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Comparative pharmacokinetics and metabolism of doxorubicin and 4-demethoxy-4'-O-methyldoxorubicin in tumor-bearing mice*

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Summary. It has been reported that 4-demethoxy-4'-O-methyldoxorubicin (4-dm-4'-O-methylDX) is more potent than doxorubicin (DX), equally active in some murine leukemias and solid tumors, and almost devoid of cardiotoxicity. We used HPLC to investigate the metabolism and the disposition of this drug in comparison with DX in mice bearing colon 38 adenocarcinoma SC and treated with IV doses of the two drugs that were equiactive and equitoxic (4-dm-4'-O-methylDX 1 mg/kg; DX 10 mg/kg). 4-Dm-4'-O-methylDX was metabolized to a polar metabolite, presumably 4-demethoxyDX, which was eliminated more slowly than the parent drug from all the organs and accounted for 25%–50% of total fluorescence; traces of two metabolites less polar than the parent drug (2% of total fluorescence) were found only at early times in the liver. In DX-treated mice traces of doxorubicinol (1%–3% of total fluorescence) were found in tumor and organs, and two aglycones were detected only at early times in the liver. In plasma both drugs declined biexponentially and 4-dm-4'-O-methylDX was eliminated slightly faster than DX. The rate of elimination of the new analogue from lung, kidney, spleen, and small intestine was faster than that of DX; in heart and liver 4-dm-4'-O-methylDX was detectable for only up to 24 h, while DX was detectable for up to 7 days. In the tumor the kinetics and the elimination patterns of the two drugs were similar. The distribution of 4-dm-4'-O-methylDX, as a percentage of the administered dose, was 1.3–2 times higher than that of DX in the organs and 3 times higher in the tumor, which suggests an improved selectivity of the new analogue for the tumor compared with DX.

Introduction

Among the most recently synthesized anthracycline derivatives, the class of 4-demethoxy anthracyclines shares several interesting properties that the parent antibiotics do not have [12], and recently it has been reported that the most interesting of these compounds is 4-demethoxy-4'-*O*-methyldoxorubicin (4-dm-4'-*O*-methylDX), an analogue of doxorubicin (DX), which besides lacking the methoxy group in position C4 in the chromophore, is modified at C4' in the aminosugar [9]. This compound, compared with the parent drug DX, was found to be almost ten times more cytotoxic in vitro and almost ten times more potent in vivo in terms of both antitumor and toxic effect. Tested on experimental leukemias, it was as active as DX against P388 and ascitic and disseminated Gross leukemias, and more effective than DX against L1210 leukemia. In the case of disseminated Gross leukemia, the new analogue was also active after oral treatment. In solid tumors, compared with DX, it was as active against C3H mammary carcinoma, more active against early colon 26, and significantly more effective against advanced colon 38. When tested against several human tumors transplanted in athymic mice, it was found to be slightly less active than DX. Administered at doses equiactive with the parent drug DX, it turned out to be almost devoid of cardiotoxicity [9, 12].

In an effort to improve our understanding of the biological properties of this analogue, we compared its pharmacokinetics and metabolism with those of the parent drug, DX, in tumor-bearing mice treated with equiactive doses of the two drugs.

Materials and methods

Drugs. 4-Dm-4'-O-methylDX and DX were kindly supplied as hydrochlorides by Farmitalia-Carlo Erba (Milan, Italy). Aqueous drug solutions were freshly prepared immediately before use. Equiactive doses of the two drugs, which are also equitoxic doses (e.g., 1 mg/kg for 4-dm-4'-*O*-methylDX and 10 mg/kg for DX [9]) were injected IV in a volume of 10 ml/kg body weight.

Animals and tumor. Adult female BDF1 mice were supplied by Charles River Breeding Laboratories (Calco, CO, Italy). The animals were 2–3 months old, weighed 20–25 g, and were maintained under standard laboratory conditions. The animals received SC transplants of colon 38 adenocarcinoma into the right flank and were used for tissue distribution studies when the tumors were palpable (5–10 mm diameter). Three animals per point were treated and killed with ether at 5, 10, and 30 min and 1, 2, 3, 6, 15, 24, 48, 72, 120, and 168 h after treatment. Tumor and tissues (heart, liver, lung, kidney, spleen, and small intestine) were removed, rinsed in cold saline, and stored at −70 °C until drug extraction. The gallbladder was always removed

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from the liver and the small intestine was always emptied before being rinsed in saline. Blood was collected from the retro-orbital plexus into cold heparin-coated glass tubes. After the blood had been centrifuged the plasma was removed and stored at −70 °C until analysis.

Biochemical assay of drugs and metabolites. The injected drugs and their metabolites were assayed from the supernatants of plasma and tissues homogenates obtained by precipitation of proteins according to Brown et al. [4] with slight modifications. To release drugs bound to DNA [14] and to precipitate proteins [4], plasma and tissues were processed as follow: 0.01 ml KH_2PO_4 buffer 0.2 *M* pH 3.8, 0.02 ml $AgNO_3$ (33%) and 0.13 ml $CH_3CN:H_2O$ (8:2) were added to 0.1 ml plasma; 0.1 ml $AgNO_3$ (33%) and 0.5 ml $CH_3CN:H_2O$ (8:2) were added to 0.5 ml tissue homogenates in KH_2PO_4 buffer 0.02 *M* pH 3.8 (1:5). The samples were vortex-mixed and centrifuged to pellet the precipitated proteins and the supernatants were analyzed by HPLC. Then 20 µl of the supernatants was injected directly into a C_{18} reverse-phase column (Perkin Elmer HS-5 150 × 4.6 mm), and the peaks were compared with those from reference standard curves set up for plasma, tumor, and organs with both drugs. The chromatographic standards used were: the 13-dihydroderivative of DX (DXol), the 13-dihydroderivative of 4-dm-4'-O-methylDX (4-dm-4'-O-methylDXol), doxorubicinone (DXone), 7-deoxydoxorubicinone (7-deoxyDXone), and 4-demethoxyDX (4-dmDX). Owing to the limited amounts of the standard 4-dmDX it was not possible to set up reference standard curves for this compound, and we assumed that extraction efficiency and fluorescence were the same as for the parent drug 4-dm-4'-O-methylDX. For this reason the concentrations of the metabolite in the organs are reported as '4-dm-4'-O-methylDX fluorescent equivalents'. The standards were kindly supplied by Ricerca Chimica Farmitalia Carlo Erba (Milan, Italy). The mobile phase was CH_3CN:0.01 *M* H_3PO_4 pH 2.2 (40:60) for 4-dm-4'-O-methylDX and CH_3CN:0.01 *M* KH_2PO_4 pH 3.8 (34:66) for DX at a flow rate of 1,5 ml min_{-1}. Detection was by fluorometry on a Perkin-Elmer MPF 44A spectrofluorometer with excitation wavelength of 487 nm and emission wavelength of 570 nm for 4-dm-4'-O-methylDX and excitation wavelength of 500 nm and emission wavelength of 590 nm for DX. The recoveries were 75%–85% and the sensitivities were 50 ng/g tissue and 10 ng/ml plasma for both drugs. These recoveries were higher than those obtained in preliminary trials run in parallel with $AgNO_3$ and *n*-butyl alcohol [8] and with chloroform:methanol (4:1 v/v) [11].

The presence in the samples of glucoronides and sulfates of the injected drugs was checked by comparing HPLC chromatograms before and after incubation of liver homogenates for 4 h at 37 °C with Sigma β-glucoronidase and arysulfatase.

Pharmacokinetics analysis. The experimental results, represented by the average of the concentrations of three animals per time (coefficients of variation ≤20%), were analyzed according to a single-, two-, or three-compartment model by means of programs implemented on a UNIVAC 1106 computer which calculates the pharmacokinetics parameters by means of an iterative calculation process where the convergence is obtained by a 'steepest descent' method [10]. The fit of the experimental data was good, with the highest standard errors of the estimates equal to 10%. The total areas under the curve (AUC) were calculated by trapezoidal rule from zero to the last sampling time and then by first-order extrapolation to infinite time with the experimentally determined terminal half-life values.

Results

Metabolism

In the liver of animals treated with DX (Fig. 1) three metabolites were found, which presumably are DXol, DXone, and 7-deoxyDXone, since they coeluted with the corresponding standards. DXol was constantly found as a small percentage (1%–3%) of the total fluorescence in the tumor and in all the organs examined at every time point. It has to be pointed out that HPLC analysis of the DX solution used for treatment showed an impurity, representing about 1% of total fluorescence, with the same retention time as DXol. The two aglycones were found in the liver only after as little as 5 min, and they were no longer detectable 2 h after treatment. In animals treated with 4-dm-4'-*O*-me-

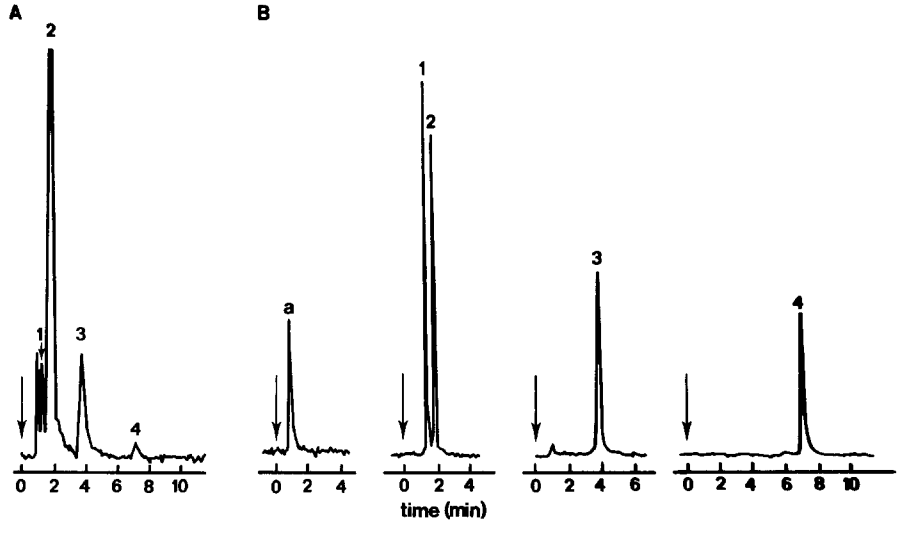

Fig. 1. **A** Chromatographic profiles of liver supernatant of DX treated mice; **B** chromatographic profiles of standard compounds. *1*, DXol; *2*, DX; *3*, DXone; *4*, 7-deoxyDXone; *a*, supernatant of precipitated livers from nontreated mice

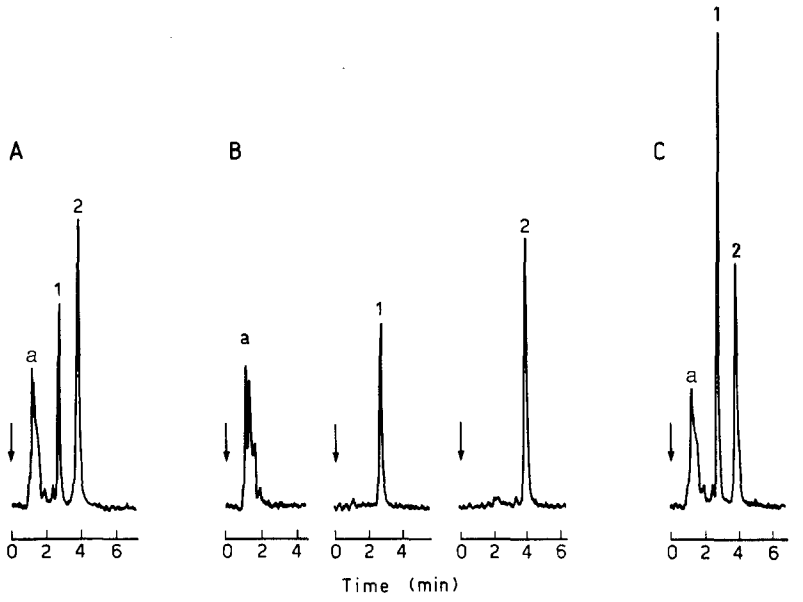

Fig. 2. **A** Chromatographic profiles of liver supernatant of 4-dm-4'-*O*-methylDX-treated mice; **B** chromatographic profiles of standard compounds. *1*, 4-dmDX; *2*, 4-dm-4'-*O*-methylDX; *a*, supernatant of precipitated livers from nontreated mice; **C** chromatographic profiles obtained by adding standard 1 **(B)** to the supernatant of precipitated livers of 4-dm-4'-*O*-methylDX-treated mice **(B)**

thylDX, a metabolite more polar than the parent drug was found in the liver (Fig. 2A), tumor, and all other organs. This metabolite is not 4-dm-4'-*O*-methylDXol, since it was found to be less polar than the reference standard (data not shown) and is not a conjugation product with glucoronic acid or sulfate ion, as confirmed when incubation of tissue homogenates with β-glucoronidase and arysulfatase did not alter the chromatographic pattern. In the chromatographic comparison this metabolite had the same retention time as 4-dmDX (Fig. 2B) and the two coeluted when present together in the same sample (Fig. 2C). The same results were obtained in other elution systems with CH_3CN and KH_2PO_4 at different pH values (from 2.2 to 5.1) and with different percentages of CH_3CN (from 30% to 40%) and in an elution system (data not shown) with $CH_3CN:CH_3OH:KH_2PO_4$ pH 5.1 (22:26:52). To check whether this metabolite is the result of a modification of the parent drug only in the aminosugar moiety, we compared its aglycone with the aglycones of the parent drug and of the standard 4-dmDX, all obtained by hydrolysis with HCl 0.1 *N* at 70 °C for 1 h. The three aglycones obtained had the same retention time. Small amounts (2% of total fluorescence) of two metabolites less polar than the parent drug, presumably aglycones, were found in the liver up to 10 min after treatment.

Pharmacokinetics

Figures 3–5 display the levels of DX, 4-dm-4'-*O*-methylDX, and its polar metabolite, and the fitted curves calculated by means of the constants reported in Tables 1 and 2.

Plasma drug levels (Fig. 3) in animals treated with the new analogue were detectable only up to 6 h after treatment, and they were about eight times lower than DX levels. The plasma elimination of both drugs was best described by a two-exponential curve (Table 1), and the half-lives of 4-dm-4'-*O*-methylDX were slightly lower than those of DX.

In the organs and the tumor (Figs. 4 and 5) DX was detectable up to last experimental point (168 h), while 4-dm-4'-*O*-methylDX and its metabolite were detectable only for 24–120 h in the organs and for 168 h in the tumor.

DX levels were best described by a three-exponential curve in heart, liver, lung, kidney, and small intestine

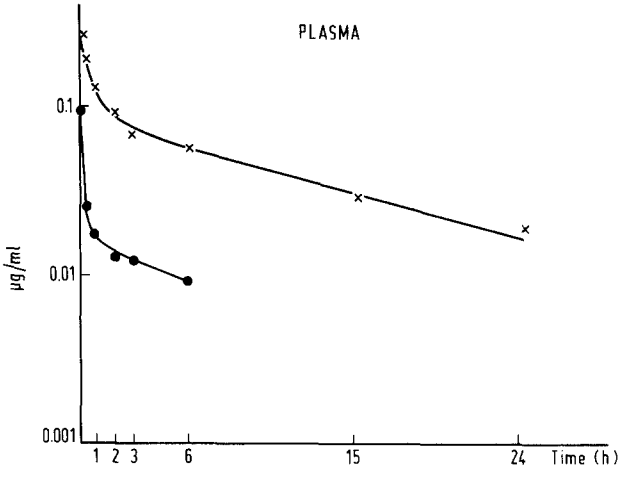

Fig. 3. Drug levels and relative fitted curves found in plasma of BDF1 mice bearing colon 38 SC treated IV with 4-dm-4'-*O*-methylDX 1 mg/kg (●) and with DX 10 mg/kg (X)

Table 1. Plasma pharmacokinetics parameters of DX and 4-dm-4'-*O*-methylDX in BDF1 mice bearing colon 38 SC

	DX	4-dm-4'-*O*-methylDX
$t_{1/2}$ (α) [a]	0.420	0.110
$t_{1/2}$ (β) [a]	10.230	6.300
AUC 0 → ∞ [b]	1.463	0.2018
Clearance [c]	150.370	109.120
Vdss [d]	2009.00	815.11

[a] Half-life of α and β phases (h)
[b] Area under the curve from 0 to ∞ (μg h/ml)
[c] Clearance (ml/h)
[d] Apparent steady-state distribution volume (ml)

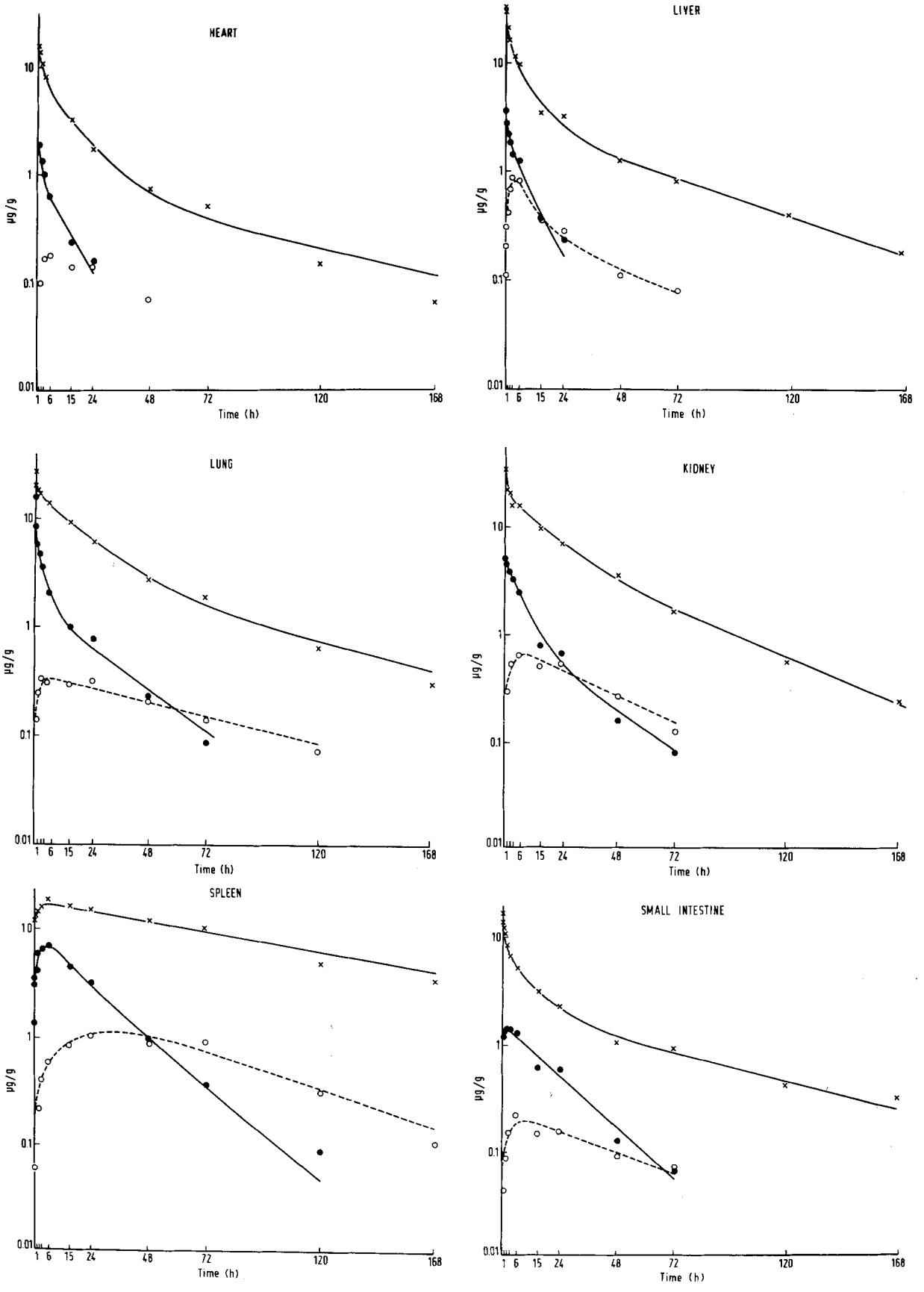

Fig. 4. Drug levels and relative fitted curves found in organs of BDF1 mice bearing colon 38 SC treated IV with 4-dm-4'-*O*-methylDX 1 mg/kg (●) and with DX 10 mg/kg (X) and its metabolite 4-dmDX (○). For the metabolite drug levels are reported as '4-dm-4'-*O*-methylDX fluorescent equivalent µg/g' (●)

(Fig. 4). In the spleen DX had an evident absorption phase which lasted about 6 h, then its elimination was monoexponential with a very long half-life ($t_{1/2} = 80$ h).

The kinetics of 4-dm-4'-*O*-methylDX in the organs cannot be described so exhaustively as for DX, owing to its low levels, undetectable at later times, and comparison of its kinetics with that of DX requires caution. In heart and liver, a two-compartment model was fitted to the levels of 4-dm-4'-*O*-methylDX, which were detectable only for 24 h. A comparison within 24 h between 4-dm-4'-*O*-methylDX and DX shows that the constant rates α and β are very similar, but nothing can be said about the γ-phase, which was found only for DX. The metabolite of 4-dm-4'-*O*-methylDX was eliminated from the liver at approximately the same rate as DX. As in the case of DX, a three-compartment model best fitted the levels of 4-dm-4'-*O*-methylDX in lung and kidney and the elimination of the analogue was found to be faster than that of DX. In these organs the levels of the metabolite followed a one-compartment model with elimination rates similar to those of DX. In spleen and small intestine 4-dm-4'-*O*-methylDX showed an absorption phase followed by bi- and monoex-

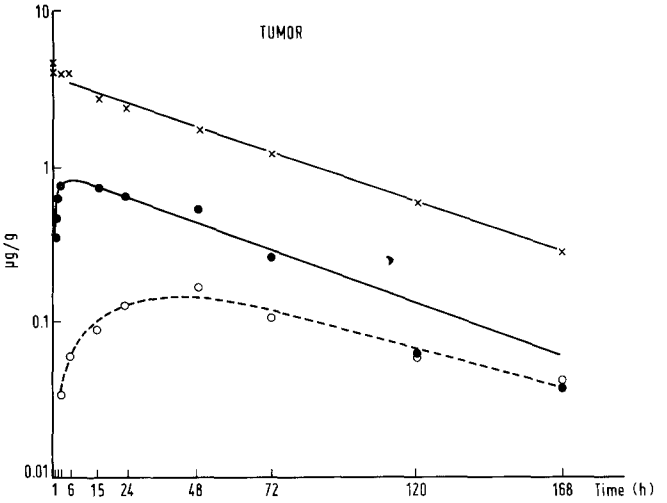

Fig. 5. Drug levels and relative fitted curves found in SC colon 38 tumor of BDF1 mice treated IV with 4-dm-4'-*O*-methylDX 1 mg/kg (●) and with DX 10 mg/kg (X) and its metabolite 4-dmDX (○). For the metabolite drug levels are reported as 4-dm-4'-*O*-methylDX fluorescent equivalent (µg/g)

Table 2. Pharmacokinetics parameters of DX and 4-dm-4'-*O*-methylDX in tumor and organs of BDF1 mice bearing colon 38 SC [a]

	Drug	k[b]	$t_{1/2}$[c]			AUC $0 \rightarrow \infty$ [d]	% Met.[e]	AUCs ratio[f]
			α	β	γ			
Tumor	DX		45.95			239.26		
	4-dm-4'-*O*-methylDX	0.77	51.37			58.80	25	3.28
	4-dm-4'-*O*-methylDX M[g]	0.04	46.97	55.70		19.69		
Heart	DX		1.17	8.98	58.00	195.73		
	4-dm-4'-*O*-methylDX		1.01	7.67		16.32	35	1.29
	4-dm-4'-*O*-methylDX M		n. d.			9.01		
Liver	DX		0.77	6.33	43.05	323.70		
	4-dm-4'-*O*-methylDX		0.31	6.30		22.23	51	1.40
	4-dm-4'-*O*-methylDX M	0.31	3.20	33.11		23.05		
Lung	DX		0.14	12.38	57.38	557.90		
	4-dm-4'-*O*-methylDX		0.14	2.22	19.64	67.00	27	1.64
	4-dm-4'-*O*-methylDX M	0.52	50.84			24.39		
Kidney	DX		0.40	10.06	33.59	594.02		
	4-dm-4'-*O*-methylDX		3.54	6.61	19.67	53.38	34	1.37
	4-dm-4'-*O*-methylDX M	0.35	29.50			28.04		
Spleen	DX	1.80	80.58			1996.41		
	4-dm-4'-*O*-methylDX	0.48	13.44	21.04		200.80	35	1.56
	4-dm-4'-*O*-methylDX M	0.04	36.69			110.70		
Small Intestine	DX		0.84	7.84	52.74	246.04		
	4-dm-4'-*O*-methylDX	3.21	15.00			36.30	27	2.03
	4-dm-4'-*O*-methylDX M	0.33	35.26			13.60		

[a] BDF1 female mice bearing colon 38 SC were treated IV with equiactive doses of 4-dm-4'-*O*-methylDX (1 mg/kg) and of DX (10 mg/kg). Experimental results, average of three animals per time, were analyzed according to a one- or a two- and three-compartment model

[b] k, absorption rate constant for DX and 4-dm-4'-*O*-methylDX and metabolism rate costant for 4-dm-4'-*O*-methylDX M

[c] $t_{1/2}$ half-life of α, β, and γ phases (h)

[d] AUC areas under concentration versus times curves from 0 to ∞

[e] Percentage of flourescene due to the metabolite = AUC M/total AUC

[f] AUCs ratio = (total 4-dm-4'-*O*-methylDX AUC/4-dm-4'-*O*-methylDX dose)/(total DX AUC/DX dose)

[g] M, metabolite

ponential elimination phase, respectively. The elimination of both 4-dm-4'-*O*-methylDX and its metabolite from these organs was found to be faster than that of DX. The elimination of the metabolite of 4-dm-4'-*O*-methylDX from all the organs was slower than that of the parent drug.

In the tumor (Fig. 5) the kinetics of DX, of 4-dm-4'-*O*-methylDX, and of its metabolite followed a one-compartment model with similar rates of elimination.

The percentage of fluorescence due to the metabolite of 4-dm-4'-*O*-methylDX was high, ranging from 25% of the total fluorescence in the tumor to 50% in the liver (Table 2).

For comparison of the distribution of the two drugs, the ratios between the AUCs of 4-dm-4'-*O*-methylDX divided by the administered dose and the AUCs of DX divided by the administered dose are reported in Table 2 (last column). The distribution of 4-dm-4'-*O*-methylDX was 1.3–2.0 times higher in all the organs and 3 times higher in the tumor than that of DX.

Discussion

The results reported in this paper show that the removal of the methoxyl group in position 4 of the aglycone and the presence of a methoxyl group in position 4' of the amino sugar in the molecule of the anthracycline DX lead to a different metabolic and pharmacokinetic behavior between the two drugs. 4-dm-4'-O-methylDX was extensively converted into a polar metabolite. No mass spectrometry analysis of this metabolite was possible because of the very low amounts found, and we are therefore not completely sure about its structure. All the chromatographic assays performed suggest that this metabolite is 4-dmDX, i.e., the result of demethylation in position 4' of the amino sugar. The activity of 4-dmDX has already been studied both in vitro and in vivo [6, 7, 15], and its biological properties have been summarized together with those of other 4-demethoxy anthracyclines [12].

This compound shares with the other 4-demethoxy derivatives the properties of being more cytotoxic, more potent, and less cardiotoxic at equiactive doses than the parent compound DX, in addition to which it is as cytotoxic and as active as 4-dm-4'-*O*-methylDX in experimental mouse tumor systems [12]. Since 4-dm-4'-*O*-methylDX is metabolized to a compound which has antitumor activity, we might ask whether the new analogue is active in itself or because of its metabolite.

In studies on HeLa and MS-2 cells grown in vitro, we found that 4-dm-4'-*O*-methylDX is not metabolized to 4-dmDX during a 24 h incubation, but that a small percentage (2%–10%) is metabolized to the 13-dihydroderivative (data not shown). These results suggest that this drug is cytotoxic in itself and that its antitumor activity in mice is therefore due to the drug itself and is sustained by its active metabolite. In contrast to 4-dm-4'-*O*-methylDX, DX was scarcely metabolized. In fact, besides the aglycone, which was detected in the liver only and only at early times, a small percentage (1%–3%) of total fluorescence was due to the 13-dihydroderivative and we are not sure that it was a result of metabolism of the drug in vivo, since it was already present in a small percente (1%) in the injected drug. These findings confirm previous studies showing that DX is metabolized in mice to a limited extent and yields only aglycones [3], unlike other species like rabbit, hamster, monkey, and also man, where DXol is the chief metabolite [1, 2, 5, 13, 16]. This fact shows up a species differences in anthracycline metabolism and it raises the question of the reliability of mouse tumors as experimental models for prediction of the activity of anthracycline in man.

Our findings on the pharmacokinetics of the two drugs show that the new analogue, like DX, is rapidly cleared from plasma and extensively deposited in tissues. The pattern of distribution of the two drugs in tumor and organs was similar except in the small intestine, where it was possible to show an absorption phase only for 4-dm-4'-*O*-methylDX. The rate of elimination of the new analogue was higher than that of DX in all the organs tested, while the two drugs had similar behavior in the tumor.

It has to be pointed out that after administration of equiactive doses of the two drugs, while DX was still present in the heart 7 days after treatment the new analogue was detectable only up to 24 h after, which may explain its lower cardiotoxicity compared with equiactive doses of DX [9]. In addition, the new analogue's property of accumulating to a higher extent than DX in the tumor than in organs suggests improved selectivity for the tumor and might explain its higher potency.

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