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Transport mechanism of anthracycline derivatives in human leukemia cell lines: uptake and efflux of pirarubicin in HL60 and pirarubicin-resistant HL60 cells

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Abstract We studied the transport mechanism of pirarubicin (THP) in HL60 and its THP-resistant (HL60/THP) cells, which showed no expression of *mdr*1 mRNA on Northern blot analysis. Under physiological conditions, the uptake of THP by both types of cell was time- and temperature-dependent. The amount of drug transport in the resistant cells was significantly less than that in the parent cells within 3 min of incubation. THP uptake was significantly higher in the presence than in the absence of 4 m*M* 2,4-dinitrophenol (DNP) in glucose-free Hanks' balanced salt solution in both HL60 and HL60/THP cells and the increases were approximately equal. In the presence of DNP, the uptake of THP by both types of cell was concentration-dependent, and there were no significant differences in the apparent kinetic constants (Michaelis constant (K_m), maximum velocity (V_{max}) and V_{max}/K_m) for THP uptake between HL60 and HL60/THP cells. Additionally, THP transport was competitively inhibited by its analogue doxorubicin. The efflux of THP from HL60/THP cells was significantly greater than that from HL60 cells, and the release from both types of cell was completely inhibited by decreasing the incubation temperature to 0°C and by treatment with DNP in glucose-free medium. In contrast, the P-glycoprotein inhibitors verapamil and cyclosporin A did not inhibit THP efflux. However, genistein, which is a specific inhibitor of multidrug resistance-associated protein (MRP), increased the THP remaining in the resistant cells, and the value was approximately equal to that of the control group in the sensitive cells. These results suggest that THP is taken up into HL60 and HL60/THP cells via a common carrier by facilitated diffusion, and then pumped out in an energy-dependent manner. Furthermore, the accelerated efflux of THP by a specific mechanism, probably involving MRP, other than the expression of P-glycoprotein, resulted in decreased drug accumulation in the resistant cells, and was responsible, at least in part, for the development of resistance in HL60/THP cells.

Keywords Pirarubicin · HL60 cell lines · Transport

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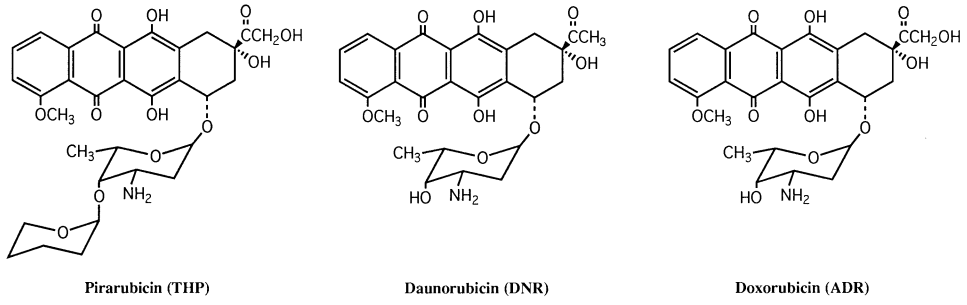
Introduction

Anthracycline antibiotics are very potent broad-spectrum antitumor agents [2]. However, these drugs induce dose-dependent cardiac myopathy, alopecia, etc. which are dose-limiting factors in their clinical use [1, 20]. To preclude these adverse effects, analogue compounds and pharmaceutically modified drugs have been developed [7, 10, 32].

Different transport mechanisms in normal cells and in tumor cells, should enable drugs to be delivered selectively into tumor cells. Therefore, we examined the transport mechanisms of anthracycline derivatives using rat polymorphonuclear leukocytes (PMN) as normal cells and cultured leukemia cells as tumor cells to determine a strategy to reduce the incidence and intensity of side effects of these agents in patients with leukemia. We have reported previously that a common carrier-mediated transport system seems to be involved in the uptake of pirarubicin (THP), daunorubicin (DNR) and doxorubicin (ADR) by PMN [23, 24]. The chemical structures of these drugs are illustrated in Fig. 1.

In clinical cancer chemotherapy, however, the development of multidrug resistance (MDR) to antitumor agents such as anthracyclines is a severe problem.

Fig. 1 Chemical structures of anthracycline derivatives



Recently, there have been many studies on the mechanism of MDR using cultured cell lines ([3, 18]; references therein), and these have indicated the involvement of various physiological changes in MDR. P-glycoprotein, which is an energy-dependent multidrug transporter responsible for resistance, has been studied intensively, and the DNA sequence of its gene and physiological role have been determined [12]. Thus, there have been many reports concerning drug efflux from tumor cells ([3] and references therein), but few detailed studies of the uptake mechanism.

In this study, we examined the mechanism of THP transport in HL60 cells, a human leukemia cell line, and compared it with that in THP-resistant (HL60/THP) cells.

Materials and Methods

Chemicals

Pure THP, tetrahydropyranyldoxorubicinol (THP-OH) and DNR (Meiji Seika Kaisha, Tokyo, Japan), ADR (Kyowa Hakko Kogyo Co., Tokyo, Japan), vincristine (VCR, Sionogi Pharmaceutical Ind. Co., Osaka, Japan), and etoposide (VP-16; Nippon Kayaku Co., Tokyo, Japan) were used. Cyclosporin A was provided by Sandoz Ltd. (Tokyo, Japan). All other reagents were obtained commercially or were of analytical grade requiring no further purification. Deionized double-distilled water was used throughout the experiments.

Cell lines and culture conditions

HL60, K562 and K562/ADM cells were provided by Dr. R. C. Gallo (NIH, Bethesda, Md.), the Japanese Cancer Research Resources Bank (Tokyo, Japan) and Dr. T. Tsuruo (Tokyo University, Tokyo, Japan), respectively. K562/TPA (12-*O*-tetradecanoylphorbol 13-acetate) cells were established by the method of Takeda et al. [34]. HL60/THP cells were selected by exposure to increasing drug concentrations beginning at 2×10^{-8} *M*. The final resistant subline was established when cells were capable of surviving in 2×10^{-7} *M* THP. Table 1 shows the resistance factors to THP, DNR, ADR, VCR and VP-16 in HL60/THP cells. These cells were maintained in RPMI-1640 medium (GIBCO) containing 10% heat-inactivated fetal bovine serum, 10 μg/ml kanamycin sulfate and 25 m*M* HEPES in an atmosphere containing 5% CO_2. Exponentially growing HL60 and HL60/THP cells, which had a viability of 98% or more as determined by the Trypan blue exclusion test, were used in the experiments. We did not synchronize the cell cycle because it has been shown to have little effect on the uptake or release of anthracyclines

Table 1 IC_{50} values for various antineoplastic agents in HL60 and HL60/THP cells. The values were determined in the MTT assay. Each value represents the mean of duplicate assays

Drug	IC_{50} (μg/ml)		
	HL60	HL60/THP	Resistance factor
THP	0.086	0.406	4.7
DNR	0.028	0.095	3.4
ADR	0.146	0.492	3.4
VCR	0.0029	0.0035	1.2
VP-16	0.117	0.566	4.8

[37]. For the uptake and efflux experiments, Hanks' balanced salt solution (HBSS, pH 7.4) was used as the incubation medium.

Growth-inhibition assay

In vitro drug sensitivity was assessed by the tetrazolium dye (MTT) assay [26]. Briefly, 100 μl of cells was placed in the wells of 96-well microculture plates with six concentrations of each drug in duplicate. The drugs used and range of concentrations were THP, DNR and ADR at 0.002–2 μg/ml, VCR at 0.412–8.25 ng/ml, and VP-16 at 0.0294–0.589 μg/ml. 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 6 h under the same conditions. The tetrazolium salt MTT is reduced to a colored formazan by living but not by dead cells. The formazan crystals were dissolved with 100 μl acid isopropanol and the optical density of the wells, linearly correlated with the number of cells, was measured with 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.

RNA extraction and Northern blot analysis

RNA extraction and Northern blotting were performed as reported previously [25]. A human *mdr*1 clone was used as a probe (kindly provided by Dr. I. H. Pastan, NIH, Bethesda, Md.).

Uptake experiments

In uptake experiments, the reaction suspension of HL60 or HL60/THP cells was preincubated for 10 min with shaking (160 strokes/min) at a designated temperature (0 or 37°C) in a sterilized plastic tube, except in those experiments using inhibitors in which

the reaction suspension was preincubated without and then with the inhibitor for 10 min and for 20 min, respectively. A metabolic inhibitor, 2,4-dinitrophenol (DNP), was always used in glucose-free HBSS. The reaction was initiated by the addition of THP solution to the incubation medium and the final concentration in the medium was 0.3 μM unless otherwise stated. At appropriate time intervals (0.25, 0.5, 0.75, 1, 3, 5, 10, 15, 30 and 60 min), the reaction was terminated by the addition of ice-cold phosphate buffered saline (PBS), and the suspension was then centrifuged at 14,000 rpm for 30 s at room temperature. The supernatant was removed by aspiration, and the cell pellet was first washed with ice-cold PBS and then resuspended in 1 ml water. Samples were frozen at $-80°C$ until assay. In *cis*-inhibition experiments, THP (1 or 5 μM) and ADR as an inhibitor (10, 25, 50 or 75 μM) were added simultaneously to the reaction suspension, and the uptake of THP was determined after incubation for 1 min.

Efflux experiments

After preloading the reaction suspension of HL60 or HL60/THP cells with 0.3 μM THP for 10 min with or without the inhibitors, cells were washed twice with ice-cold HBSS. They were then incubated at 0 or 37°C for an appropriate time (0, 10, 20 or 60 min) in fresh medium with or without inhibitors. Thereafter, samples were treated in the same manner as in the uptake experiments.

Assay procedure

The THP concentrations in HL60 and HL60/THP cells were determined by high-performance liquid chromatography (HPLC) as reported previously [21, 22]. Briefly, to each 1-ml sample, 3 ml 0.1 M ammonia-ammonium chloride buffer (pH 9.5), 100 µl 0.16 μM THP-OH solution in methanol as an internal standard, and 13.5 ml of a chloroform/methanol (2:1, v/v) mixture were added and then shaken vigorously for 1 min. After centrifugation at 3000 g for 10 min, the organic layer was collected and evaporated to dryness at 30°C under a stream of nitrogen gas. The residue was dissolved in 0.3 ml of HPLC mobile phase. The HPLC system consisted of an LC-9A system (Shimadzu, Kyoto, Japan) equipped with a fluorescence detector (RF-535; Shimadzu). The analytical conditions were as follows: the column was a STR-ODS II (5 µm, 250 mm × 4 mm i.d.; Shimadzu) with a mobile phase of acetonitrile/0.2 M acetic acid-ammonium formate buffer, pH 4.0 (30:70, v/v) at a flow rate of 0.7 ml/min. Fluorescence was measured at 470 nm (excitation) and 550 nm (emission). The retention times under these conditions were 7.4, 10.9 and 21.8 min for ADR, THP-OH and THP, respectively. No interference peaks due to endogenous substances were observed.

Statistical analysis

The data are expressed as means ± SE. Comparisons between two groups and among three or more groups were performed by Student's t-test and ANOVA, respectively, and differences with a P-value of 0.05 or less were considered significant.

Results

General characteristics of cells

Table 1 shows the IC_{50} values for the anthracycline derivatives, VCR and VP-16 in HL60 and HL60/THP cells determined by the MTT assay. The IC_{50} values for these drugs in the resistant cells were higher than those in the sensitive cells. The resistance factors for THP, DNR, ADR, VCR and VP-16 in HL60/THP cells were 4.7, 3.4, 3.4, 1.2 and 4.8, respectively. To clarify the expression of P-glycoprotein, its mRNA (*mdr*1) in HL60/THP cells was analyzed by Northern blotting (Fig. 2). There was no band (28S) corresponding to *mdr*1 mRNA as observed in the positive control K562/ADM cells, in HL60/THP cells and in HL60, K562 or K562/TPA cells as a negative control.

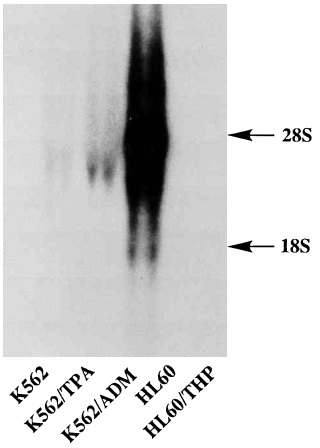


Fig. 2 Northern blot for *mdr*1 gene expression in various leukemia -cell lines. A 20 µ g sample of total RNA was loaded in each lane

Kinetics and effect of temperature on THP uptake under physiological conditions

The time courses of THP uptake by HL60 and HL60/THP cells were examined to determine the general transport characteristics. As shown in Fig. 3, the THP uptake (0.3 μM) in both types of cell at 37°C was extremely rapid and reached equilibrium in about 3–5 min. Thereafter, the accumulation of THP in these cells tended to decrease up to 60 min, but their 60-min values were not significantly different compared with the corresponding maximum values. The resistant cells showed significantly less THP uptake than the sensitive cells within 3 min of incubation, and the value at 60 min in HL60/THP cells was 13% lower than that in HL60 cells. To calculate the initial adsorption rate of THP to the cell membrane surface, the Y section (Y value) was obtained by extrapolation of the regression line to time zero (inset of Fig. 3). Consequently, the adsorption rates in HL60 and HL60/THP cells were 11% and 14% of each equilibrium value, respectively. To examine whether THP was metabolized in both types of cell, cell extracts were analyzed by HPLC. Neither ADR nor THP-OH were found even after 60 min of incubation in agreement with the results of previous studies on PMN [23, 24]. Figure 4 shows the

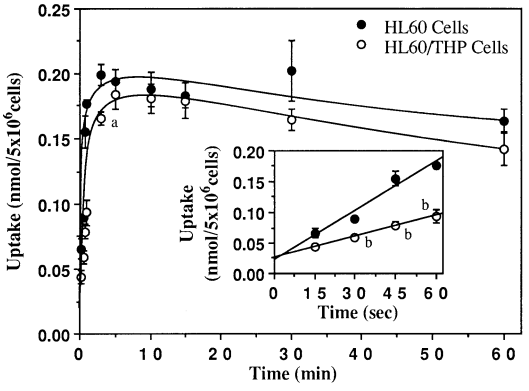


Fig. 3 Time courses of THP uptake by HL60 and HL60/THP cells under physiological conditions. Cells were incubated with 0.3 μ*M* THP for the indicated times at 37°C. Each point represents the mean ± SE of three experiments. The *inset* shows linear regression of the initial four time points (up to 1 min). *a* and *b* $P < 0.05$ and 0.01, respectively, with respect to the corresponding value for HL60 cells

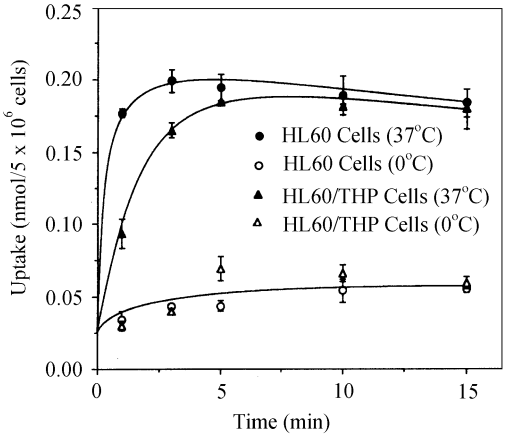


Fig. 4 Effect of temperature on THP uptake by HL60 and HL60/THP cells. Cells were incubated with 0.3 μ*M* THP for the indicated times at 0 or 37°C. Each point represents the mean ± SE of three experiments

effect of temperature on THP uptake by HL60 and HL60/THP cells. The uptake of THP by both types of cell at 0°C was significantly lower than that at 37°C, and there was a clear temperature dependency in both cell types. There was also no significant difference between the accumulation in the parent and resistant cells at 0°C, and the amount of uptake was approximately equal to the *Y* value described above (Fig. 3, inset).

Kinetics and effect of temperature on THP efflux

Figure 5 shows the kinetics and effect of temperature on THP efflux from HL60 and HL60/THP cells. THP appeared to be released rapidly from both cell types, but efflux of THP from the resistant cells was significantly greater than that from the parent cells beyond

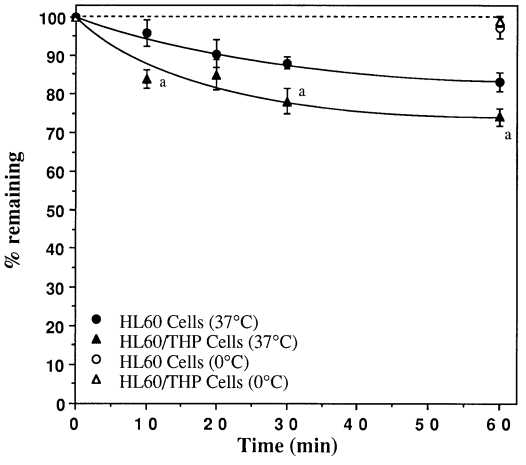


Fig. 5 Time courses of THP efflux from HL60 and HL60/THP cells at 0 and 37°C. After cells were loaded with 0.3 μ*M* THP for 10 min at 37°C, they were washed twice with ice-cold HBSS. The cells were then resuspended with an appropriate volume of HBSS cooled to 0°C or warmed to 37°C, and incubated for the indicated times at the designated temperature. Each point represents the mean ± SE of three to six experiments. *a*, $P < 0.05$ with respect to the corresponding value for HL60 cells

10 min after the start of the reaction. The percentages of THP remaining in HL60 and HL60/THP cells at 60 min were 83% and 74%, respectively, and the value for the resistant cells was significantly lower than that of the sensitive cells ($P < 0.05$). No efflux of THP from either cell type was observed at 0°C, indicating that there was a strong temperature dependency in the efflux process as well as the uptake process in both types of cell.

Effects of various compounds on THP efflux

Table 2 shows the effects of DNP, verapamil, cyclosporin A and genistein on the THP efflux from HL60 and HL60/THP cells. On treatment of the cells with 4 m*M* DNP in glucose-free HBSS, the percentage of THP retained in both cell types was significantly increased in comparison with the respective control group ($P < 0.01$). On the other hand, in the presence of 10 μ*M* verapamil or cyclosporin A, which was used only in the case of HL60/THP cells, the amounts of THP remaining in the sensitive and resistant cells were approximately equal to that in the respective control group (Table 2). However, the addition of 50 μ*M* genistein resulted in an increase in the THP remaining in HL60/THP cells, and the value was equal to those of the control and genistein groups in the sensitive cells (Table 2).

Effect of a metabolic inhibitor on THP uptake

The amount of THP uptake into HL60 and HL60/THP cells treated with 4 m*M* DNP in glucose-free

Table 2 Effects of various agents on THP efflux from HL60 and HL60/THP cells. Cells were loaded with 0.3 μ*M* THP in glucose-free HBSS with or without 4 m*M* DNP, or 10 μ*M* verapamil, cyclosporin A or 50 μ*M* genistein in HBSS for 10 min at 37°C. After washing the cells twice with the respective ice-cold medium they were incubted with or without the inhibitors in an appropriate medium for 60 min at 37°C. Each value represents the mean ± SE or three to six experiments. *$P < 0.01$ versus control group (*ND* not determined)

	THP remaining at 60 min (%)	
	HL60 cells	HL60/THP cells
Control	83.0 ± 2.3	74.0 ± 2.3
4 m*M* DNP in glucose-free HBSS	111.0 ± 3.6*	97.3 ± 3.8*
10 μ*M* verapamil	83.5 ± 5.7	74.0 ± 3.0
10 μ*M* cyclosporin A	N.D.	77.2 ± 4.9
50 μ*M* genistein	81.3 ± 1.5	81.7 ± 3.2

Table 3 Effect of metabolic inhibitor on THP uptake by HL60 and HL60/THP cells. Cells were pretreated with or without 4 m*M* DNP in glucose-free HBSS for 20 min, and then incubated with 0.3 μ*M* THP for 1 min at 37°C. Each value represents the mean ± SE of three experiments. *$P < 0.01$ versus control

	THP uptake (nmol/5 × 10^6 cells/min)	
	HL60 cells	HL60/THP cells
Control	0.171 ± 0.007	0.119 ± 0.007
4m*M* DNP in glucose-free HBSS	0.250 ± 0.015*	0.203 ± 0.010*

HBSS for 20 min was significantly higher than that in their corresponding control groups ($P < 0.01$, respectively; Table 3), with increases of about 46% and 71%, respectively. On the other hand, the amount of uptake by HL60/THP cells was approximately equal to that by HL60 cells in the groups treated with DNP in glucose-free HBSS (Table 3).

Concentration dependency of THP uptake

Figure 6 shows the relationships between the drug concentration and the initial uptake rate in HL60 and HL60/THP cells treated with 4 m*M* DNP in glucose-free HBSS. The uptake by each type of cell was a saturable concentration-dependent process, indicating that a carrier-mediated transport system was involved. Table 4 summarizes the kinetic constants estimated using a Lineweaver-Burk plot (Fig. 6, inset). The apparent Michaelis constant (K_m), maximum velocity (V_{max}) and V_{max}/K_m values in HL60 cells were 115.6 μ*M*, 78.1 nmol/5 × 10^6 cells per min and 0.676 ml/5 × 10^6 cells per min and in HL60/THP cells, 91.6 μ*M*, 55.9 nmol/5 × 10^6cells per min and 0.610 ml/5 × 10^6 cells per min respectively. These values were comparable between the two cell types and

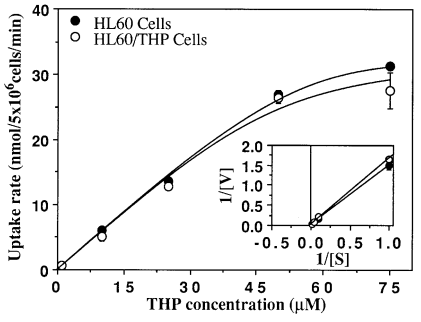


Fig. 6 Concentration dependency of THP uptake by HL60 and HL60/THP cells treated with 4 m*M* DNP in glucose-free HBSS. Cells were preincubated with 4 m*M* DNP in glucose-free HBSS for 20 min, and then incubated with the indicated concentrations of THP for 1 min at 37°C. The *inset* shows the Lineweaver-Burk plot. Each point represents the mean ± SE of three experiments.

Table 4 Apparent kinetic constants for THP uptake by HL60 and HL60/THP cells. Each kinetic constant was calculated on a Lineweaver-Burk plot as shown in Fig. 5. (*Km* Michaelis constant, *Vmax* maximum velocity)

Parameter	HL60 cells	HL60/THP cells
Km (μ*M*)	115.6	91.6
Vmax (nmol/5 × 10^6 cells/min)	78.1	55.9
Vmax/Km (ml/5 × 10^6 cells/min)	0.676	0.610

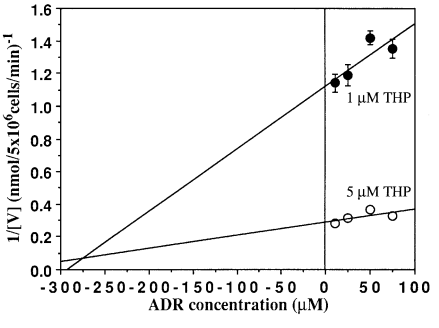


Fig. 7 Dixon plot of THP uptake showing inhibition by ADR in HL60 cells treated with 4 m*M* DNP in glucose-free HBSS. Cells were preincubated with 4 m*M* DNP in glucose-free HBSS for 20 min, and then incubated with the indicated concentrations of THP and ADR for 1 min at 37°C. Each point represents the mean ± SE of three experiments

there were no significant differences between the values for sensitive and resistant cells.

Effect of a THP analogue on THP uptake

The Dixon plot (Fig. 7) shows the effect of an analogue compound, ADR, on THP uptake by HL60 cells. The reciprocals of the THP uptake rate increased with increasing doses of ADR with either 1 or 5 μ*M* THP as the substrate. In addition, the *Y* coordinate at the intersection of the two extrapolated regression lines

was positive in this plot. The results obtained in HL60/THP cells were similar to those obtained in the HL60 cells (data not shown).

Discussion

In studies of MDR, the acceleration of drug efflux due to the overexpression of P-glycoprotein or other proteins on the plasma membrane, qualitative and/or quantitative changes in the target enzyme topoisomerase II, and increases in cellular levels of the drug metabolic enzymes glutathione-*S*-transferases have been shown in resistant cells [18]. However, the decisive factors in MDR are as yet unclear. In the elucidation of the mechanisms of MDR, the identification of the transport mechanisms and the intracellular distribution of antitumor agents are considered to be important [6, 8, 11, 14, 16, 27–31]. Therefore, we examined the characteristics of the uptake and efflux of THP in HL60 and HL60/THP cells.

The results of cytotoxicity experiments in HL60/THP cells indicated that they have an MDR phenotype because they expressed resistance against DNR, ADR, VCR and VP-16 as well as THP (Table 1). On Northern blot examination, no *mdr*1 mRNA, a band at 28S, was detected in the HL60/THP cells, although it was observed in K562/ADM cells as positive controls. Furthermore, there was no expression of P-glycoprotein in HL60/THP cells as indicated by the result of the Western blotting using the JSB-I P-glycoprotein-specific monoclonal antibody (data not shown). Therefore, expression of P-glycoprotein might not be involved in the mechanisms of the observed resistance in HL60/THP cells.

Under physiological conditions, the accumulation of THP in HL60/THP cells was significantly lower than that in HL60 cells within 3 min of incubation (Fig. 3), and the area under the intracellular concentration-time curve for the drug accumulation from 0 to 60 min (AUC) in the resistant cells was decreased by about 13% compared with that in the sensitive cells, although this was not statistically significant. The size (diameter) of HL60/THP cells was approximately equal to that of HL60 cells as observed under a light microscope. The volume of the parental and resistant cells as determined with a model ZBI Coulter counter equipped with a channelizer (Coulter Electronics, Hialean, Fl.) were 1266 μm^3 and 1324 μm^3, respectively. Therefore, cell volume appeared to be unrelated to the change in cellular THP accumulation. These results indicate that decreased accumulation of THP resulted, at least in part, in the observed resistance of these cells. On the other hand, the time- and temperature-dependent uptake of THP by both types of cell suggested the involvement of a carrier in these transport systems (Figs. 3 and 4).

To evaluate these transport mechanisms in more detail, we examined the THP efflux from HL60 and HL60/THP cells. There was a similarity in the efflux pattern between both cell types, and the efflux was time-dependent. In addition, the efflux of THP was completely inhibited by decreasing the reaction temperature to 0°C and by the addition of a metabolic inhibitor, DNP, in glucose-free HBSS (Fig. 5 and Table 2, respectively). Therefore, THP might be actively pumped out of the cells in an energy-dependent fashion. The efflux of THP from the resistant cells was significantly greater than that from the sensitive cells (Fig. 5), which suggests that the decreased accumulation of THP in HL60/THP cells described above was due to accelerated efflux. Furthermore, verapamil and cyclosporin A, specific inhibitors of P-glycoprotein [35, 38, 39], showed no inhibitory effects on the efflux (Table 2). These results indicate that P-glycoprotein does not contribute to the enhanced efflux of THP from HL60/THP cells, and this was supported by the results of the Northern blot analysis described above (Fig. 2). Human cells can change to MDR cells by an increase in the expression of the *mdr*1 P-glycoprotein or by other, as yet unknown mechanisms, referred to as non-P-glycoprotein-mediated MDR [4, 5, 19, 28, 33].

Recently, the overexpression of MDR-associated protein (MRP), a 190-kDa membrane ATP-binding glycoprotein, in a non-P-glycoprotein MDR phenotype has been noted, and MRP appears to be an energy-dependent multidrug efflux pump similar to P-glycoprotein [5, 15, 17, 19, 41]. It has been reported that the MDR isolate HL60/Adr of HL60 cells is defective in cellular drug accumulation, and contains a 190,000 Da ATP-binding protein, which is thought to be MRP, in the endoplasmic reticulum and plasma membrane, but no amplified *mdr*1 or *mdr*3 [15, 17, 19]. In our study, we used the same parent HL60 cell line and its isolate HL60/THP cells selected by THP, an analogue of ADR. The efflux of THP from HL60/THP cells was increased compared with that from HL60 cells, and this accelerated efflux was energy-dependent. Furthermore, HL60/THP cells expressed cross-resistance to DNR, ADR, VCR and VP-16, all of which are substrates of MRP. Thus, we examined the involvement of MRP in HL60/THP cells using its specific inhibitor genistein [40, 41]. Versantvoort et al. reported that another significant aspect of the action of genistein is its effect on cellular ATP levels, that is, it is decreased by genistein in a concentration-dependent manner [40]. They recommended that genistein should be used at a concentration of 200 μM which results in the complete inhibition of MRP function, but concentrations of 100 μM and 200 μM decrease the cellular ATP levels. Genistein at the concentration of 100 μM and 200 μM completely inhibited the THP efflux from HL60/THP cells as well as in the case following DNP treatment (data not shown). So we used it at 50 μM in this study, because it was thought that there would be

no decrease in cellular ATP levels at this concentration. As shown in Table 2, 50 μM genistein apparently increased the percentage of THP remaining in the resistant cells, and furthermore the value (81.7 ± 3.2%) was approximately equal to those of the control (83.0 ± 2.3%) and the genistein-treated HL60 cells (81.3 ± 1.5%). Therefore, it may be that the enhanced drug efflux in HL60/THP cells is due to overexpression of MRP in the plasma membrane. Detailed studies on MRP are currently in progress in our laboratory, which should clarify this.

Next, the mechanisms of THP uptake by both types of cell were evaluated by treatment with 4 m*M* DNP in glucose-free HBSS in a similar manner to the previous study by Inaba et al. [13]. As shown in Table 3, the amount of THP incorporated was significantly increased by the addition of DNP in both sensitive and resistant cells ($P < 0.05$), and their increased values were approximately equal. These were considered to be due to the inhibition of drug efflux in both types of cell (Table 3). Accordingly, the process of THP uptake by both types of cell was suggested to be energy-independent. Moreover, THP uptake was concentration-dependent and expressed clear saturation kinetics, indicating that it might involve carrier-mediated transport (Fig. 6). On the basis of these findings, we attempted to calculate the kinetic constants for THP uptake using a Lineweaver-Burk plot. Although we used an equation where two uptake processes were assumed in calculating the kinetic constants, the nonsaturable process was negligible. This was supported by the observation that the amounts of uptake at 0°C for 15 min by both types of cell were approximately equal to the *Y* values, as shown in Fig. 4. There were no differences in kinetic constants between HL60 and HL60/THP cells, suggesting that THP might be taken up by facilitated diffusion utilizing a common carrier. In addition, to identify the involvement of a carrier-mediated transport system, we performed *cis*-inhibition experiments using ADR, an analogue of THP. THP uptake by HL60 cells appeared to be inhibited competitively by the addition of ADR (Fig. 7), and this effect was also observed in HL60/THP cells (data not shown). These results suggest that HL60 and HL60/THP cells both contain a specific transport system for the uptake of THP, in agreement with findings obtained previously in other tumor cell lines [3, 9, 13, 36]. In our previous studies [23, 24], it appeared that THP is taken up by rat PMN via a carrier-mediated transport system requiring ATP as an energy source, and the kinetic constants for this uptake are as follows: $K_m = 13.4\ \mu M$, $V_{max} = 4.84\ \text{nmol}/5 \times 10^6$ cells per min and $V_{max}/K_m = 0.361\ \text{ml}/5 \times 10^6$ cells per min. Therefore, the development of a specific drug delivery system for cancer chemotherapy using these differences may be possible, as there was a marked difference in THP uptake systems between normal PMN and HL60 tumor cells. However, these differences might have been due to a difference in cell species, and therefore not conclusive.

In conclusion, it was indicated that THP was taken up into HL60 and HL60/THP cells by facilitated diffusion mediated by a common carrier and was pumped out in an energy-dependent fashion. Furthermore, the accelerated efflux of THP by a specific mechanism, probably involving MRP, other than the expression of P-glycoprotein in the resistant cells resulted in decreased drug accumulation, and was responsible, at least in part, for the development of resistance in these cells. However, this defect in drug accumulation (about 13% in AUC) cannot account fully for the resistance to THP (five-fold) in HL60/THP cells. Therefore, additional studies are in progress to clarify the expression of MRP, the intracellular distribution, particularly the uptake of THP by the nuclei of HL60 and HL60/THP cells and the mechanism of THP transport in normal human leukocytes.

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