were time-dependent and expressed saturation kinetics as did the uptake of THP, as shown in Fig. 2. In addition, both initial rate and the amount at equilibrium of these uptakes were large in the order of $\text{THP} > \text{DNR} > \text{ADR}$.

Figures 3 and 4 show the relationships between drug concentration and the initial uptake rate for DNR and ADR, respectively. Uptake of these by PMN cells was a saturable concentration-dependent process. These results indicated that DNR and ADR were transported into

PMN cells *via* a carrier-mediated system.

The apparent kinetic constants for THP,¹⁸⁾ DNR and ADR uptake by PMN cells calculated by using Lineweaver-Burk plots shown in Figs. 3 and 4, inset, are summarized in Table I. The Michaelis constant (K_m), the maximum velocity (V_{max}) and V_{max}/K_m values for THP uptake were 13.4 μM , 4.84 nmol/ 5×10^6 cells/min and 0.361, for DNR uptake 9.22 μM , 0.870 nmol/ 5×10^6 cells/min and 0.094, and for ADR uptake 45.0 μM , 1.64 nmol/ 5×10^6 cells/min and 0.036, respectively. Thus, the uptake rate of THP was the highest, and that of DNR was higher than ADR; these results are supported by those in Fig. 2. Since there was a correlation between these V_{max}/K_m values and the lipophilicity of these drugs (THP > DNR > ADR), it was speculated that the uptake of drugs by PMN cells might correspond to their lipophilic charac-

Fig. 4. Concentration Dependency of ADR Uptake by PMN Cells

Each medium was incubated with the indicated concentration of ADR for 1 min at 37°C. The inset shows the Lineweaver-Burk plot. Each point represents the mean $\pm$ S.E. of three experiments.

TABLE I. Apparent Kinetic Constants for THP, DNR and ADR Uptake by PMN Cells

Parameter	THP	DNR	ADR
K_m (μM)	13.4	9.22	45.0
V_{max} (nmol/ 5×10^6 cells/min)	4.84	0.87	1.64
V_{max}/K_m	0.361	0.094	0.036
K_i for THP uptake (μM)	—	31.2	48.9
K_i for DNR uptake (μM)	—	—	11.3
K_i for ADR uptake (μM)	—	34.4	—

K_m , Michaelis constant; V_{max} , maximum velocity; K_i , inhibition constant.

teristics. This speculation was supported by the findings of Kleeberger and Rottinger²²⁾ and Levin,²³⁾ in which the lipophilicity of a drug might dominate its uptake by cells. However, specific details are still unclear, because it was reported that anthracycline derivative uptake by Ehrlich ascites tumor cells did not follow to the derivatives' lipophilicity.²⁴⁾

In addition, in order to investigate the mutual competitive inhibition of THP, DNR and ADR transport, the cellular uptake of each drug as a substrate was observed after both the substrate and inhibitor reacted with

Fig. 5. Dixon Plot of THP Uptake Showing Inhibition by ADR

Each medium was incubated with the indicated concentration of THP and ADR for 1 min at 37°C. Each point represents the mean $\pm$ S.E. of three experiments.

Fig. 6. Dixon Plot of THP Uptake Showing Inhibition by DNR

Each medium was incubated with the indicated concentration of THP and DNR for 1 min at 37°C. Each point represents the mean $\pm$ S.E. of three experiments.

Fig. 7. Dixon Plots of ADR Uptake Showing Inhibition by DNR (A), and DNR Uptake Showing Inhibition by ADR (B)

Each medium was incubated with the indicated concentration of ADR and DNR for 1 min at 37°C. Each point represents the mean $\pm$ S.E. of three experiments.

Fig. 8. Effect of Preloaded ADR on THP Uptake by PMN Cells

Each medium was preincubated with the indicated concentration of ADR for 15 min at 37°C, and then washed three times with ice-cold Ca^{2+} -free medium. Thereafter, the cells were suspended in gel-HBSS warmed at 37°C containing 0.3 μM of THP and incubated for 1 min. Each bar represents the mean ± S.E. of three experiments. a) and b) $p < 0.01$ and 0.001 significantly different from the value of 0 μM of ADR concentration, respectively.

PMN cells simultaneously. Besides, we could not perform the experiments using THP as an inhibitor, because THP was contaminated by a small amount ($<0.5\%$) of ADR (unpublished data), and the effect of ADR contained in THP on the uptake of a substrate drug could not be disregarded. The Dixon plots for each case are depicted in Figs. 5, 6 and 7. We found the inhibition of each substrate's uptake by inhibitors, and this inhibition was competitive in all cases.

As shown in Table I, the apparent inhibition constant (K_i) values of DNR and ADR for the inhibition of the THP uptake were 48.9 and 34.4 μM, respectively, and the K_m value for THP uptake was 13.4 μM. For DNR uptake ($K_m = 9.22$ μM), the K_i value of ADR was 11.3 μM, and for ADR uptake ($K_m = 45.0$ μM), the K_i value of DNR was 34.4 μM. These K_m and K_i values were almost comparable, suggesting that THP, DNR and ADR were transported into PMN cells *via* a common carrier.

To further clarify the suggestion described above, the effect of a trans-stimulation on THP uptake was examined. In general, when performing the experiments for a trans-stimulation effect, the cells were preloaded by the RI-unlabeled substrate, and then the RI-labeled substrate was used for measurement of the drug uptake. However, we could not obtain RI-labeled THP. So we used ADR instead of THP as the preloading drug, because the results of the cis-inhibition experiments suggested that ADR and THP were taken up *via* a common carrier. As a result, it appeared that the THP uptake by PMN cells was significantly elevated by increasing the preloaded amount of ADR (Fig. 8). Thus, it was indicated that there

was a trans-stimulation effect on THP transport in PMN cells.

In conclusion, considering the above results (saturation, cis-inhibition and trans-stimulation), it was suggested that carrier-mediated transport system might be involved in the uptake of anthracycline derivatives, THP, DNR and ADR, by PMN cells.

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