

Contribution of the Nucleoside Transport System to Doxorubicin Transport in HL60 Cells but Not in Mononuclear Cells

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Previously, we reported that pirarubicin (THP), an anthracycline, was transported, at least in part, via a nucleoside transport system in human leukemic HL60 cells, but not in mononuclear cells (MNCs). In this study, the contribution of the nucleoside transport system to the transport of other anthracyclines, doxorubicin (DOX), daunorubicin (DNR) and idarubicin (IDA), in HL60 cells and MNCs was investigated. The experiments were performed after both types of cells had been pretreated with a metabolic inhibitor, 2,4-dinitrophenol, to deplete cellular ATP. The DOX uptake by HL60 cells was partially inhibited by inhibitors of equilibrative nucleoside transporters. In HL60 cells, moreover, the uptake of DOX depended on an inwardly directed Na⁺-gradient, and was inhibited by concentrative nucleoside transporters, but there was no change in the DNR or IDA uptake under any of these conditions. On the other hand, the uptake of the three drugs by MNCs was not affected by any inhibitors of the nucleoside transporters, and there was no dependence of the uptake on an Na⁺-gradient. These results suggested that DOX, but not DNR or IDA, was partially transported in HL60 cells via the nucleoside transport system, whereas in MNCs the system did not contribute to the uptake of any of these three drugs. Thus, nucleoside transport systems contributing to the transport of anthracyclines may be different among different derivatives and cell types.

Key words: Anthracycline — Transport mechanism — Nucleoside transport system — Human leukemia HL60 cell — Human mononuclear cell

Anthracyclines are among the most active agents used for the treatment of human malignancies. However, these drugs induce dose-dependent leukopenia, cardiac myopathy, alopecia, etc., which has restricted their clinical usefulness. These adverse effects are accounted for by the distribution of these drugs not only to tumor cells, but also to normal cells. So, it is important to regulate their distribution in order to reduce the incidence and/or intensity of adverse effects, and to increase the antitumor effects of these agents.

Findings obtained in basic studies on cellular transport systems are very useful for the development of clinically successful and safe chemotherapies. Therefore, we have been studying the transport mechanisms for four anthracyclines, doxorubicin (DOX), pirarubicin (THP), daunorubicin (DNR) and idarubicin (IDA) (Fig. 1), in human leukemia HL60 cells and human mononuclear cells (MNCs), as models of tumor and normal cells, respectively, in order to establish a strategy for selectively delivering anthracyclines to leukemia cells. A carrier-mediated system(s) was found to be involved in their uptake by both cell types, but there are differences in uptake characteristics between HL60 cells and MNCs.^{1–4)} Recently, it was

shown that THP was transported, at least in part, in HL60 cells via a nucleoside transport system,⁵⁾ which consists of equilibrative transporters (eNTs) and Na⁺-dependent concentrative ones (cNTs),^{6–9)} while MNCs did not exhibit such transport. However, whether or not the nucleoside transport system is involved in the transport of other anthracyclines remained unclear.

Therefore, in this study, the contribution of the nucleoside transport system to the transport of DOX, DNR and IDA in HL60 cells and MNCs was investigated.

MATERIALS AND METHODS

Chemicals Pure DOX (doxorubicin hydrochloride; Kyowa Hakko Kogyo Co., Tokyo), DNR (daunorubicin hydrochloride; Meiji Seika Kaisha, Ltd., Tokyo), IDA (idarubicin hydrochloride; Pharmacia K. K., Tokyo), and tetrahydropyranyldoxorubicin (internal standard for the assay; Meiji Seika Kaisha, Ltd.) were used. Nitrobenzylthioinosine (NBMPR), nitrobenzylthioguanosine (NBTGR), dilazep, formycin B and FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone) were purchased from Sigma Chemical Co. (St. Louis, MO), and 2,4-dinitrophenol (DNP), uridine, thymidine, cytosine arabinoside (Ara-C) and monensin from Wako Pure Chemical Ind. (Osaka). All other reagents were obtained commercially or were of ana-

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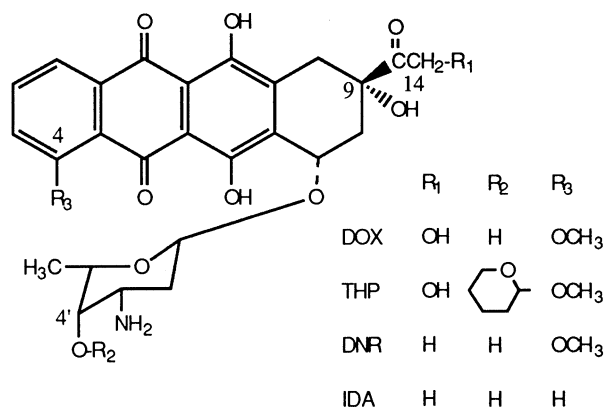


Fig. 1. Chemical structures of the anthracyclines used.

lytical grade requiring no further purification. Deionized double-distilled water was used throughout the experiments.

Cell line and culture conditions HL60 cells were provided by Dr. R. C. Gallo (NIH, Bethesda, MD). This line was maintained in RPMI1640 medium (GIBCO Laboratories, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum, 10 $\mu\text{g/ml}$ kanamycin sulfate and 25 mM HEPES under an atmosphere containing 5% CO₂. P-Glycoprotein was not detectable in these cells at either the protein or RNA level.¹ Exponentially growing cells, with a viability of 98% or more, as determined by means of the trypan blue exclusion test, were used in the experiments. We did not synchronize the cell cycle because this has been shown to have little effect on the uptake or release of anthracyclines.¹⁰ As the incubation media in the uptake experiments, glucose-free Hanks' balanced salt solution (HBSS) (pH 7.4) was used as the Na buffer, and choline-replaced, glucose-free HBSS (pH 7.4) as the choline buffer.

Preparation of MNCs The isolation and purification of MNCs from human peripheral blood were performed by the methods described previously.^{3,4} The recovered cells accounted for about 27% of the whole leukocytes in the blood, and consisted of about 90% MNCs, 10% polymorphonuclear leukocytes and a negligible amount of platelets, as determined by differential counting and observation under a light microscope, respectively.^{3,4} Their viability was 98% or more, as determined by means of the trypan blue exclusion test. For the experiments involving MNCs, glucose-free 0.1% gelatin-containing HBSS (pH 7.4, Na buffer) or choline-replaced, glucose-free 0.1% gelatin-containing HBSS (pH 7.4, choline buffer) was used as the incubation medium, as indicated.

Uptake experiments Uptake experiments were performed by the methods previously reported.⁵ The concen-

tration of each nucleoside transport inhibitor used was based upon our previous report,⁵ so as to give approximately the maximum inhibitory effect.⁶⁻⁹ It has been reported that anthracyclines (5.5–7.5 μM), and high concentrations of purine (>2–6 mM) and pyrimidine (>85–100 mM) nucleoside derivatives form complexes in aqueous solution,¹¹ and this might result in a decrease in their uptake. Therefore, we confirmed that there was no change in the ultraviolet spectrum when each compound was mixed in the transport buffer, indicating that there is no interaction among them (data not shown). Because of the low levels of uptake of DOX and DNR by MNCs, the sampling procedures and the substrate concentrations for the experiments were different for HL60 cells and MNCs. However, we think that comparison of the transport mechanisms for DOX and DNR between the two cell types is valid, since the substrate concentrations for the uptake (0.3 μM in HL60 cells and 1 μM in MNCs) used in this study were much lower than the Michaelis constants for the uptake of DOX and DNR, which are 230 and 10.0 μM in HL60 cells and MNCs, respectively, for DOX, and 25.9 and 54.0 μM , respectively, for DNR.^{2,3} We determined the rates of uptake of DOX, DNR and IDA at 3 min, 1 min and 45 s, respectively, because there were apparent differences among their uptake rates (DOX<DNR<IDA).¹⁻⁴

Assay procedures The DOX, DNR and IDA concentrations in cells were determined by high-performance liquid chromatography as reported previously.^{2,4}

Statistical analysis For HL60 cells, the data were expressed as means \pm SE. For MNCs, each experiment was carried out in triplicate with MNCs obtained from a male donor, and the data were averaged. Then, the same experiments as described above were performed with MNCs obtained from three or more separate donors, and the data were expressed as means \pm SEM. Comparisons between two groups and among three or more groups were performed by means of Student's unpaired or paired *t*-test, and one-way analysis of variance followed by Scheffe's *S* or Bonferroni-Dunn (Control) test, respectively, a difference with a *P* value of 0.05 or less being considered statistically significant.

RESULTS

Effects of eNT inhibitors Fig. 2, A–C shows the effects of inhibitors of eNTs on the uptake of DOX, DNR and IDA by HL60 cells. The DOX uptake in the presence of an inwardly directed Na⁺-gradient at 3 min was significantly increased by the simultaneous addition of 10 μM NBMPR, 10 μM NBTGR or 100 μM dilazep. However, in the absence of the gradient, they all significantly decreased the DOX uptake (Fig. 2A). On the other hand, there was no change in the uptake of DNR or IDA under any conditions (Fig. 2, B and C).

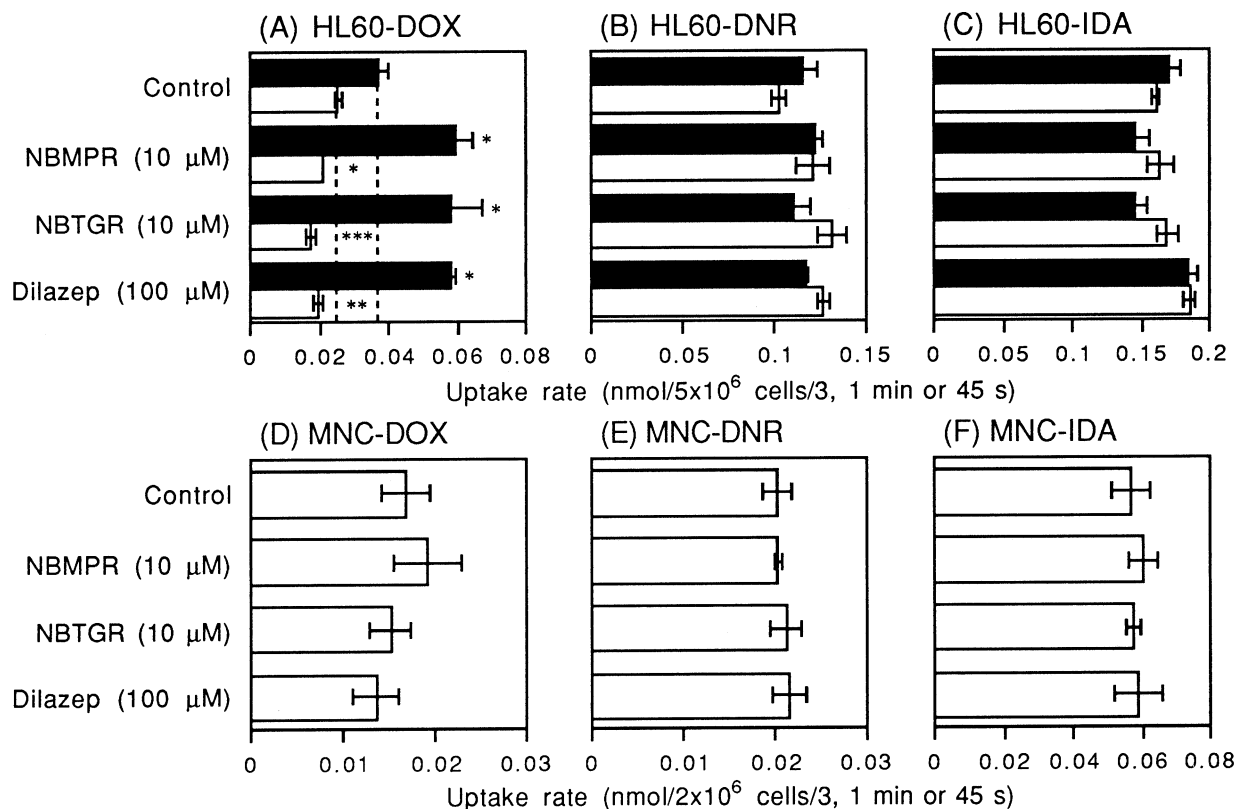


Fig. 2. Effects of eNT inhibitors on the uptake of DOX, DNR and IDA by HL60 cells and MNCs in the presence and absence of an inwardly directed Na⁺-gradient. A–C: After HL60 cells had been pretreated with 4 mM DNP for 20 min in choline buffer, they were incubated with 0.3 μM DOX, DNR or IDA and the indicated concentration of an inhibitor in Na (closed bars) or choline (open bars) buffer containing 4 mM DNP for 3 min, 1 min or 45 s, respectively, at 37°C. D–F: MNCs were preincubated with 4 mM DNP in a choline buffer for 20 min, and then the reaction was initiated by adding the same volume of choline buffer containing 1 μM DOX or DNR, or 0.3 μM IDA, the indicated concentration of an inhibitor and 4 mM DNP (final concentration) for 3 min, 1 min or 45 s, respectively, at 37°C. Each bar represents the mean ± SE or SEM for three experiments with HL60 cells or experiments with MNCs obtained from three separate donors, respectively. *, ** and *** significantly different from each control group, $P < 0.05$, 0.01 and 0.001, respectively.

The effects of eNT inhibitors on the uptake of DOX, DNR and IDA by MNCs are shown in Fig. 2, D–F. In the absence of an inwardly directed Na⁺-gradient, their uptake by MNCs was not affected by any of the inhibitors.

Effect of an inwardly directed Na⁺-gradient The time courses of the uptake of DOX, DNR and IDA by HL60 cells with or without an inwardly directed Na⁺-gradient are shown in Fig. 3, A–C. The uptake of DOX by HL60 cells in the absence of a Na⁺-gradient was significantly less than that in the presence of a gradient. As shown in Table I, the DOX uptake by HL60 cells pretreated with 10 μg/ml monensin was significantly ($P < 0.001$) lower than that in the cases of non- and FCCP-treated cells. In contrast to the case of DOX, the uptake of DNR and IDA was independent of an inwardly directed Na⁺-gradient.

As shown in Fig. 3, D–F, the uptake of these three anthracyclines by MNCs in the presence and absence of an

inwardly directed Na⁺-gradient was approximately equal at each time point. Table I shows the effects of ionophores on the uptake by MNCs. Their uptake was not affected by pretreatment with monensin or FCCP.

Effects of cNT inhibitors Fig. 4 shows the effects of inhibitors of cNTs on the DOX uptake by NBMPR-pretreated HL60 cells in the presence or absence of an inwardly directed Na⁺-gradient. One hundred micromolar formycin B, or 500 μM uridine or Ara-C significantly decreased the DOX uptake in the presence of the gradient, although 500 μM thymidine had no effect. On the other hand, in the absence of a Na⁺-gradient, formycin B, uridine and thymidine did not affect the DOX uptake by NBMPR-pretreated HL60 cells (Fig. 4). Furthermore, the uptake of DOX in the presence of the inhibitors, except for thymidine, with a Na⁺-gradient was equivalent to that of the control without the gradient (Fig. 4). Ara-C

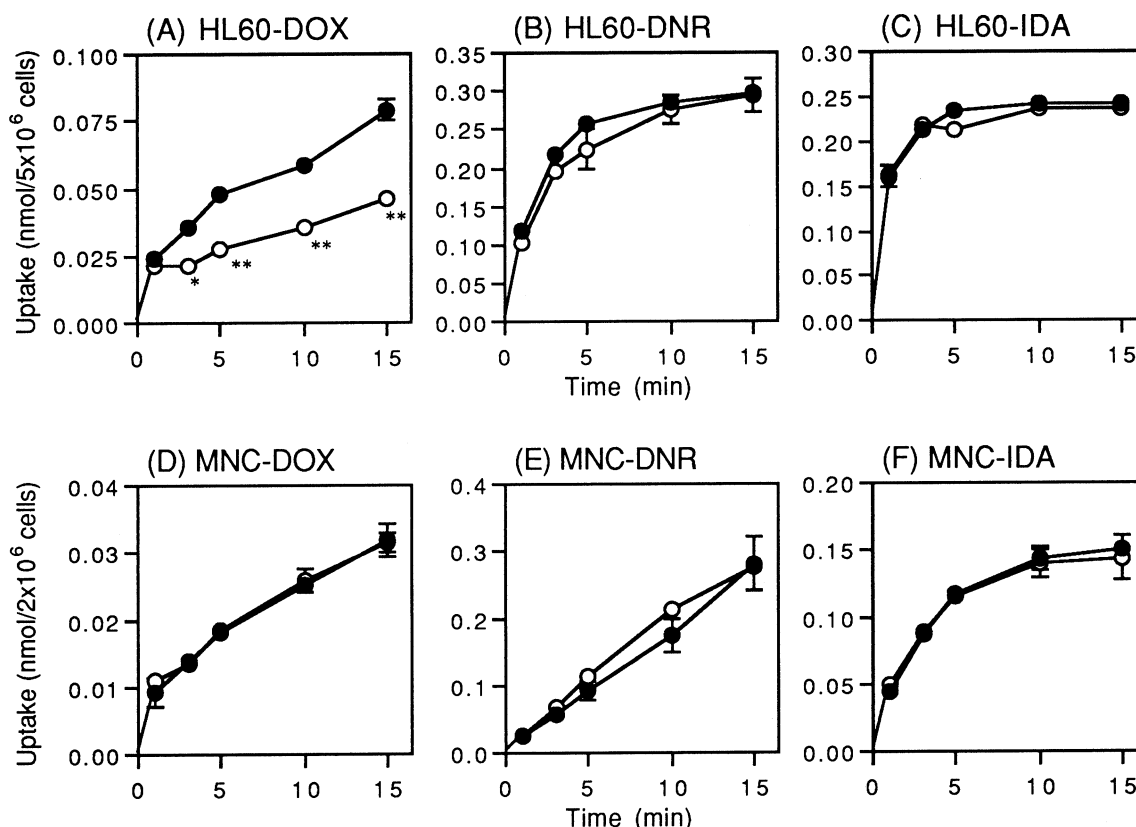


Fig. 3. Time courses of the uptake of DOX, DNR and IDA by HL60 cells and MNCs in the presence and absence of an inwardly directed Na⁺-gradient. A–C: After HL60 cells had been pretreated with 4 mM DNP for 20 min in choline buffer, they were incubated with 0.3 μM DOX, DNR or IDA in Na (closed circles) or choline (open circles) buffer containing 4 mM DNP for the indicated times at 37°C. D–F: MNCs were preincubated with 4 mM DNP in a choline buffer for 20 min, and then the reaction was initiated by adding the same volume of Na (closed circles) or choline (open circles) buffer containing 1 μM DOX or DNR, or 0.3 μM IDA and 4 mM DNP (final concentration) for the indicated times at 37°C. Each point represents the mean±SE or SEM for three experiments with HL60 cells or experiments with MNCs obtained from three separate donors, respectively. * and ** significantly different from each value for the Na buffer group at the corresponding time point, P<0.05 and 0.001, respectively.

Table I. Effects of Ionophores on Uptake of DOX, DNR and IDA by HL60 Cells and MNCs

	Uptake rate					
	HL60 (nmol/5×10 ⁶ cells/3 min, 1 min or 45 s)			MNC (nmol/2×10 ⁶ cells/3 min, 1 min or 45 s)		
	DOX	DNR	IDA	DOX	DNR	IDA
Control	0.042±0.003	0.122±0.006	0.169±0.008	0.014±0.001	0.028±0.002	0.051±0.010
Monensin	0.033±0.001*	0.127±0.011	0.175±0.010	0.012±0.001	0.024±0.004	0.045±0.009
FCCP	0.044±0.002	0.128±0.001	0.190±0.011	0.016±0.001	0.026±0.003	0.067±0.008

HL60 cells were pretreated with 4 mM DNP and 10 μg/ml ionophore for 20 min, and then incubated with 0.3 μM DOX, DNR or IDA for 3 min, 1 min or 45 s, respectively, in Na buffer at 37°C. MNCs were pretreated with 4 mM DNP and 10 μg/ml ionophore for 20 min, and then incubated with 1 μM DOX or DNR, or 0.3 μM IDA for 3 min, 1 min or 45 s, respectively, in Na buffer at 37°C. Each value represents the mean±SE or SEM for three experiments with HL60 cells, and experiments with MNCs obtained from three separate donors, respectively. * significantly different from each control, P<0.001.

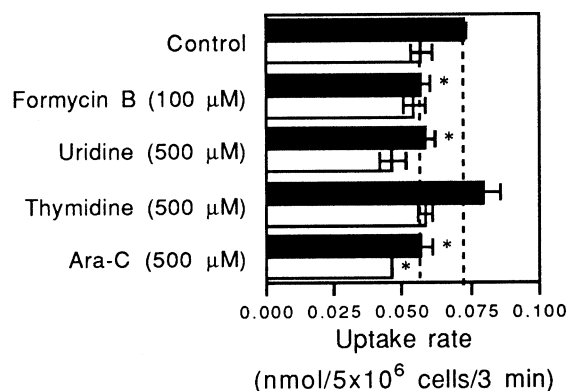


Fig. 4. Effects of cNT inhibitors on DOX uptake by NBMPR-pretreated HL60 cells in the presence and absence of an inwardly directed Na⁺-gradient. After cells had been pretreated with 4 mM DNP and 10 μM NBMPR for 20 min in choline buffer, they were incubated with 0.3 μM DOX and the indicated concentration of an inhibitor in Na (closed bars) or choline (open bars) buffer containing 4 mM DNP and 10 μM NBMPR for 3 min at 37°C. Each bar represents the mean ± SE for three to six experiments. * significantly different from each control group, $P < 0.05$.

decreased the uptake significantly in the absence of an Na⁺-gradient.

DISCUSSION

As shown in Fig. 2A, the DOX uptake apparently increased on the addition of certain eNT inhibitors, NBMPR, NBTGR and dilazep, in the presence of an Na⁺-gradient, indicating that DOX may be a substrate for the nucleoside transport system in HL60 cells. When these inhibitors were used in the absence of an Na⁺-gradient, they significantly inhibited the uptake (Fig. 2A). It has been reported that under physiological conditions, the eNTs which show directional symmetry act mainly as efflux systems, while the cNTs act as influx systems, whereas in the absence of an inwardly directed Na⁺-gradient, the eNTs act as influx systems.^{12,13} So, the results in Fig. 2A can be accounted for as follows. The increased uptake in the presence of an Na⁺-gradient was possibly due to the inhibition of the efflux (via eNTs) of DOX taken up via cNTs and others, and the decreased uptake in the absence of an Na⁺-gradient was due to the inhibition of influx via eNTs. That is, DOX might be transported via eNTs. On the other hand, since the uptake of DNR and IDA was not affected by eNT inhibitors under any conditions (Fig. 2, B and C), they might not be substrates for eNTs.

Next, we examined the contribution of cNTs to the uptake of DOX, DNR and IDA by HL60 cells. DOX uptake was apparently stimulated in the presence of an

inwardly directed Na⁺-gradient (Fig. 3A). However, the DOX uptake did not show an overshoot in the presence of the gradient, contrary to the case of THP reported previously.⁵ This might be explained by assuming that the rate of uptake of DOX was much lower than that of THP.^{1,2} That is to say, an overshoot phenomenon might be theoretically observed for DOX uptake if this experiment were continued for an appropriately longer time, but we could not confirm this because of the decrease in cell viability. Thus, it appeared that the DOX uptake by HL60 cells partially depended on an inwardly directed Na⁺-gradient, and this was supported by the finding that an Na⁺-selective ionophore, monensin, but not a protonophore, FCCP, significantly decreased its uptake (Table I). On the other hand, it appeared that the uptake of DNR and IDA was independent of an Na⁺-gradient (Fig. 3, B and C, and Table I).

In 10 μM NBMPR-pretreated HL60 cells, there appeared to be significant inhibition of DOX uptake by cNT inhibitors, formycin B and uridine, in the presence of an Na⁺-gradient (Fig. 4). Furthermore, the DOX uptake in the formycin B and uridine groups with the gradient was found to be approximately equal to that in the control group without the gradient. It is known that 10 μM NBMPR almost completely inhibits eNTs.^{8,12,14} Thus, DOX might be taken up, at least in part, into HL60 cells via cNTs, and DOX uptake depending on an Na⁺-gradient might be accounted for by the uptake via cNTs. We cannot clearly explain the results for thymidine in this study, although it has been reported that it might have an inhibitory effect on eNTs.^{8,12,14} Ara-C caused significant inhibition of DOX uptake under both sets of experimental conditions examined (Fig. 4). So, we speculate that Ara-C might inhibit other system(s) of DOX transport in addition to eNTs and cNTs. In clinical leukemia chemotherapy, especially for acute myeloid leukemia, the combination of anthracycline and Ara-C exhibits synergistic antitumor activity.¹⁵⁻¹⁸ So, there is a discrepancy between the clinical efficacy and our basic results. However, we only examined the uptake of DOX, i.e., we did not assess the uptake of Ara-C or their pharmacological interaction. Thus, we are now examining the interaction between DOX and Ara-C in more detail in order to explain this discrepancy.

On the basis of these results, the nucleoside transport system might contribute, at least in part, to the transport of DOX, but not DNR or IDA, in HL60 cells, although DOX uptake via eNTs and cNTs was a minor component in its total uptake under physiological conditions.² In our previous report, THP was suggested to be transported in HL60 cells via the nucleoside transport system.⁵ As shown in Fig. 1, R₁ at position 14 of DOX and THP is a hydroxy group, while that of DNR and IDA is a hydrogen atom. The recognition of anthracyclines as substrates by the

nucleoside transport system might be related to the nature of the moiety at position 14. Previously, we speculated from the results of *cis*-inhibition and *trans*-stimulation experiments that the difference in the structure at position 14 might be related to the recognition of the anthracyclines by the carrier.^{1,2,4)} This speculation needs to be tested by additional studies with greater numbers of derivatives and gene expression systems, etc.

We also examined whether the nucleoside transport system contributed to the transport of DOX, DNR and IDA in MNCs. As shown in Fig. 2, D–F, eNT inhibitors had no effect on the uptake of DOX, DNR or IDA by MNCs in the absence of an inwardly directed Na⁺-gradient. Moreover, their uptake by MNCs appeared to be independent of an Na⁺-gradient, because their uptake with the gradient was equal to that without a gradient (Fig. 3, D–F), and monensin had no effect on their uptake (Table I). Therefore, we think that the uptake of DOX, DNR and IDA by MNCs does not depend on an inwardly directed Na⁺-gradient, and that they are not transported in MNCs via both eNTs and cNTs, as in the case of THP.⁵⁾

Goh *et al.* reported that the total amounts of NBMPR binding sites in various human tumor tissues were 1.5- to 5-fold higher than those in the respective normal tissues of the same patient, in spite of the fact that the NBMPR

binding affinities for both tumor and normal tissues were statistically insignificant.¹⁹⁾ In addition, nucleoside transporters are suggested to be heterogeneous and functionally dissimilar among tissues.^{20–22)} These findings might explain the differences in the results for DOX transport between HL60 cells and MNCs in this study, although the details of the mechanisms are unclear. More detailed studies are in progress in our laboratory to clarify the expression levels and substrate specificities of these transporters.

In conclusion, it was suggested that DOX, but not DNR or IDA, is at least partly transported via both eNTs and cNTs in HL60 cells, while none of the three is transported by the nucleoside transport system in MNCs. The contribution of the nucleoside transport system to the transport of the anthracyclines appears to be different among different derivatives and cell types.

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