RAPID UPTAKE BY CULTURED TUMOR CELLS AND INTRACELLULAR BEHAVIOR OF 4′-*O*-TETRAHYDROPYRANYLADRIAMYCIN

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Intracellular levels of 4′-*O*-tetrahydropyranyladriamycin (THP) and adriamycin (ADM) were measured by a fluorospectroscopic method, and the former was shown to be taken up by L5178Y cells much faster than ADM; the uptake velocity of THP at 1 μg/ml was calculated to be about 170 times faster than that of ADM.

High performance liquid chromatography of cell extracts indicated that THP exists mainly in nuclei intact without hydrolysis. The effect of THP in inhibiting [^{3}H]thymidine incorporation into DNA also indicated that THP taken up by cells rapidly went to nuclei and inhibited DNA synthesis. Fifty percent inhibition concentrations of THP or ADM on [^{3}H]-thymidine incorporation during a 60-minute period, 15 minutes after the addition, were 0.1 μg/ml and 4.2 μg/ml, respectively. Similar results were obtained when L1210 cells were used in place of the L5178Y cells.

In the screening for new anthracyclines, baumycins were discovered[1,2]; and one of them, baumycin A1, showed a strong effect in prolonging the survival period of mice bearing L1210 mouse leukemia. Structurally, baumycins are 4′-*O*-acetal derivatives of daunomycin. Therefore, 4′-*O*-acetal derivatives of adriamycin and daunomycin were synthesized, and 4′-*O*-(α-tetrahydropyranyl)adriamycin (THP) was found to have a stronger therapeutic effect against L1210 than adriamycin[3]. Moreover, THP was found by DANTCHEV *et al.* to have lower cardiac and skin toxicity in hamsters than ADM and aclacinomycin[4]. Thus, a clinical study was started, and although not yet conclusive, THP has been suggested to also have low cardiac toxicity in cancer patients. Side effects such as alopecia and vomiting are very slight with THP. Furthermore, with its clinical use, complete regression has been observed in the treatment of lymphomas, ovarial tumors, mesotheliomas, *etc.* It seems that tumors sensitive to THP treatment show a rapid response. Therefore we studied the therapeutic mechanism of THP and found that it is very rapidly incorporated into tumor cells and goes into their nuclei where it inhibits DNA synthesis.

In this paper, we report on the uptake of THP by L5178Y cells and inhibition of DNA synthesis in L5178Y and L1210 cells. We also confirmed that THP is not hydrolyzed to ADM within the cells.

Materials and Methods

Chemicals

[Methyl-^{3}H]thymidine (53 Ci/mmole) and [5-^{3}H]uridine (30 Ci/mmole) were products of RCC Amersham, England. Calf thymus DNA was purchased from P-L Biochemicals, Milwaukee, Wisc., U.S.A.

THP was prepared in our laboratory[3]. ADM was supplied by the National Cancer Institute, Bethesda, Md., U.S.A.

Culture of and Polynucleotide Synthesis in L5178Y and L1210 Cells

Mouse lymphoma L5178Y and leukemia L1210 cells were cultured in RPMI 1640 medium sup-

plemented with 10% horse serum. Cell growth was determined by counting the cell number using a Coulter counter. Cells (5×10^5 cells/ml) in the logarithmic phase of growth were preincubated with different concentrations of THP or ADM at 37°C for 15 minutes, and then 0.05 μCi/ml of [methyl-^{3}H]-thymidine or [5-^{3}H]uridine was added. One hour thereafter, the reaction mixture was processed according to the method described previously[5], and the radioactivity of the 5% TCA-insoluble fraction was measured by a liquid scintillation counter.

Determination of Uptake of THP and ADM

Cell suspensions (2×10^6/ml) were incubated with THP or ADM at various concentrations in 1 ml of RPMI 1640 medium without serum at 37°C. Uptake was terminated by centrifugation at $12,000\times g$ for 30 seconds in an Eppendorf microcentrifuge. After the removal of the supernatant, the cells were washed twice with 1 ml of cold phosphate-buffered saline (pH 7.2) and disrupted by ultrasonication in 1 ml of the same buffer. THP and ADM were extracted from aliquots of sonicated cell suspension (0.8 ml) by adding 0.2 ml of water, 2 ml of 40% TCA, and 0.1 ml of 10% bovine serum albumin according to the method of NOEL *et al.*[6]. After centrifugation, fluorescence intensity of the supernatant was measured and calculated by comparison with standard solutions treated in the same manner. The same experiments were also performed at 0°C. As shown later, the uptake at 0°C was very small. The uptake values at 0°C were subtracted from those at 37°C to calculate the uptake velocity at 37°C.

Separation of Cytoplasm and Nuclei and Analysis of Cellular THP and ADM

Cytoplasm and nuclei were separated by the method of FOURCADE *et al.*[7]. After incubation of L5178Y cells (10^7 cells/5 ml of RPMI 1640 medium supplemented with 10% horse serum) at 37°C with THP or ADM (10 μg/ml), the cells were washed twice with cold HANKS' balanced salt solution (pH 7.2), resuspended in 2 ml of cold HANKS' solution containing 0.1% Nonidet P-40, and allowed to stand at 0°C for 5 minutes. The nuclear and cytoplasmic fractions were separated by centrifugation at 800 rpm for 15 minutes. The nuclei were resuspended with 2 ml of HANKS' solution containing 0.1% Nonidet P-40. To both nuclear and cytoplasmic fractions, 6 ml of 0.1 M ammonium hydroxide-ammonium chloride buffer (pH 9.0) were added; and THP, ADM, and their possible metabolites were extracted by shaking with 24 ml of chloroform - methanol (2 : 1, v/v). The aqueous layer was reextracted with 8 ml of chloroform at pH 9.0 and the organic layers were combined and evaporated *in vacuo*. The residue was dissolved in water - acetonitrile (65 : 35, v/v) containing daunomycin (0.5 μg/ml) as an internal standard (2 ml for THP and 0.4 ml for ADM), and 50 μl were subjected to high performance liquid chromatography using a Shimadzu LC-3A equipped with a Shimadzu fluorescence monitor (Shimadzu Corporation, Kyoto, Japan). A Waters μBondapak phenyl column (Waters Associates, Milford, Massuchusetts U.S.A., 3.9 mm inner diameter $\times$ 30 cm) was used in this analysis. Elution was performed with acetonitrile - 0.035 M formic acid/ammonium formate buffer, pH 3.0 (35 : 65, v/v). The details of this method will be published by MATSUSHITA *et al.*[8]

Fluorometric Titration of THP and ADM Binding to DNA

The quenching of THP and ADM fluorescence (590 nm excited by 500 nm) by DNA was measured in reaction mixtures containing 2.59×10^{-6} M THP or ADM and calf thymus DNA varying in amount between 2×10^{-6} M and 6.66×10^{-5} M. A Hitachi MPF-4 spectrofluorometer thermostated at 20°C was used. The concentrations of DNA were expressed in terms of nucleotide concentrations calculated from an extinction coefficient of $\varepsilon_{260}=6600\ \text{M}^{-1}\ \text{cm}^{-1}$.

Results

THP and ADM Uptake by L5178Y Cells

As shown in Fig. 1, uptake of THP and ADM (10 μg/ml in the initial concentration used) by L5178Y cells was temperature-dependent. Especially, there was a marked difference between 37°C and 0°C in the case of THP. The uptake of THP reached a plateau within 5 minutes, but the intracellular concentration of ADM continued to rise gradually. The concentration of ADM at 20 minutes was lower than one fifth of that of THP. The ratio of the concentration in cells to that in the medium at 20 minutes

Fig. 1. Time course of uptake of THP and ADM by L5178Y cells.

Initial drug concentration was 10 μg/ml.

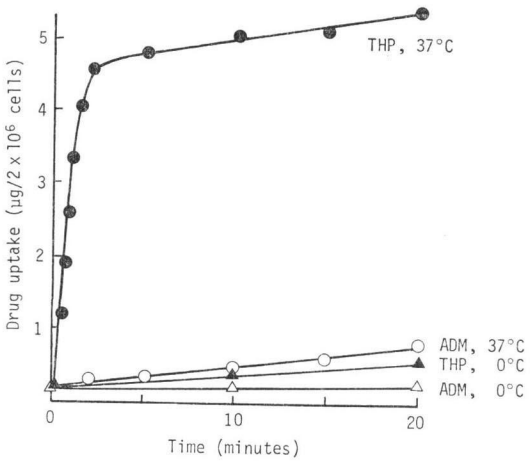
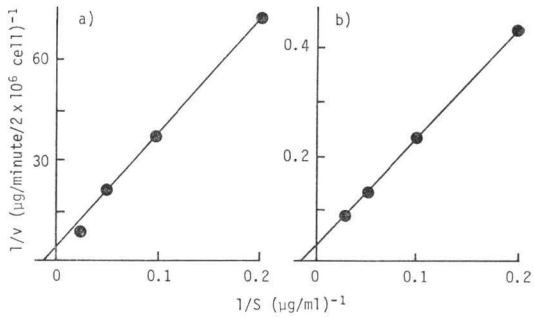


Fig. 2. LINEWEAVER-BURK plot of THP and ADM uptake.

Initial rate of uptake (v) was calculated from data derived from incubating THP or ADM and cells at 37°C for 45 seconds or 3 minutes, respectively.

a) ADM uptake. b) THP uptake.



was calculated to be 462 for THP and 35.4 for ADM. Thus, assuming a volume of an average L5178Y cell as 1273 ± 28 cu μm[9], THP was shown to be more effectively transported into L5178Y cells than ADM.

Table 1. Calculated velocity (v) of uptake.

Drug concentration	THP (a)	ADM (b)	a/b
	(μg/minute/2$\times 10^6$ cells)		
1 μg/ml	0.524	0.003	174
0.1 μg/ml	0.053	0.0003	176

$v = V_{max} \times [S/(S+Km)]$

The LINEWEAVER-BURK plot of the initial rate of uptake (Fig. 2) indicated simple saturation kinetics, suggesting a carrier-mediated transport. The *Km* was 88.3 μM for THP and 143.7 μM for ADM. The V_{max} (nmole/minute/2$\times 10^6$ cells) was 47.0 for THP and 0.44 for ADM. As shown by these values, the apparent *Km* of THP was approximately the same of that of ADM, but the V_{max} of THP was 107 times higher than that of ADM. Using these values, the uptake velocity/minute by 2×10^6 cells exposed to 1 or 0.1 μg/ml was calculated to be about 170 times faster for THP than for ADM (Table 1).

Subcellular Localization of THP and ADM in L5178Y Cells

After a 10- or 60-minute incubation of L5178Y cells with THP or ADM, the subcellular localization of these drugs was determined. As seen from the results shown in Table 2, 69~74% of the THP incorporated into cells was located in the nuclear fraction. No metabolites were detected except for a

Table 2. Subcellular localization of THP and ADM in L5178Y cells.

Drug	Incubation time (minutes)	Fraction	μg/10^7 cells		Total
			THP	ADM	
THP	10	Cytoplasmic	7.28	0.0370	27.7
		Nuclear	20.36	0.0659	
THP	60	Cytoplasmic	8.57	0.0869	28.0
		Nuclear	19.16	0.151	
ADM	10	Cytoplasmic		0.143	0.661
		Nuclear		0.518	
ADM	60	Cytoplasmic		0.393	2.593
		Nuclear		2.20	

Table 3. Effect of THP and ADM on nucleic acid synthesis in cultured L5178Y cells.

Addition		Incorporation of			
		[^{3}H]Thymidine		[^{3}H]Uridine	
		dpm	Inhibition %	dpm	Inhibition %
None		3478		548	
THP	5 μg/ml	34	99	135	75
	1.25	94	97	154	73
	0.31	911	74	242	56
	0.078	1810	48	468	15
	0.020	3081	11	543	0.9
ADM	5	1682	52	297	46
	1.25	2377	35	344	37
	0.31	2922	16	572	−4
	0.078	2994	14	635	−6

small amount of ADM. The amount of ADM produced from THP in nuclei during a 10-minute exposure was 0.32%; and after 60 minutes, 0.79%, thus indicating a very slow hydrolysis in nuclei. In the case of ADM, the amounts taken into the cytoplasm and nucleus were very small compared with THP that is, at 10 minutes, 50 times less ADM than THP had entered.

Inhibition of Nucleic Acid Synthesis in L5178Y and L1210 Cells by THP and ADM

The effect of THP and ADM on the incorporation of precursors of DNA and RNA into the TCA-insoluble fraction of L5178Y cells was determined and is shown in Table 3. The IC_{50} of THP and ADM on DNA synthesis was 0.1 μg/ml and 4.2 μg/ml, respectively. THP at a concentration 42 times lower than that of ADM showed the same effect as the latter. In the case of the inhibition of RNA synthesis, the IC_{50} of THP was 0.23 μg/ml; and that of ADM, 6.6 μg/ml. Also in the case of L1210 cells, THP showed inhibition of DNA and RNA synthesis at its very low concentration compared with ADM. The IC_{50} of THP and ADM on DNA synthesis was 0.24 μg/ml and 13 μg/ml, respectively, when [^{3}H]-thymidine was added at the same time as the addition of THP or ADM and 30 minutes thereafter the reaction was stopped.

The Binding of THP and ADM to Calf Thymus DNA

The degree of binding of THP and ADM to DNA was examined by spectrofluorometric analysis. The fluorescence spectrum of THP and its steady state quenching by calf thymus DNA are shown in Fig. 3. Fluorescence titrations with various concentrations of DNA were performed and analyzed by a SCATCHARD plot (Fig. 4). The binding parameters of these drugs to DNA obtained from Fig. 4 were as follows: the apparent association constant (*Ka*) was 5.87×10^6 M^{-1} for THP and 4.21×10^6 M^{-1} for ADM. The number of binding sites per nucleotide (n) was 0.226 for THP and ADM. Thus, both *Ka* and number of binding sites per nucleotide (n) were very similar; and with respect to the interaction with DNA, there was no significant difference between THP and ADM.

Discussion

Uptake of both THP and ADM was faster at 37°C than at 0°C. Especially, there was a marked difference in THP uptake at these two temperatures. This difference and a markedly faster uptake than ADM suggests that different carrier may be involved in THP and ADM uptake or the transport of THP

Fig. 3. Fluorescence spectra resulting from the interaction of THP with calf thymus DNA.

Free THP (2.59×10^{-6} M) was titrated with varying concentrations of calf thymus DNA.

DNA concentration: a) free, b) 1.0×10^{-4} M, c) 1.66×10^{-4} M, d) 9.14×10^{-4} M.

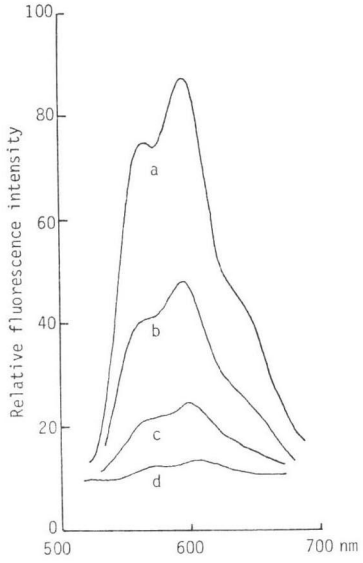
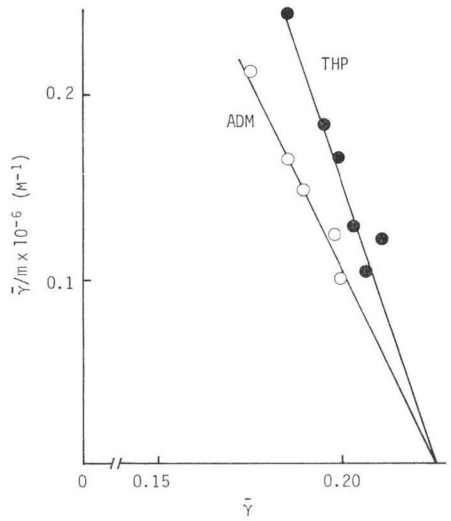


Fig. 4. SCATCHARD analysis of calf thymus DNA binding with THP and ADM.



by the carrier may be more effective than ADM. Moreover, it is interesting that once taken into the cells, THP goes rapidly into nuclei. The affinity of THP for DNA is almost the same as that of ADM; therefore, DNA is not involved in the rapid uptake of THP. Although the experiments are not described in this paper, THP has almost the same activity as ADM in inhibiting RNA polymerase of *E. coli* (IC_{50} of THP, 1.8×10^{-5} M; and of ADM, 8.8×10^{-6} M).

ADM is gradually taken up by cells, and therefore when the growth inhibition concentrations were determined after 2 days of culture, there was not a marked difference from THP: 50% growth inhibition of L5178Y cells was caused by 0.005 μg/ml of THP and 0.014 μg/ml of ADM. However, the values for inhibiting DNA synthesis during 30 to 60 minutes of exposure to THP or ADM may be more related to their therapeutic activity than the growth inhibitory concentrations observed after 1 or 2 days of culture. As reported clinically, $t_{1/2}$ of the α phase of blood level was 0.78 minutes for THP and 3.19 minutes for ADM. The results of the experiments described above indicate that even after one minute, a very large amount of THP has been taken up by cells sensitive to the compound. The calculated uptake values after 30 seconds exposure to a 1 μg/ml concentration was 0.262 μg for THP and 0.0015 μg for ADM.

The high uptake of THP by L5178Y and L1210 cells indicates that tumors susceptible to THP treatment would show a rapid response.

It should also be noted here that in L5178Y cells, THP was not metabolized to 13-dihydro THP or adriamycinone. It is interesting that this is a characteristic of this tumor cell or tumor cells sensitive to THP in general. The amount of ADM produced from THP in cells is 0.37% after 10 minutes and 0.85% after 60 minutes. This fact and the effect of THP in inhibiting RNA synthesis *in vitro* indicates that THP itself is involved in cell destruction.

Acknowledgments

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