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Comparative Uptake, Metabolism and Retention of Anthracyclines by Tumors Growing *In Vitro* and *In Vivo**

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Abstract—*Adriamycin, daunomycin and their metabolites were differentially taken up* in vitro *by L*1210 *lymphocytic leukemia cells in culture. An intracellular drug equivalent concentration of about* 0.20 μg/10^7 *cells* (20 μg/g) *was required to attain equivalent cell-kill for daunomycin, daunomycinol or adriamycin but, in order to attain this intracellular concentration, the extracellular concentrations of daunomycinol and adriamycin were* 3 *and* 2 *times that of daunomycin. Intracellular concentrations of daunomycin comparable to those* in vitro *were attained in L*1210 *lymphocytic leukemia, P*388 *leukemia, and B*16 *melanoma* in vivo *when both drug and tumor were present in the intraperitoneal cavity, but only a fraction of that concentration was attained if the tumor was growing as a solid mass at a site distal to the site of drug administration. Daunomycin was quantitatively metabolized by the reductase in homogenates of L*1210/*NSC* 38280, *L*1210, *P*388 *or B*16 *tumors; however, the rate of metabolism by the lymphocytic leukemias was much slower than that by B*16 *melanoma. Daunomycinol effluxed more slowly than daunomycin from cells of all responsive tumors. In practice, chemotherapeutic response to anthracycline drugs may depend upon several dynamic factors: uptake, metabolism and retention of the drug by tumors and the pharmacokinetics of the drug and metabolites in the mammalian host.*

INTRODUCTION

ADRIAMYCIN (NSC 123127) and daunomycin (NSC 82151) are glycosidic anthracyclines having clinical chemotherapeutic utility [1, 2]. The pharmaco-kinetics of these anthracyclines are generally similar among several animal species [3–8] and man [9–12]. Adriamycin and daunomycin administered i.v. rapidly leave the plasma compartment, are distributed into tissues within the first few minutes according to blood flow through the tissues, and are further compartmentalized within the tissues. Thereafter, the anthracyclines are metabolized, varying in rate for tissues [13], and for several animal species [14]. Whole-body radioautographic studies in rats indicate that the anthracyclines and possibly metabolites extensively enter the lymphatic circulation [15]. The excretory pathways for the anthracycline drugs and their metabolites are primarily bile [3, 6, 15–18] possibly gastric secretion [15], and secondarily, via urine [3, 6, 9, 10, 17, 18] and rates are generally slow.

The anthracycline-metabolizing enzymes which effect the reduction of the 13-carbonyl to carbinol, are located in the cytoplasm of many tissues, and those which effect the reductive cleavage of the amino sugar, daunosamine, are located in the microsomes [13, 19–29].

Daunomycin (13-carbonyl) is metabolized *in vivo* to daunomycinol (13-carbinol) which has the long half-life in tissues, similar to that of adriamycin [6]. In those animal species, e.g., rabbits and monkeys, which metabolize adriamycin (13-carbonyl) to adriamycinol (13-carbinol) [3], the half-life of adriamycin in tissue is relatively short.

In our experimental chemotherapeutic studies, it has been observed that tumors respond differentially to anthracycline therapy. For

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example, B16 melanoma appears to be more responsive to anthracycline therapy than L 1210 lymphocytic leukemia. In addition, excellent chemotherapy response is obtained when the drug is administered proximal to the tumor, e.g., drug and tumor are present in the intraperitoneal cavity, whereas minimal chemotherapy is obtained when the drug is administered distal to the tumor and has to be delivered to the tumor via the systemic circulation.

In these studies, we have evaluated the uptake, metabolism, and retention of the anthracycline drugs by cells of several tumor lines and have evaluated the importance of the pharmacokinetics of the anthracycline drugs with respect to the site of the tumor and the route of administering the drug.

MATERIALS AND METHODS

The clinical dose forms of daunomycin and of adriamycin were dissolved in physiological saline at various concentrations. Adriamycin was supplied by Farmitalia, Societa Farmaceutica Italia, 20146 Milan, Italy. Daunomycin and daunomycinone were supplied by the National Cancer Institute. The metabolites, daunomycinol, D_x, D_y, A_x and A_y were enzymatically prepared and then isolated and purified using methods which have previously been reported [19, 20].

L 1210 lymphocytic leukemia cells were grown in suspension in Fisher's medium [30], supplemented with 10% horse serum, penicillin and streptomycin. For growth inhibition assays, $8–9 \times 10^5$ cells per ml and 4 ml per tube were incubated with varying concentrations of the anthracycline compounds for 48 hr. Growth was determined by cell count [31]. In a second experimental design, the L 1210 cells were exposed to drug and aliquots from each drug treatment were taken for cell counts and for assay of colony-forming efficiency [32]. In a third experimental design, larger volumes of L 1210 cells were exposed to drug and then were centrifuged out of drug-containing medium, were washed twice with 25 ml of cold physiological saline, and the anthracycline drugs were extracted from them as described below.

P388, L 1210 or L 1210/NSC 38280 ascitic cells ($10^5–10^6$) were inoculated i.p. in male BDF_1 hybrid mice. Daunomycin or adriamycin was administered at 10 mg/kg i.p. on day 5. Tumor cells were harvested at several times after dosing, washed in physiological saline and frozen at −20°C. L 1210 solid tumors were implanted i.m. and s.c. in male BDF_1 hybrid mice. Daunomycin and adriamycin were injected at 10 mg/kg i.p. on day 16. Tumor was harvested from mice at several times after dosing, washed with physiological saline and frozen at −20°C.

The methods for extracting and quantitating anthracycline equivalents and for separating parent drug and its metabolites by TLC have been described [6]. In brief, tumors and tissues were extracted with $CHCl_3$:MeOH (2:1; v/v) and the extracts made biphasic with water. Anthracycline equivalents in the non-aqueous phase were quantitated by fluorescence spectrophotometry and separated into parent drug and metabolites by TLC on activated silica-gel H with a $CHCl_3$:MeOH:HOAc:H_2O solvent system. The drug species were eluted from the silica gel and quantitated by fluorescence spectrophotometry.

RESULTS

The relative cytotoxicity of adriamycin, daunomycin and their metabolites to L 1210 using the 48-hr growth inhibition assay is shown in Table 1. Adriamycin, daunomycin, and daunomycinol were very active, and comparable concentrations of all three resulted in comparable chemotherapy. In contrast, the aglycone metabolites (A_y, A_x, D_y and D_x) are considerably less active, requiring a 100-fold greater concentration.

Table 1. *L* 1210 *cytotoxicity assay of anthracycline compounds* (48-hr *growth inhibition*)*

Compound	ID_{50} (μg/ml)
Adriamycin	0.009–0.020
Daunomycin	0.004–0.013
Daunomycinol	0.02–0.08
Daunomycinone	0.6
Reduced Aglycones	
A_y	2.0
A_x	2.4
D_y	2.3
D_x	3.5

*See Materials and Methods for details.

The relative cytotoxicity of adriamycin, daunomycin and daunomycinol to L 1210 using a colony-forming assay is shown in Table 2. These results are consistent with the well established observation that cancer chemotherapy is related to the concentration of the

Table 2. L 1210 cytotoxicity assay of daunomycin, daunomycinol, and adriamycin (8–10 days *soft-agar colony-forming assay*)

Concentration (μg/ml)	Surviving fraction (%) Exposure to drug (hr) 1	2	4	8
Daunomycin				
0.015	88	46	41	6
0.05	62	9	0.1	—
Daunomycinol				
0.05	—	91	50	
0.1	75	64	1	
0.14	62	6	—	
Adriamycin				
0.03	—	—	81	
0.1	64	12	0.5	
0.3	1	0.05	<0.01	

antitumor agent and to the duration of exposure, i.e., Cxt. At each extracellular concentration for each anthracycline, there was a progressive decrease in surviving fraction as the duration of drug exposure increased. Increasing the extracellular concentration of each drug resulted in greater cell-kill, as seen by the decrease in surviving fraction. Daunomycin was much more cytotoxic than daunomycinol or adriamycin. At 0.14 μg/ml of daunomycinol, the cell-kill was comparable to that seen with 0.05 μg/ml of daunomycin. Similarly, at 0.1 μg/ml of adriamycin, cell-kill was comparable to that seen with 0.05 μg/ml of daunomycin. Thus, comparable cell-kill at equivalent times of exposure can be obtained with daunomycin, daunomycinol and adriamycin, if their extracellular concentrations are in the ratio 1:3:2, respectively.

The relationship between the relative cytotoxicity of daunomycin, daunomycinol and adriamycin and the intracellular concentration of each drug was determined (Table 3). For each drug, a decrease in the surviving fraction corresponded to an increase in the intracellular concentration of total drug equivalents. To attain comparable intracellular concentrations of the three drugs and comparable cytotoxicity, the extracellular concentrations of daunomycinol and adriamycin were three and two times that of daunomycin, respectively. Cells exposed to daunomycin

Table 3. Relationship between cell-kill and uptake of daunomycin, daunomycinol and adriamycin

Drug exposure (μg/ml × hr)	Surviving fraction (%)	Uptake and metabolism Total (μg/10^7 cells)	Daunomycin (%)	Daunomycinol (%)
Daunomycin				
0.5 × 1	62	0.04		
0.05 × 2	9	0.12	70	30
0.05 × 4	0.1	0.18	86	14
Daunomycinol				
0.14 × 1	62	0.07		100
0.14 × 2	6	0.20	0	100
Adriamycin				
0.1 × 1	64	<0.01		
0.1 × 2	12	0.07		
0.1 × 4	0.5	0.20		

contained mostly daunomycin and 15–30% daunomycinol. This metabolism occurred within 1–4 hr and further metabolism of daunomycin probably occurred during the bioassay, which takes 8–10 days. The cells exposed to daunomycinol or adriamycin contained only the parent compound, suggesting that further metabolism of these drugs may not occur.

The uptake, metabolism, and retention of daunomycin was determined in several tumor lines implanted i.p. in mice after administration of daunomycin i.p. (Table 4). L 1210/NSC 38280 resistant cell line, which is cross-resistant to daunomycin, took up daunomycin but there was a subsequent efflux from the cells. Sensitive cell lines, L 1210 and P388, took up equivalent concentrations of daunomycin and retained the drug over 24 hr. These results agree with the findings described by Kessel *et al.* [33]. B16 melanoma, the most chemotherapeutically responsive tumor, took up less daunomycin and although it metabolized daunomycin to daunomycinol to a greater extent than the other tumors, the concentration of daunomycinol was initially equivalent among the four tumor lines. However, daunomycinol was preferentially retained in responsive tumors.

Huffman and Bachur [25] have shown that the level of anthracycline reductase in leukemic myeloblasts is related to the chemical response to daunomycin therapy. We have characterized the kinetic constants for anthracycline reductase from several tumor lines (Table 5). All four tumor lines extensively metabolize daunomycin to daunomycinol; however, the velocities were relatively slow for the lymphocytic leukemias in comparison to B16 melanomas which is the most responsive tumor. We have also evaluated the reductive metabolism of adriamycin to adriamycinol and the reductive cleavage of daunomycin and adriamycin to their reduced aglycones in L 1210 and L 1210/NSC 38280 cell lines. In all instances the velocities of these reactions were extremely slow.

Minimal chemotherapy (<120 T/C%) was obtained with either adriamycin or daunomycin administered at 10 mg/kg i.p. or i.v. to mice bearing L 1210 implanted i.m. or s.c., or 5-day B16 melanomas implanted s.c. or i.m. These chemotherapeutic results differ from those found for treatment of i.p. tumors (Table 4). The uptake and retention of daunomycin and adriamycin by L 1210 and by B16 melanoma, growing distally from the i.p. administration of drug, are shown in Table 6 and Figs. 1 and 2, respectively. In general, the uptake of either drug by tumor growing s.c. or i.m. was only a fraction ($<5\%$) of that taken up by the tumors grow-

Table 4. Uptake, metabolism and retention of daunomycin by tumors growing intraperitoneally in mice

Tumor line*	T/C%†	Hr after treatment‡	Total	Daunomycin (μg/g)	Daunomycinol
L 1210/R§	100	0.5	50	48	2
		4	50	46	4
		24	5	*ca.* 5	*ca.* 0.5
L 1210	150	0.5	44	43	1
		4	52	49	3
		24	24	21	3
P388	227	0.5	62	60	2
		4	57	53	4
		24	20	17	3
B16 Melanoma	>300	0.5	9	8	1
		4	15	11	4
		24	13	9	4
		48	7	4	3

*An inoculum of 10^5–10^6 ascites cells (L 1210/R, L 1210 or P388) or a brei of B16 melanoma tumor was implanted i.p. on day 0.
†Treatment (i.p.) occurred on days 1–9 following inoculation of tumor (i.p.) at the optimum dose which generally was 1 or 2 mg/kg.
‡The dose (i.p.) was 10 mg/kg on day 5 or 6 following inoculation of tumor (i.p.).
§Resistant to the terephthalanilide, NSC 38280.

Table 5. Comparison of kinetic constants for anthracycline reductase from several tumor lines

Tumor Line	Mean ± (S.D.) K_m*	V_{max}†
L 1210/R	0.042 (0.006)	0.045 (0.007)
L 1210	0.059 (0.006)	0.067 (0.016)
P388	0.142 (0.010)	0.078 (0.015)
B16 Melanoma	0.168 (0.014)	0.302 (0.062)

*K_m = mmole/liter/mg protein.
†$V_{max} = 10^{-5}$ mmole/min/mg protein.
These estimates were made from Lineweaver–Burk double reciprocal plots (1/velocity vs 1/substrate).

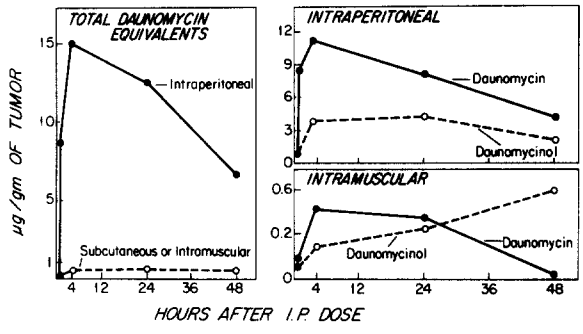

Fig. 1. Uptake and metabolism of daunomycin by B16 melanoma growing i.p., i.m. and s.c. in mice.

ing i.p. when drug was administered i.p. in all cases. The efflux of drug or the metabolism of daunomycin did not, however, differ from tumors growing i.p., s.c. or i.m.

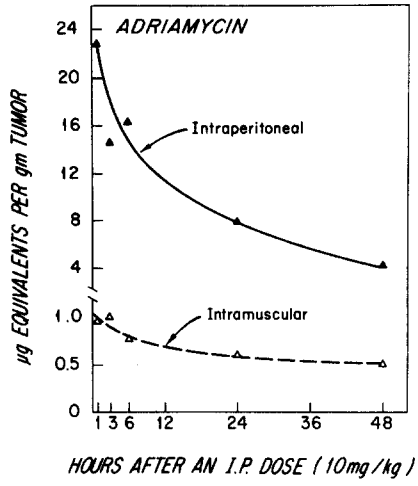

Fig. 2. Uptake and retention of adriamycin by B16 melanoma growing i.m. and i.p. in mice.

DISCUSSION

Adriamycin, daunomycin and its metabolite, daunomycinol, are chemotherapeutically active against L 1210 growing *in vitro*. Cytotoxicity with these drugs depended both on the extracellular concentration and on the duration of exposure. When an intracellular concentration of 0.20 μg/10^7 cells (10^9 cells is approximately 1 gm which is comparable to 20 μg/g) was attained for each anthracycline drug, significant cytotoxicity was observed. In order to attain this intracellular concentration and comparable cell-kill, the required extracellular concentrations of daunomycin, daunomycinol and adriamycin were in a molar ratio of 1:3:2, respectively.

Adriamycin and daunomycin, when administered i.p. to mice bearing i.p. tumors, result in comparable chemotherapeutic response. The intracellular concentration of each drug in L 1210 cells exceeds 20 μg/g during a 24-hr

*Table 6. Uptake and retention of anthracycline equivalents in L 1210 tumors growing i.p. or s.c. following treatment of mice i.p.**

Hr after treatment (i.p.)	μg/g of L 1210 cells		
	Ascites (i.p.)	Solid (s.c.)	
	Adriamycin	Daunomycin	Adriamycin
0.5	44	0.8	1.4
1	41	0.9	1.6
2	33	1.0	2.0
4	52	1.0	2.0
6	—	1.0	1.2
8	34	—	—
24	24	0.2	0.8

*A $10^5 - 10^6$ cell inoculum was injected i.p. or s.c. and drug was administered i.p. at 10 mg/kg on day 6 or 7.

period. A similar pattern was found for daunomycin administered to P388 or B16 melanoma-bearing mice. L1210/NSC 38280 cells showed uptake of daunomycin comparable to that for sensitive cell lines but there was a subsequent efflux of daunomycin and daunomycinol from the resistant cells. In the sensitive cell line, there appeared to be a differential efflux of daunomycin compared to daunomycinol, the latter being the slower. Thus, the rate of metabolism of daunomycin to daunomycinol by tumors also becomes an important parameter in chemotherapy in addition to uptake.

Tumors that are growing at a site distal to the site of administration of drug are generally non-responsive to anthracycline chemotherapy and was probably due to the extensive uptake of the anthracycline drugs by tissues, which results in a decreased concentration of drugs in plasma and a decreased availability of drug for uptake of tumor. Adriamycin and daunomycin are rapidly and extensively taken up by tissues. Thus, non-responsiveness may be due to a decreased availability and uptake of drugs by tumors. This appeared to be so; therefore, the maintenance of anthracyclines within the plasma compartment for a longer period of time, e.g., by liposome encapsulation of adriamycin [34], may be necessary to effect chemotherapy of tumors growing distally to the site of administration of drug.

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