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ORIGINAL ARTICLE

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Relationships between the in vitro cytotoxicity and transport characteristics of pirarubicin and doxorubicin in M5076 ovarian sarcoma cells, and comparison with those in Ehrlich ascites carcinoma cells

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Abstract *Purpose*: We sought to determine whether the de novo resistance of M5076 ovarian sarcoma cells, which show sensitivity to pirarubicin (THP), to doxorubicin (DOX) is due to differences in the transport characteristics between THP and DOX, and the results were compared with those for drug-sensitive Ehrlich ascites carcinoma cells. *Methods*: The in vitro cytotoxicity of the drugs was assessed by means of the tetrazolium dye assay. Transport experiments were performed by the rapid centrifugation method. *Results*: In an in vitro cytotoxicity experiment, M5076 cells showed lower sensitivity to DOX than to THP, and the cytotoxicity of THP and DOX toward M5076 cells was lower than toward Ehrlich cells, and these results were similar to those of an in vivo experiment. This was due to the much lower expression of topoisomerase II in M5076 cells than in Ehrlich cells. The amount of intracellular DOX was found to be significantly lower than that of THP in both cell types, and furthermore, little free intracellular DOX was observed in M5076 cells, indicating that the low sensitivity of M5076 cells to DOX was partially a result of the low amount of intracellular DOX. There was no difference in the efflux rate, but there was an apparent difference in the uptake efficiency of the carrier between THP and DOX. *Conclusions*: These findings suggest that the cytotoxicities of THP and DOX toward M5076 and Ehrlich cells depend, at least in part, on the uptake efficiency of the carrier.

Keywords Anthracycline · De novo resistance · Cytotoxicity · Transport · M5076 cell

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Introduction

The anthracycline antibiotics pirarubicin (THP) and doxorubicin (DOX) are very potent broad-spectrum antitumor agents [1, 16]. The main molecular target of anthracyclines is intracellular topoisomerase II [10], and thus permeation through the plasma membrane is the rate-limiting step in their cytotoxicity. We have investigated the transport mechanisms for anthracyclines in normal and tumor cells, and have found that the membrane transport of anthracyclines occurs, in part, via a system or systems that are carrier-mediated, and that the transport characteristics differ among cell types and anthracycline analogues [4, 5, 6, 7, 8].

M5076 ovarian sarcoma cells are transplantable murine reticulum sarcoma cells originating from the ovaries of C57BL/6 mice, and are highly invasive and metastatic [15]. We have previously found in in vivo experiments that a clinical dose of THP significantly reduces tumor growth, but that DOX does not [13, 14]. Although many factors contribute to the difference in cytotoxic activity between THP and DOX toward M5076 cells, they have not yet been clarified.

The purpose of the present in vitro study was to clarify the correlation between the cytotoxicity and

transport characteristics of THP and DOX in M5076 cells. We also compared the results for M5076 cells with those for Ehrlich ascites carcinoma cells, which are sensitive to THP and DOX in vivo [12].

Materials and methods

Chemicals

Pure THP (pirarubicin; Meiji Seika Kaisha, Tokyo, Japan), DOX (doxorubicin hydrochloride; Kyowa Hakko Kogyo Company, Tokyo, Japan), and tetrahydropyranyldoxorubicinol (internal standard for the HPLC assay; Meiji Seika Kaisha) were used. Sodium azide (AZ), 2-deoxy-D-glucose (DOG), and 2,4-dinitrophenol (DNP) were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were of commercial or analytical grade requiring no further purification.

Preparation of M5076 and Ehrlich cells

M5076 and Ehrlich cells were grown in the abdominal cavity of male C57BL/6 and ddY mice (SLC, Hamamatsu, Japan), respectively, aged approximately 5–7 weeks, and were isolated and purified by washing with Hanks' balanced salt solution (HBSS, pH 7.4) or glucose-free HBSS. Their density and viability (>90%) were determined by the trypan blue exclusion test.

Measurement of cell volume

The cell volume was determined using a model ZBI coulter counter equipped with a channelizer. The mean volumes of M5076 and Ehrlich cells were 817 ± 12 and 1142 ± 19 μm^3, respectively.

Cytotoxicity assay

The in vitro sensitivities of the cells to a drug were assessed by means of the tetrazolium dye (MTT) assay as described previously [5]. Briefly, cells (0.5×10^5 cells/ml) were placed in the wells of 12-well microculture plates with 11 concentrations of each drug in the range 0.0002–20 μg/ml in duplicate. Untreated control cells were set up in six wells. After incubation in a humidified atmosphere containing 5% CO_2 for 3 days at 37°C, 10 μl MTT solution was added and the plates were incubated for a further 4 h under the same conditions. The tetrazolium salt MTT is reduced to the colored product formazan by living but not by dead cells. The formazan crystals were dissolved in 100 μl acid isopropanol and the optical density of the wells, which is linearly correlated with the number of cells, was measured using a microplate reader at 540 nm. Cell survival was calculated as the optical density of the treated well divided by the mean optical density of control wells ×100%. IC_{50} represents the drug concentration lethal to 50% of the cells [11], and was estimated by an iterative nonlinear least-squares method using the MULTI program [17].

Table 1 IC_{50} values (μ*M*) of THP and DOX in M5076 and Ehrlich cells. The IC_{50} values were determined by means of the MTT assay after the cells (0.5×10^5/ml) had been treated with or without THP or DOX at concentrations in the range 0.0002–20 μg/ml for 3 days

Drug	M5076 cells	Ehrlich cells	M5076/Ehrlich ratio
THP	0.366	0.078	4.69
DOX	1.300	0.095	13.7
THP/DOX ratio	0.28	0.82	–

Uptake experiments

The experiments were performed using the method of Nagasawa et al. [4, 5, 6, 7, 8].

Assay procedure

The THP and DOX concentrations in M5076 and Ehrlich cells were determined by HPLC following the method of Nagasawa et al. [9].

Western blot analysis

Proteins from M5076 and Ehrlich cells dissolved in Laemmli sample buffer [3], were subjected to 4–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and blotted onto an Immobilon membrane (Millipore, Bedford, Mass.). The Immobilon membrane was incubated with phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 5% dehydrated skim milk (Difco Laboratories, Detroit, Mich.) to block nonspecific protein binding. The Immobilon membrane was incubated with monoclonal antibody to topoisomerase II (TopoGEN) diluted 1:1000. Subsequently, membrane-bound horseradish peroxidase-labeled antibodies were detected using the enhanced chemiluminescence detection system (ECL kit, Amersham). Protein bands reacting with the antibodies were detected on radiographic film (X-Omat JB-1, Kodak) 5 min after exposure, and the integrated optical densities of the 170-kDa protein band recognized by the anti-topoisomerase II antibody were determined.

Statistical analysis

The data are expressed as means ± SE. Comparisons between two or more groups were performed by means of Student's unpaired *t*-test or analysis of variance (ANOVA), followed by Scheffe's test or Bonferroni-Dunn (control) test, respectively. Differences with a *P*-value of 0.05 or less were considered statistically significant.

Results

Cytotoxicity of THP and DOX

Table 1 shows the IC_{50} values of THP and DOX for M5076 and Ehrlich cells determined with the MTT assay. The IC_{50} values for these drugs in M5076 cells were higher than in Ehrlich cells, and in both cell types, the values for THP were lower than for DOX.

Kinetics and temperature dependence of THP and DOX uptake

To clarify the correlation between their cytotoxicity and intracellular levels, the amounts of THP and DOX taken up by M5076 and Ehrlich cells were measured (Fig. 1A, B). Uptake of THP by both cell types was significantly greater than the uptake of DOX, and the uptake of both drugs by M5076 and Ehrlich cells reached equilibrium at 5–10 min. The uptake of THP and DOX by both cell types at 0°C was significantly lower than that at 37°C. Using the cell volumes and intracellular amounts of a drug, the cell to medium drug concentration (C/M) ratios were calculated (Fig. 1C, D). There were no

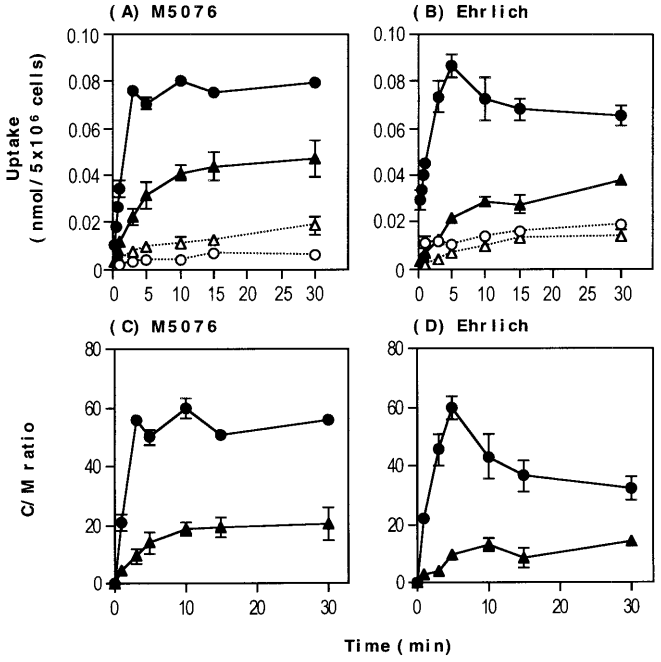

Fig. 1A–D Time courses of THP and DOX uptake (**A**, **B**) and the C/M ratios (**C**, **D**) in M5076 and Ehrlich cells at 0°C and 37°C. Cells were preincubated for 10 min, and then incubated with 0.3 μ*M* THP (*circles*) or DOX (*triangles*) for the indicated times at 0°C (*open symbols*) and 37°C (*closed symbols*). The C/M ratios were calculated by subtracting the uptake at 0°C from that at 37°C and using the mean cellular volume. Each point represents the mean ± SE from three to six independent experiments

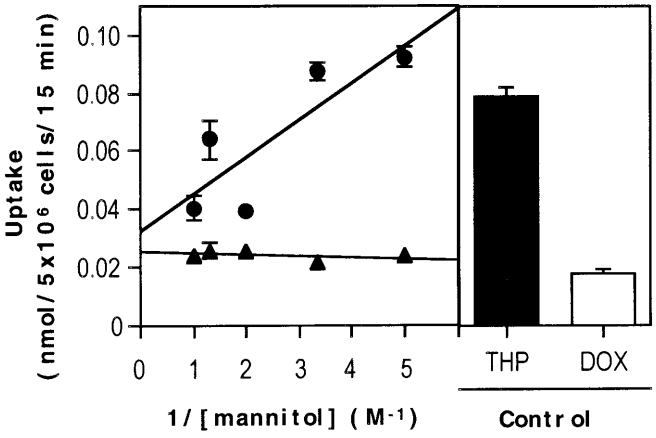

Fig. 2 Effect of osmolarity on THP and DOX uptake by M5076 cells. Cells were preincubated at the indicated osmolarity for 10 min, and then incubated with 0.3 μ*M* THP (*circles*) or DOX (*triangles*) for 15 min at 37°C. Osmolarity is expressed as the reciprocal of the mannitol molar concentration. Each point represents the mean ± SE from three independent experiments

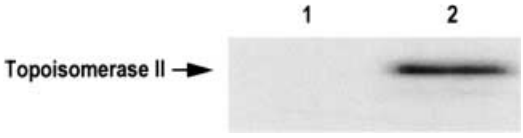

Fig. 3 Western blot analysis of topoisomerase II in M5076 and Ehrlich cells. Aliquots of whole cell extracts containing 50 μg protein were prepared from each cell type, electrophoresed on a 4–20% SDS-PAGE, and then transferred onto an Immobilon membrane. The membrane was incubated with a monoclonal antibody against topoisomerase II followed by HRP-labeled anti-mouse IgG (*lane 1* M5076 cells, *lane 2* Ehrlich cells)

apparent differences in the C/M ratios between M5076 and Ehrlich cells for either THP or DOX.

Effect of osmolarity on THP and DOX uptake

The osmotic sensitivity of THP and DOX uptake by M5076 cells was investigated (Fig. 2). The uptake of THP by M5076 cells decreased linearly with increasing extracellular osmolarity. In contrast, the uptake of DOX by M5076 cells did not change with increasing concentrations of mannitol.

Expression level of topoisomerase II in M5076 and Ehrlich cells

The expression levels of topoisomerase II in M5076 and Ehrlich cells are shown in Fig. 3. The protein band for topoisomerase II was not detectable in M5076 cells but was detectable in Ehrlich cells.

Kinetics and temperature dependence of THP and DOX efflux

Figure 4 shows the kinetics for THP and DOX efflux from M5076 and Ehrlich cells. THP and DOX were released rapidly from both cell types. The intracellular levels remaining did not differ between the drugs and cell types. The percentages of THP and DOX remaining in M5076 and Ehrlich cells at 0°C were significantly greater than at 37°C, and their efflux from both cell types was negligible.

Effects of metabolic inhibitors on THP and DOX efflux and influx

The effects of metabolic inhibitors on the efflux and uptake of THP and DOX in M5076 and Ehrlich cells are shown in Table 2. On treatment of cells with 10 m*M* AZ plus 10 m*M* DOG or 4 m*M* DNP in glucose-free HBSS, THP and DOX efflux from both cell types was completely inhibited. On the other hand, the rates of uptake of THP and DOX by both cell types significantly increased following pretreatment of the cells with 10 m*M* AZ plus 10 m*M* DOG. Based upon these results, the following experiments were performed using M5076 and Ehrlich cells pretreated with 10 m*M* AZ plus 10 m*M* DOG.

Concentration dependence of THP and DOX uptake

To examine the kinetics of THP and DOX uptake by M5076 and Ehrlich cells, the initial rates of uptake of THP and DOX were measured over the concentration range 1–200 μ*M* at 0°C and 37°C (Fig. 5). The initial rates of uptake of THP and DOX by M5076 and Ehrlich cells at 0°C exhibited a linear increase over the concentration range examined. In contrast, at 37°C, the initial uptake rates in all groups tended to become saturated as the drug concentration was increased. The initial rate of uptake of a saturable component showed apparent saturable concentration dependency.

Table 3 shows the kinetic parameters for THP and DOX uptake by M5076 and Ehrlich cells, which were estimated using the MULTI program [17]. The Michaelis constant (Km) and maximum uptake velocity (Vmax) for THP uptake by M5076 cells were three- and sixfold greater, respectively, than those for DOX uptake. In Ehrlich cells, there was no difference in the Km value between THP and DOX uptake, but the Vmax value for THP uptake was significantly greater than that for DOX. The Vmax/Km value (uptake clearance) for THP uptake was greater than that for DOX uptake in both cell types. The nonsaturable transport rate constants (Kd) for THP and DOX uptake by Ehrlich cells were higher than for uptake by M5076 cells.

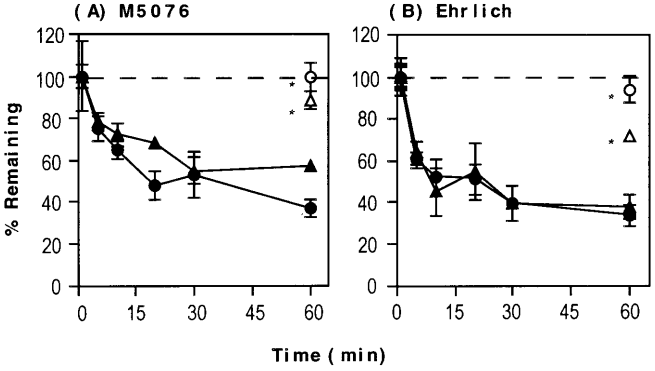

Fig. 4A, B Time courses of THP and DOX efflux from M5076 (**A**) and Ehrlich (**B**) cells at 0°C and 37°C. After the cells had been loaded with 0.3 μ*M* THP (*circles*) or DOX (*triangles*) for 15 min at 37°C, they were incubated in drug-free HEPES-HBSS for the indicated times at 0°C (*open symbols*) and 37°C (*closed symbols*). Each point represents the mean ± SE from three independent experiments. **P* < 0.01 vs the respective drug at the corresponding time point at 37°C

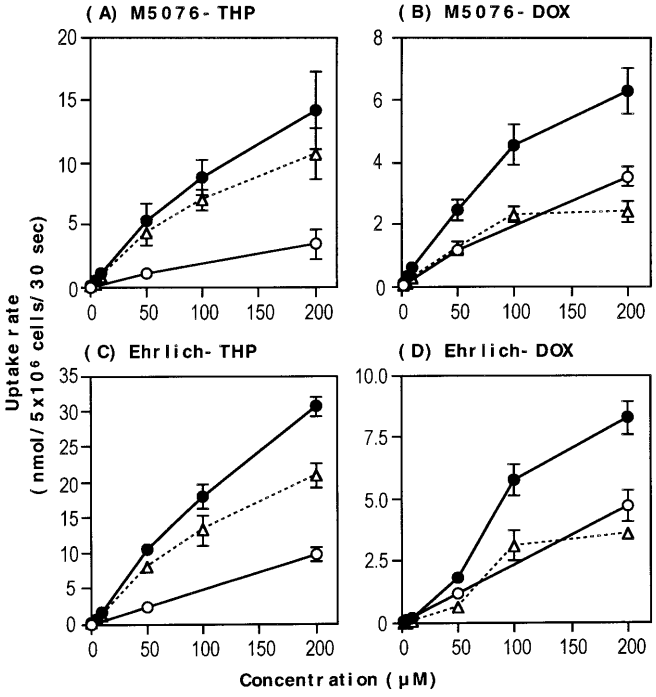

Fig. 5A–D Concentration dependence of THP and DOX uptake by M5076 and Ehrlich cells. Cells were preincubated with 10 m*M* AZ plus 10 m*M* DOG in glucose-free HEPES-HBSS for 20 min, and then incubated with the indicated concentrations of THP or DOX for 30 s at 37°C (*closed circles*) or 0°C (*open circles*). Each point represents the mean ± SE from three or four independent experiments. The saturable component (*open triangles*) was calculated by subtracting the rate of uptake at 0°C from that at 37°C

Table 2 Effects of metabolic inhibitors on efflux and uptake. Efflux experiments: after the cells had been loaded with 0.3 μ*M* THP or DOX for 15 min, they were incubated in drug-free medium with or without 10 m*M* AZ plus 10 m*M* DOG or 4 m*M* DNP for 60 min at 37°C. Uptake experiments: cells were preincubated with or without 10 m*M* AZ plus 10 m*M* DOG for 20 min, and then incubated with 0.3 μ*M* THP or DOX for 30 s at 37°C. Each value represents the mean ± SE from three to six independent experiments

	Treatment	M5076 cells		Ehrlich cells	
		THP	DOX	THP	DOX
Drug remaining at 60 min (%)	Control	61.2 ± 2.3	66.3 ± 4.4	42.0 ± 4.7	42.9 ± 4.0
	10 m*M* AZ + 10 m*M* DOG	107.6 ± 7.0**	104.5 ± 3.0**	95.9 ± 0.7**	104.3 ± 0.6**
	4 m*M* DNP	105.8 ± 3.7*	80.3 ± 1.1**	72.4 ± 3.6**	104.5 ± 3.9*
Uptake rate (nmol/5×106 cells/30 s)	Control	0.0196 ± 0.0013	0.0099 ± 0.0009	0.0224 ± 0.0033	0.0062 ± 0.0005
	10 m*M* AZ + 10 m*M* DOG	0.0391 ± 0.0049**	0.0158 ± 0.0013**	0.0492 ± 0.0045**	0.0084 ± 0.0003*

**P* < 0.05,
***P* < 0.01, vs respective control

Table 3 Apparent kinetic constants for THP and DOX uptake in M5076 and Ehrlich cells. The kinetic constants were estimated by an iterative nonlinear least-squares method using the data shown in Figs. 5 and 6. Each value represents the mean ± SE from three or four independent experiments

Parameter	M5076 cells[a]		Ehrlich cells[b]	
	THP	DOX	THP	DOX
C/M ratio at 30 s[c]	36.9	13.7	34.5	5.0
Km (μ*M*)	198.5 ± 23.8	62.3 ± 7.2*	288.0 ± 36.2**	293.5 ± 54.0**
Vmax (nmol/5×10^6 cells/30 s)	21.99 ± 4.51	3.99 ± 0.59	50.04 ± 1.73*,**	9.55 ± 1.06***
Vmax/Km (ml/5×10^6 cells/30 s)	0.112 ± 0.017	0.070 ± 0.018	0.180 ± 0.026**	0.034 ± 0.004***
Kd (ml/5×10^6 cells/30 s)	0.0167 ± 0.0061	0.0174 ± 0.0015	0.0496 ± 0.0049*,**	0.0234 ± 0.0030***
Carrier-mediated uptake (%)	87.7 ± 3.0	78.2 ± 2.8	77.7 ± 4.0	58.9 ± 5.4
Ki for THP uptake (μ*M*)	–	116.2 ± 29.5	–	–
Vmax (nmol/mm^3 cell volume/30 s)	5.39 ± 1.11	0.98 ± 0.14*	8.75 ± 0.30*,**	1.67 ± 0.19*,***
Vmax/Km (ml/mm^3 cell volume/30 s)	0.0273 ± 0.0042	0.0172 ± 0.0046	0.0315 ± 0.0045	0.0059 ± 0.0007*,***

*$P < 0.05$, vs THP uptake by M5076 cells
**$P < 0.05$, vs DOX uptake by M5076 cells
***$P < 0.05$, vs THP uptake by Ehrlich cells
[a]Cell volume 817 μm^3
[b]Cell volume 1142 μm^3
[c]The C/M ratio at 30 s was calculated using the data shown in Table 2

Effect of DOX on THP uptake

To examine the *cis*-inhibitory effect of DOX on THP uptake by M5076 and Ehrlich cells, the cellular uptake of THP as a substrate was studied after THP and DOX, as an inhibitor, had reacted with the cells simultaneously (Fig. 6). The uptake of THP by M5076 cells was competitively inhibited by DOX. On the other hand, in Ehrlich cells, the addition of DOX caused no or little inhibition of THP uptake. As shown in Table 3, the apparent inhibition constant (Ki) of DOX for the inhibition of THP uptake by M5076 cells was 116 μ*M*, and this value was comparable to the Km value for DOX uptake by M5076 cells (62 μ*M*).

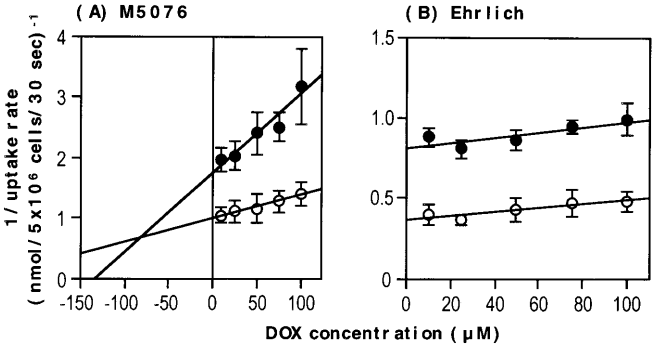

Fig. 6A, B Dixon plots of THP uptake showing inhibition by DOX in M5076 (**A**) and Ehrlich (**B**) cells. Cells were preincubated with 10 m*M* AZ plus 10 m*M* DOG in glucose-free HEPES-HBSS for 20 min, and then incubated with 5 μ*M* (*closed symbols*) or 10 μ*M* (*open symbols*) of substrate (THP) and the indicated concentration of inhibitor (DOX) for 30 s at 37°C. Each point represents the mean ± SE from three independent experiments

Figure 7 shows the *trans*-stimulatory effect of DOX on THP uptake by M5076 and Ehrlich cells. The uptake rate of THP in M5076 cells significantly increased with increasing amounts of preloaded DOX. On the other hand, DOX preloading had no effect on the rate of uptake of THP in Ehrlich cells.

Discussion

In the in vitro cytotoxicity experiments, THP exhibited greater cytotoxicity than DOX in Ehrlich cells and

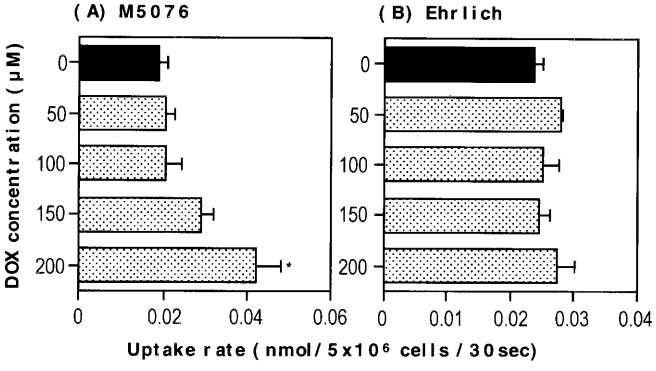

Fig. 7A, B Effect of preloaded DOX on THP uptake by M5076 (**A**) and Ehrlich (**B**) cells. Cells were loaded with the indicated concentrations of DOX in glucose-free HEPES-HBSS containing 10 m*M* AZ plus 10 m*M* DOG for 15 min, and then washed once with PBS at 37°C. The cells were then suspended in glucose-free HEPES-HBSS containing 0.3 μ*M* THP and 10 m*M* AZ plus 10 m*M* DOG at 37°C, and then incubated for 30 s at 37°C. Each bar represents the mean ± SE from three to six independent experiments. *$P < 0.001$ vs the value for 0 μ*M* DOX

particularly in M5076 cells (Table 1). The cytotoxicities of THP and DOX in M5076 cells were less than in Ehrlich cells (Table 1). These results are consistent with in vivo results reported previously [12, 13, 14], confirming that in vitro cytotoxicity experiments predict well the in vivo cytotoxicity of these drugs. Thus, in order to clarify the difference in cytotoxicity among drugs and cell types, we examined the transport characteristics, which are a major factor determining cellular incorporation.

The intracellular accumulation and the C/M ratio of THP in both cell types were significantly greater than those of DOX, while the C/M ratios of THP and DOX in M5076 cells were approximately the same as in Ehrlich cells (Fig. 1). These results could explain the difference in cytotoxicity between THP and DOX in the two cell types. Furthermore, the findings of the osmolarity experiment indicate that DOX was not taken up into the intracellular free space, except for intracellular binding to organelles, in M5076 cells, in contrast to THP (Fig. 2). For these reasons, the cytotoxicity of DOX is thought to be less than that of THP in M5076 cells. However, the cellular accumulation of THP and DOX did not agree with the difference in cytotoxicity of THP and DOX between M5076 and Ehrlich cells, indicating that the difference in cytotoxicity between the two cell types could not be fully explained only by the amount of cellular accumulation of the drug. We considered that this may have been due to differences in the expression levels of topoisomerase II between the two cell types. We therefore performed Western blot analysis of topoisomerase II in M5076 and Ehrlich cells. The expression level of topoisomerase II in M5076 cells was much lower than in Ehrlich cells (Fig. 3). Because the level of topoisomerase II is generally decreased in anthracycline-resistant cells [2], the difference in cytotoxicity between the cell types may have been the result, at least in part, of the low level of topoisomerase II in M5076 cells.

Based upon the data obtained in this study, it was thought that in the same cells, the intracellular drug concentration was a more important factor for cytotoxicity than the level of expression of topoisomerase II. This was because a greater amount of THP was incorporated into M5076 cells, in which topoisomerase II could not be detected, than DOX, and THP showed a higher cytotoxicity than DOX. A similar tendency was also observed in Ehrlich cells in which topoisomerase II was detectable. Thus, we examined the transport characteristics of THP and DOX in M5076 and Ehrlich cells.

The intracellular amount of a drug is generally known to be determined by its membrane transport, that is efflux and influx. We therefore first examined the efflux of THP and DOX from M5076 and Ehrlich cells. THP and DOX appeared to be actively pumped out of the cells (Fig. 4 and Table 2). THP and DOX showed similar efflux patterns in the two cell types (Fig. 3), indicating that the differences in the intracellular amounts of THP and DOX in M5076 and Ehrlich cells are not due to the differences in the efflux rate.

Next, we examined the characteristics of THP and DOX influx by the two cell types. As shown in Table 2, the rates of uptake of THP and DOX significantly increased on pretreatment of the M5076 and Ehrlich cells with 10 m*M* AZ plus 10 m*M* DOG. This finding was considered to be due to inhibition of active drug efflux in both cell types, and suggested that the uptake of THP and DOX by the two cell types was partially energy-independent. Thus, we performed uptake experiments involving suppression of the efflux system by pretreatment of M5076 and Ehrlich cells with 10 m*M* AZ plus 10 m*M* DOG in glucose-free medium. The uptake of THP and DOX was concentration-dependent and showed saturation kinetics (Fig. 5 and Table 3), indicating the contribution of one or more carrier-mediated systems.

To confirm the involvement of a carrier in the uptake of THP and DOX by M5076 and Ehrlich cells more clearly, we performed *cis*-inhibition and *trans*-stimulation experiments. The uptake of THP by M5076 cells appeared to be inhibited competitively following the addition of DOX with an apparent Ki value of 116 μ*M*, which was comparable to the apparent Km value for DOX uptake by M5076 cells (62 μ*M*) (Fig. 6 and Table 3), and to be stimulated following preloading of M5076 cells with DOX (Fig. 7), indicating that THP and DOX are taken up into M5076 cells via the same carrier system(s). In Ehrlich cells, on the other hand, DOX resulted in no or little inhibition of THP uptake (Fig. 6), and did not stimulate THP uptake (Fig. 7). These results suggest that the uptake of THP and DOX is mediated by different systems in Ehrlich cells.

Finally, we compared the kinetic constants for THP and DOX uptake in the two cell types. It appeared that the carrier-mediated uptake of THP and DOX by M5076 cells was low-affinity high-capacity, and high-affinity low-capacity, respectively, while that of THP and DOX by Ehrlich cells was low-affinity high-capacity, and low-affinity low-capacity, respectively. The uptake clearance (Vmax/Km) of THP was greater than that of DOX in both M5076 and Ehrlich cells, and the contribution of carrier-mediated uptake to total cellular uptake was estimated to be 87.7% for THP by M5076 cells, 78.2% for DOX by M5076 cells, 77.7% for THP by Ehrlich cells, and 58.9% for DOX by Ehrlich cells. Therefore, it appears that the cellular uptake of THP and DOX in both types of cell was determined primarily by the uptake clearance of the carrier, which was dominated by the affinity and capacity in M5076 cells, and by the capacity in Ehrlich cells of the two drugs. Thus, the difference in cytotoxicity toward the two cell types between THP and DOX was suggested to be dependent, at least in part, on the uptake efficiency of the carrier(s).

We also compared the kinetic constants between the cell types. Since cell volume is generally known to affect the uptake capacity, we used the Vmax value corrected by the respective cell volume in this comparison. The ratios of the Vmax/Km in M5076 cells of THP and DOX to those in Ehrlich cells, which were calculated to

be 0.9 and 2.9, respectively, were comparable to the ratios of the C/M ratio at 30 s of THP and DOX uptake in M5076 cells to those in Ehrlich cells, which were 1.1 and 2.7, respectively. Therefore, the difference in incorporation between the two cell types was also indicated to be dependent on the uptake efficiency of the carrier.

In conclusion, our findings indicate that: (1) the in vitro cytotoxicity of THP was greater than that of DOX in M5076 and Ehrlich cells, and that the cytotoxicities of the two drugs against M5076 cells were less than against Ehrlich cells, and this was partially explained by the much lower expression levels of topoisomerase II in M5076 cells than in Ehrlich cells; (2) the resistance of M5076 cells to DOX might be due to an extremely low intracellular free drug concentration compared with THP; and (3) the cytotoxicities of THP and DOX depend, at least in part, on the uptake efficiency of the carrier in both cell types.

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