CELLULAR UPTAKE AND EFFLUX AND CYTOSTATIC ACTIVITY OF 4′-*O*-TETRAHYDROPYRANYLADRIAMYCIN IN ADRIAMYCIN-SENSITIVE AND RESISTANT TUMOR CELL LINES

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Cellular uptake and cytostatic activity of 4′-*O*-tetrahydropyranyladriamycin (THP) in various sublines resistant to anthracycline antibiotics of mouse lymphoblastoma L5178Y, Chinese hamster ovary (CHO) and mouse leukemia P388 cells were studied. All the sublines resistant to adriamycin (ADR) showed slightly decreased uptake of THP as compared with each sensitive lines, but THP was still taken up much more quickly than ADR by each of the ADR-resistant cell lines. Efflux of both anthracycline glycosides from the ADR-resistant P388 cells was faster than that from the ADR-sensitive P388 cells. The percentage of THP retained at equilibrium was higher than that of ADR in both ADR-resistant and -sensitive P388 cells. Cytotoxicity of THP to ADR-resistant cell lines was considerably lower compared with that for each of the sensitive lines but THP inhibited growth of ADR-resistant tumor cells at a concentration about 10 times lower than that for ADR. Thus THP was taken up more quickly, effluxed more slowly than ADR from the ADR-resistant cells, and showed stronger cytostatic activity than ADR on the cells.

A screening study for compounds inhibiting L1210 or P388 leukemia has led us to the discovery of new anthracyclines[1,2] which show therapeutic effects against leukemia, lymphomas, ovarian tumors, *etc*. Among them aclacinomycin A (ACL) has been shown to have no genotoxic activity on microorganisms[3] and animals[4] unlike most anthracycline glycosides. More recently, we found 4′-*O*-tetrahydropyranyladriamycin (THP)[5], a semisynthetic anthracycline glycoside, which showed lower cardiac toxicity[6] without any loss of tumoricidal activity. THP showed a stronger therapeutic effect than adriamycin (ADR) against L1210 tumors[5]. Previously we reported that THP was taken up much more rapidly than ADR by mouse leukemia L1210 and lymphoma L5178Y cells[7].

Unfortunately long term treatment of neoplastic diseases by a drug often results in the selection of drug-resistant neoplastic cells. Therefore, drugs active against these resistant neoplasms are highly desirable. We studied cellular uptake and efflux and cytostatic activity of THP using various ADR-resistant cell lines.

Materials and Methods

Culture of L5178Y, P388 and Chinese Hamster Ovary (CHO) Cells

Parental, ADR-resistant and ACL-resistant sublines of mouse lymphoblastoma L5178Y cells[8,9] were supplied by Prof. N. TANAKA, Institute of Applied Microbiology, University of Tokyo, Tokyo. They were cultured in RPMI 1640 medium supplemented with 10% horse serum. ADR-resistant

(P388/ADR (Arthur D. Little)), and vincristine-resistant (P388/VCR (NSC-67574)) were obtained from Dr. M. SUFFNESS, National Cancer Institute, Bethesda. Sensitive and the corresponding ADR-resistant lines[10] were obtained from Dr. M. INABA, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo. They were maintained by serial ip transplantation in DBA mice. Ascitic cells 7 days after transplantation to CDF_1 mice were used for our experiments. They were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 10 μM 2-mercaptoethanol. Two mutant lines of chinese hamster ovary (CHO) cells, AUXB1 (parental auxotrophic mutant) and CH^RC5 (multi-drug resistant mutant made by colchicine treatment)[11] were supplied by Prof. V. LING, University of Toronto, Toronto. They were grown in monolayer culture in α-MEM medium supplemented with 10% fetal bovine serum.

Cell growth was determined by counting cell number using a Coulter counter.

Determination of THP and ADR Uptake

Drug uptake by L5178Y and P388 cells were determined as previously described[7]. CHO cells (about 6×10^5/35 mm dish) were incubated with 10 μg/ml of THP or ADR in 1 ml of RPMI 1640 medium without serum at 37°C. After removal of the medium, adherent cells on dishes were washed with cold phosphate buffered saline (PBS) without Ca^{++} and Mg^{++}, and harvested by treatment with 0.6 ml of trypsin - EDTA solution (Gibco Laboratories) at 37°C for 15 minutes. PBS (1 ml) was then added and an aliquot (0.8 ml) was extracted with TCA and the drug quantity was determined by the fluorometric procedure as described before[7].

Determination of Efflux from P388 Cells Previously Loaded by THP or ADR

P388 sensitive cells (P388/S, 4×10^6/ml) were preloaded by incubation for 10 minutes at 37°C with PBS containing 10 mM NaN_3 and 1 μg/ml of THP or 15 μg/ml of ADR. P388/ADR from Cancer Chemotherapy Center (4×10^6/ml) were preloaded by the same method except that drug concentration were 1.5 μg/ml for THP and 22.5 μg/ml for ADR. After centrifugation cells were suspended in fresh RPMI 1640 medium and incubated at 37°C. Time-dependent increase of drug concentration which had effluxed from the cells into the medium was determined by the fluorometric method.

Results

Uptake of THP and ADR by L5178Y Cells and Their Cytotoxicity

Uptake of THP by L5178Y parental cells in RPMI 1640 medium is much faster than the uptake of ADR[7]. As shown in Fig. 1, THP was again rapidly incorporated into the ADR-resistant L5178Y cells but the maximum quantity of THP taken up by ADR-resistant cells was slightly lower being

Fig. 1. Uptake of THP and ADR by parental and drug-resistant L5178Y cell lines.

Initial concentration of THP (solid line) and ADR (broken line): 10 μg/ml.

○ Parental, △ L5178Y/ADR, ▲ L5178Y/ACL.

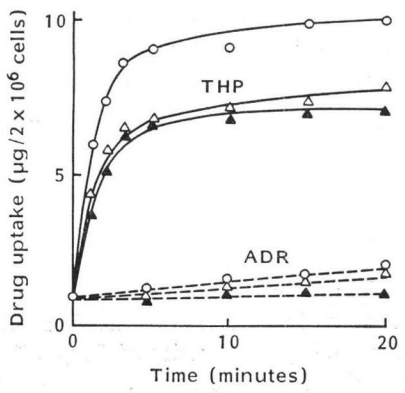


Table 1. Growth inhibition by ADR and THP against parental and resistant lines of L5178Y cells.

Cell lines	IC_{50} (μg/ml)	
	ADR	THP
Parental	0.045	0.015
ADR-resistant	1.5 (33)	0.12 (8)
ACL-resistant	2.3 (51)	0.15 (10)

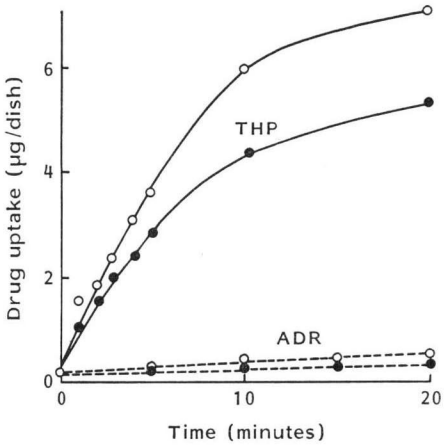
The values in brackets show the relative resistance expressed by the ratio of IC_{50} values for resistant to parental cell line.

Cells (5×10^4/ml) were incubated in RPMI 1640 medium containing 10% horse serum for 48 hours at 37°C.

Fig. 2. Uptake of THP and ADR by CHO cells.

CHO cells (10^5/2 ml) were cultured in 35×10 mm dishs. After 2 days average cell number was 6.08×10^5/dish for AUXB1 and 6.40×10^5/dish for CH^RC5. After removal of culture medium, cells were incubated with 1 ml of 10 μg/ml of THP or ADR in RPMI 1640 medium at 37°C.

○ AUXB1, ● CH^RC5.



7.8 μg/2×10^6 cells as compared with 9.9 μg/2×10^6 cells for parental cells. ADR was poorly incorporated into the parental cells and the uptake of ADR by the resistant cells was even slower. THP was also taken up much more rapidly than ADR by ACL-resistant L5178Y cells.

Table 2. Effect of ADR and THP on the growth of parental and colchicine-resistant mutant lines of CHO cells.

Cell lines	IC_{50} (μg/ml)	
	ADR	THP
AUXB1 (Parental)	0.03	0.003
CH^RC5	1.0 (33)	0.07 (23)

The values in brackets show the relative resistance expressed by the ratio of IC_{50} values for resistant to parental cell line. Cells (5×10^4/ml) were incubated in the α-MEM medium supplemented with 10% fetal bovine serum.

Fig. 3. Uptake of THP at a initial concentration of 1 μg/ml by sensitive and ADR-resistant P388 cell lines.

○ P388/S, ● P388/ADR (Cancer Chemotherapy Center).

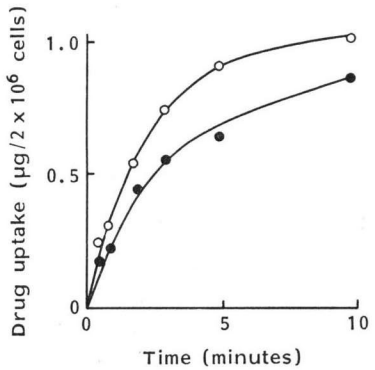
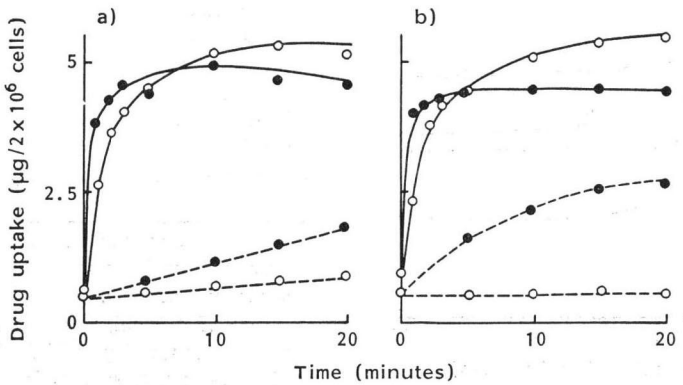


Table 1 shows 50% inhibition concentrations (IC_{50}) of ADR and THP against cell growth of the parental and two resistant lines of L5178Y. The IC_{50}'s of ADR for ADR- and ACL-resistant lines were 33 and 51 times higher, respectively, than that for parental cells, and those of THP were only 8

Fig. 4. Effect of sodium azide on uptake of THP and ADR by sensitive and ADR-resistant P388 cells.

a) P388/S and b) P388/ADR (Cancer Chemotherapy Center) were incubated with 10 μg/ml of THP (solid lines) or ADR (broken lines) in RPMI 1640 medium (○) or in PBS with 10 mM NaN_3 (●).



and 10 times higher, respectively. Therefore, the degree of resistance to THP was significantly lower than that to ADR.

Uptake of THP and ADR by CHO Cells and Their Cytotoxicity

THP was taken up by both the parental CHO cells (AUXBl) and its colchicine-resistant derivative (CH^RC5) as shown in Fig. 2, although the rate of uptake by the resistant cells was slower. On the other hand, the colchicine-resistant line which was reported to be multi-drug resistant[11,12] was resistant to both THP and ADR as shown in Table 2. But the degree of resistance to THP was slightly lower than that to ADR.

Uptake and Efflux of THP and ADR by P388 Cells and Their Cytotoxicity

Fig. 3 shows that THP was taken up by both ADR-sensitive and -resistant P388 cells. But the rate of influx was slower again by P388/ADR cells than by P388/S cells, when the cells were incubated in 1 μg/ml of THP. The uptake of THP at equilibrium was also about 20% lower in P388/ADR (Arther D. Little) and P388/VCR cells than in the sensitive P388 cells (data not shown).

Cellular accumulation of ADR is known to be increased by inhibiting the drug efflux by sodium azide[10]. As reported before by INABA *et al.*[10] accumulation of ADR in ADR-resistant cells is enhanced by addition of sodium azide, and the enhancement is larger in the resistant cells than in the sensitive cells. On the other hand, although the rate of accumulation of THP was increased by sodium azide, there was no marked difference in between the ADR-sensitive and resistant cells, as shown in Fig. 4.

Efflux of THP and ADR from the sensitive and resistant P388 cell lines is shown in Fig. 5. P388/ADR cells released both THP and ADR more rapidly than the sensitive cells. The initial rate of efflux of THP from P388/ADR cells was as fast as that of ADR from P388/ADR cells, but the percentage of drug retained at equilibrium for THP was markedly higher than that for ADR.

Fig. 5. Efflux of THP and ADR from sensitive and ADR-resistant P388 cell lines.

Initial intracellular concentrations: THP, 0.515 μg/P388/S 1.64×10^6 cells; 1.03 μg/P388/ADR 1.79×10^6 cells, ADR, 0.553 μg/P388/S 1.52×10^6 cells; 0.619 μg/P388/ADR 1.89×10^6 cells. Cells were preloaded with drugs by incubation in PBS containing ADR or THP and 10 mM NaN_3 and resuspended in RPMI 1640 medium without washing.

○ P388/S, THP; ● P388/S, ADR; △ P388/ADR, THP; ▲ P388/ADR, ADR.

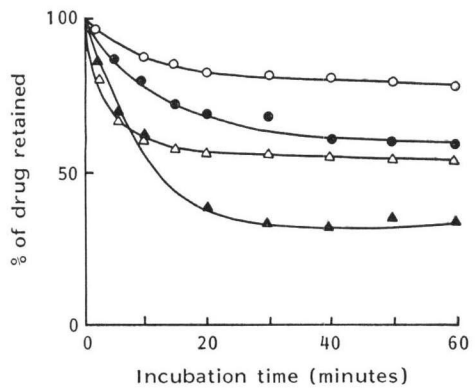


Table 3. Growth inhibition by ADR and THP against drug-sensitive and -resistant lines of P388 leukemia cells.

Cell lines	IC_{50} (μg/ml)	
	ADR	THP
Sensitive	0.01	0.006
ADR-resistant (Cancer Chemotherapy Center, Tokyo)	0.70 (70)	0.075 (13)
ADR-resistant (NCI)	0.60 (60)	0.08 (13)
Vincristine-resistant (NCI)	0.025 (2.5)	0.008 (1.3)

The values in brackets show the relative resistance expressed by the ratio of IC_{50} values for resistant to sensitive cell line.

Cells (5×10^4/ml) were incubated in RPMI 1640 medium containing 10% fetal bovine serum and 10 μM 2-mercaptoethanol for 48 hours at 37°C.

Table 3 shows the 50% growth inhibition concentrations of THP and ADR against an ADR-sensitive P388 cell line, two different ADR-resistant lines and a vincristine-resistant line. The IC_{50} of THP against the resistant cell line supplied by Cancer Chemotherapy Center was 13 times higher than that against the sensitive line, while that of ADR was 70 times higher. A similar difference was also observed for the other drug-resistant cell lines.

Discussion

Recently the mechanisms of drug resistance have been studied by using drug-resistant mutants. It is considered that one of the mechanisms of multi-drug resistance is a decrease in permeability to the drug, which may be induced by the alteration of membrane protein and be of genetic origin[13]. Recently it has been reported that the plasma membrane glycoprotein (p-glycoprotein, 170 k) in CHO cells[13] and the 230 k membrane protein in L5178Y/ACL cells recognized by a monoclonal antibody[14] (thought to be a tumor-associated antigen) may be responsible for drug resistance. On the one hand, in resistant P388 leukemia cells ADR efflux is enhanced while its influx is almost the same as that in sensitive cells[10]. TSURUO *et al.* showed that VCR resistance of P388 cells *in vivo* and *in vitro* could be diminished by the administration of calcium antagonists and calmodulin inhibitors which simultaneously inhibited drug efflux[15,16].

THP is taken up by the ADR-resistant cells much more quickly and to a higher intracellular concentration than ADR, and its efflux from the resistant cells is smaller than that of ADR. It inhibits growth of the resistant cells at lower concentrations than ADR, although the difference is smaller than in the drug uptake. Therefore, the ADR resistance has been partially overcome by THP *in vitro*.

However, intraperitoneal administration of THP was not markedly effective *in vivo* to the ADM-resistant P388 tumor (unpublished results). The drug might not be accumulated enough at the tumor site *in vivo* by intraperitoneal administration.

Alteration of the route of administration, *e.g.* intraarterial, is being studied for effects on ADR-resistant tumors in experimental animals. Moreover, screening of antitumor compounds which strongly inhibit growth of the drug-resistant cells may result in discovery of potent antitumor antibiotics.

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