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Fluorescence Assay of Tissue Distribution of 4-Demethoxydaunorubicin and 4-Demethoxydoxorubicin in Mice Bearing Solid Tumors

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Summary. *The tissue distribution of 4-demethoxydaunorubicin and 4-demethoxydoxorubicin was studied in comparison with that of their parent compounds, daunorubicin and doxorubicin, in mice bearing transplanted tumors. The doses administered were equal or equitoxic to those of their parent compounds. The levels of total fluorescence due to initial drugs and metabolites were determined on tissue extracts by fluorometry. After administration of equal doses of daunorubicin and 4-demethoxydaunorubicin, the calculated Cxt values of 4-demethoxydaunorubicin equivalents were higher than those for daunorubicin in all the organs tested except the heart. In animals treated with equitoxic doses, lower 4-demethoxydaunorubicin levels were found in all the organs tested. In mice treated with equitoxic doses of doxorubicin and 4-demethoxydoxorubicin, 4-demethoxydoxorubicin reached higher drug concentrations than doxorubicin in spleen and liver, whereas in all the other organs tested lower drug levels were found. The rate of drug disappearance from organs was slower in animals treated with 4-demethoxyderivatives than in those treated with their parent drugs.*

Introduction

4-Demethoxydaunorubicin (4-dDNR) and 4-demethoxydoxorubicin (4-dDX), two new derivatives of daunorubicin (DNR) and doxorubicin (DX) characterized by the absence of the methoxyl group at the C-4 position, are highly effective against experimental mouse tumors at doses 4–20 times lower than those effective for their parent drugs [1, 9]. On exponential-phase HeLa cells, 4-dDNR and 4-dDX were more active and were taken up in greater amounts by mouse embryo fibroblast cultures than were their parent compounds [17].

The aim of this work was to study the tissue distribution of these derivatives in comparison with that of their parent drugs. The fact that 4-dDNR and 4-dDX are active even after oral administration [8, 10] induced us also to investigate the uptake of 4-dDNR after this route of administration.

Materials and Methods

Drugs

DNR, DX, 4-dDNR and 4-dDX were supplied by Farmitalia (Milan, Italy). Aqueous drug solutions were freshly prepared immediately before use and injected IV or given PO by stomach intubation in a volume of 10 ml/kg body weight.

Animals and Tumors

Adult female C_3H/He mice and female Ha/ICR mice of the CD_1 line were supplied by the Charles River Breeding Laboratories (Calco, Italy). The animals were 2–3 months old, weighed 20–30 g, and were maintained in standard laboratory conditions.

The experimental tumors used were solid sarcoma 180 implanted SC by trocar in CD_1 mice [7] and mammary carcinoma implanted SC as 20×10^6 cells from a tumor in the second generation in C_3H/He mice [6].

All the animals were used for tissue distribution studies when the tumors were palpable. Four animals were used for each experimental point.

Biochemical Assay of the Drugs

The levels of DNR, DX, 4-dDNR, and 4-dDX equivalents in plasma and tissues were estimated by fluorometry. This method does not differentiate between parent drugs and fluorescent metabolites.

After ether anesthesia, mice from each group were bled at appropriate times via the eye plexus into cold, heparin-coated glass

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tubes. After the blood had been centrifuged, the plasma was removed and stored at −70° C until analysis. Tissues were removed, rinsed in saline, and stored at −70° C until drug extractions. The gallbladder was always removed from the liver. Stomach and small intestine were always emptied before being rinsed in saline. The tissues were homogenized with six volumes of distilled water, and 1 ml homogenate was used.

The extraction of the drugs from plasma was performed with *n*-butyl alcohol [11] and extraction from tissue homogenates with isoamyl alcohol after extraction with $AgNO_3$ to release drugs bound to DNA and RNA and to precipitate proteins, as recommended by Schwartz [15]. To each sample, 0.2 ml 33% (w/v) $AgNO_3$ was added; the tubes were shaken vigorously for 2 min at 4° C, after which 4 ml *n*-butyl alcohol was added and shaking was repeated for a further 2 min. Samples were centrifuged at room temperature for 10 min at 2,000 *g*. The organic phase was removed and the pellets were extracted again. Reference standards were set up to correct the assay for the different fluorescent intensity and the different recovery of each drug from the tissues examined.

Scalar amounts of the drugs to be tested were added to plasma and each of the examined tissue homogenates from control mice; the standards were extracted as described above, and their fluorescent intensities were read on a Perkin-Elmer MPF 44A spectrofluorometer. The optimal excitation wavelength was 467 nm for all four compounds; the maximum emission wavelengths were 592 nm for DX and DNR and 570 nm for 4-dDX and 4-dDNR. The calibration curves obtained in this way were linear. The sensitivity of the assay was 0.05 μg/ml in plasma and 0.5 μg/g in tissues for DNR and DX, and 0.02 μg/ml in plasma and 0.2 μg/g in tissues for 4-dDNR and 4-dDX.

Drug concentrations of the experimental samples were determined by comparison with the fluorescent intensity calibration curve of each drug in each organ. Drug concentrations were expressed as drug equivalents (μg/g wet tissue).

Chromatographic Analysis

The *n*-butyl alcohol extracts were dried cold under a vacuum pump, and after dilution in 50 μl methanol they were spotted on 0.25-mm silica gel thin-layer chromatography (TLC) plates. Also spotted on each plate were 50 μl each standard drug solution in methanol and extracts of organs from untreated animals. The plates were developed in an ascending fashion in diethyl ether to separate neutral lipids, then in $CHCl_3$: CH_3OH : CH_3COOH (80 : 20 : 4) [2]. After drying, the plates were examined under a 3660 Å light to locate the fluorescent areas of the drugs and their metabolites.

Statistical Analysis

The areas under the concentration-versus-time curves (Cxt values) were determined by the trapezoidal rule. To compare the areas of the different groups, the variance of each area (V area)[1] was calculated,

1 $$\text{V area} = \left(\frac{1}{2}h_1\right)^2 \frac{V(c_1)}{n_1} + \left(\frac{1}{2}h_2\right)^2 \left[\frac{V(c_1)}{n_1} + \frac{V(c_2)}{n_2}\right] + \cdots \left(\frac{1}{2}h_k\right)^2 \left[\frac{V(c_{k-1})}{n_{k-1}} + \frac{V(c_k)}{n_k}\right]$$

Where: h_1 is the height of the triangle followed by trapezoids;
$h_2 \ldots h_k$ are the heights of the trapezoids;
$c_1 \ldots c_k$ are the bases of the trapezoids;
$n_1 \ldots n_k$ are the numbers of animals for each concentration

taking into account that such a variance is a linear function of the concentrations, which are independent of each other. In fact, the concentrations refer to different animals at different times [5]. The differences among areas were tested by Sheffé's test [16].

Results

4-Demethoxydaunorubicin

Tissue Drug Levels. A first series of experiments was carried out be measuring the distribution of DNR and 4-dDNR administered at equal doses IV in Swiss CD_1 mice bearing palpable solid sarcoma 180. Figure 1 shows drug-equivalent concentrations found in several organs at different times after injection of 12 mg DNR and 4-dDNR/kg. At 1 h after treatment, drug-equivalent concentrations of DNR and 4-dDNR were equal in all the organs tested except the lung, where 4-dDNR levels were higher. At 6 h and particularly at 24 h, drug concentrations in organs of 4-dDNR-treated animals were higher than in DNR-treated animals, except in the heart, where drug concentrations were similar. 4-dDNR concentrations were longer-lasting than those of DNR in all the organs tested. In both treated groups, drug levels in kidney, lung, liver, and heart decreased very quickly during the first 6–24 h and then the rate of disappearance of the drugs from these tissues decreased. In spleen and tumor there was no decrease in drug levels during the first 6 h, after which the rate of disappearance was very slow. In spleen and tumor of 4-dDNR-treated mice, the drug concentration increased during the first 6 h. The rate of disappearance of DNR from small intestine and stomach was uniform during the first 24 h after treatment, while in 4-dDNR-treated animals drug levels increased slightly during the first 6 h, possibly because of enterohepatic recirculation, and then decreased as in DNR-treated animals.

The tissue distribution of 4-dDNR was investigated after oral administration and compared with an equitoxic dose of 4-dDNR given IV. In mice treated once IV, the LD_{50} of DNR was 18 mg/kg, and the LD_{50} of 4-dDNR was 4.5 mg/kg after IV administration and 16.8 mg/kg after oral administration (Bertazzoli, personal communication). Mice were treated with doses corresponding to two-thirds of the LD_{50}: 3 mg/kg for 4-dDNR given IV, and 11 mg/kg for 4-dDNR given PO. The levels of fluorescent drug equivalents in several tissues are shown in Fig. 2.

During the first 6 h after treatment, in kidney, lung, liver, and heart there was a decrease in drug concentrations in mice treated IV, while in mice treated PO there was an increase. In spleen and tumor, drug concentrations increased until 6 h after both treatments. Stomach and small-intestine drug levels in mice treated PO were

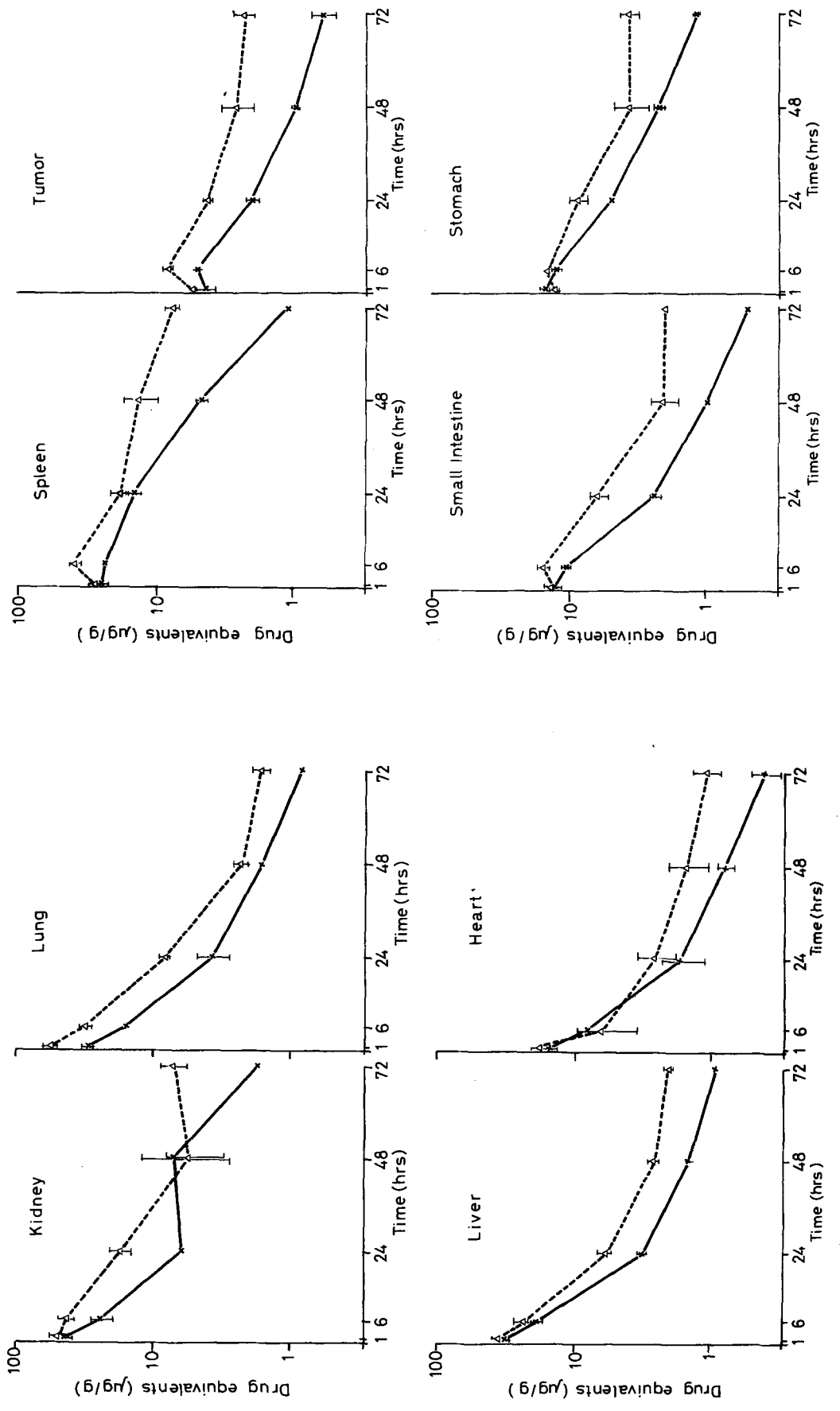

Fig. 1. Tissue distribution of daunorubicin 12 mg/kg IV (×——×) and 4-demethoxydaunorubicin 12 mg/kg IV (△– –△) in Swiss CD_1 mice bearing solid sarcoma 180

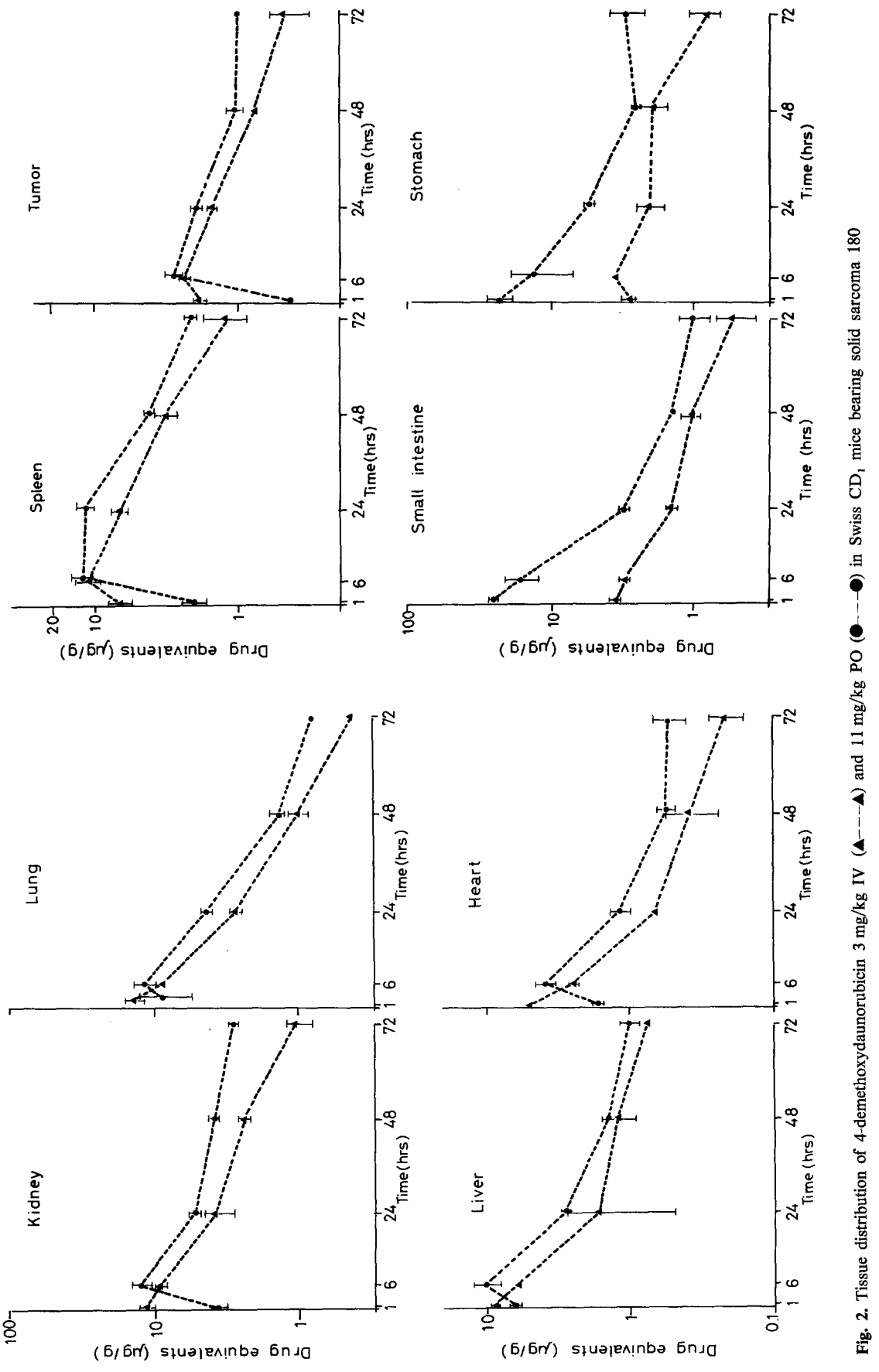

Fig. 2. Tissue distribution of 4-demethoxydaunorubicin 3 mg/kg IV (▲---▲) and 11 mg/kg PO (●---●) in Swiss CD_1 mice bearing solid sarcoma 180

Table 1. Areas under the concentration-versus-time curves (Cxt values mg/g × min ± SE) at 48 and 72 h after treatment

	Kidney	Liver	Lung	Heart	Stomach	Small intestine	Spleen	Tumor
After 48 h								
DNR (12 mg/kg) IV	36.66 ± 4.2	23.56 ± 0.8	22.46 ± 0.9	11.08 ± 0.7	19.21 ± 0.4	13.03 ± 0.4	42.01 ± 1.5	7.70 ± 0.3
4-dDNR (12 mg/kg) IV	62.69 ± 3.9[a]	31.43 ± 0.9[b]	44.60 ± 1.3[b]	12.06 ± 1.8	25.98 ± 1.3[a]	22.21 ± 1.0[b]	65.12 ± 3.4[a]	14.28 ± 0.5[b]
4-dDNR (3 mg/kg) IV	14.78 ± 0.9[a]	8.85 ± 0.3[b]	12.61 ± 0.3[b]	3.74 ± 0.1[b]	6.77 ± 0.4[b]	5.52 ± 0.2[b]	20.03 ± 1.5[b]	4.52 ± 0.1[b]
4-dDNR (11 mg/kg) PO	18.93 ± 1.1[a]	12.70 ± 1.2[b]	16.08 ± 1.1[a]	4.88 ± 0.4[b]	21.89 ± 3.5[c]	21.55 ± 2.4[c]	26.70 ± 1.8[a]	5.19 ± 0.3[a]
After 72 h								
DNR (12 mg/kg) IV	43.59 ± 5.4	25.17 ± 0.8	24.49 ± 1.0	11.95 ± 0.7	21.61 ± 0.4	14.11 ± 0.4	46.21 ± 1.5	8.82 ± 0.3
4-dDNR (12 mg/kg) IV	71.75 ± 4.4[a]	34.80 ± 1.0[b]	47.49 ± 1.3[b]	13.99 ± 1.9	31.23 ± 1.5[a]	25.13 ± 1.1[b]	80.31 ± 4.3[b]	17.83 ± 0.8[b]
4-dDNR (3 mg/kg) IV	17.27 ± 0.9[a]	10.30 ± 0.4[b]	13.63 ± 0.3[b]	4.18 ± 0.2[b]	8.46 ± 0.4[b]	6.66 ± 0.2[b]	23.28 ± 1.6[b]	5.43 ± 0.1[b]
4-dDNR (11 mg/kg) PO	23.69 ± 1.1[a,c]	14.41 ± 1.2[b]	17.65 ± 1.1[a]	5.66 ± 0.4[b]	25.91 ± 3.6[c]	23.31 ± 2.4[c]	31.27 ± 1.8[a]	6.67 ± 0.3[a]

[a] $P \leqslant 0.05$ vs daunorubicin 12 mg/kg IV
[b] $P \leqslant 0.01$ vs daunorubicin 12 mg/kg IV
[c] $P \leqslant 0.05$ vs 4-demethoxydaunorubicin 3 mg/kg IV

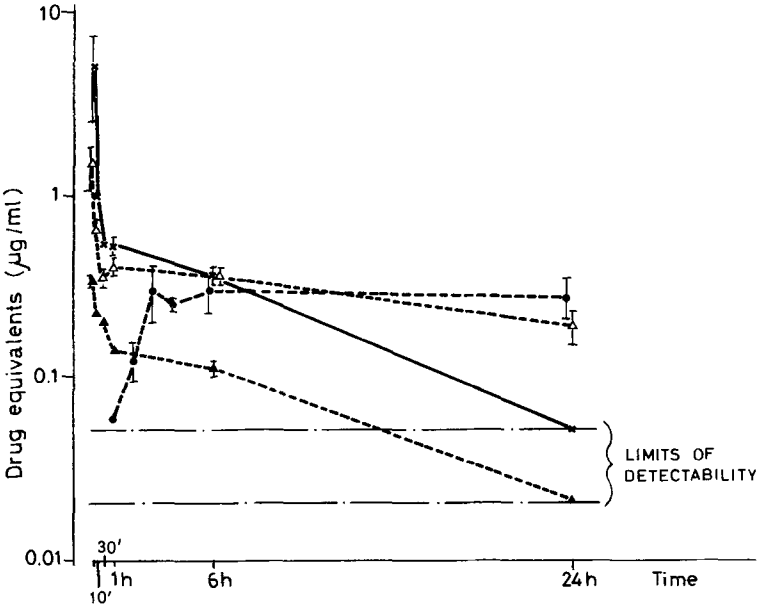

Fig. 3. Plasma clearance of daunorubicin 12 mg/kg IV (×——×) and 4-demethoxydaunorubicin 12 mg/kg IV (△– – –△), 3 mg/kg IV (▲– – –▲), and 11 mg/kg orally (●– – –●) in Swiss CD_1 mice bearing solid sarcoma 180

6–7 times higher than in animals treated IV 1 h after treatment, after which they fell to almost the same levels by 48 h after. At 72 h, in all the organs tested except liver, drug concentrations were higher after PO than after IV treatment.

From a comparison with drug levels reported in Fig. 1 after IV treatment with an equitoxic dose of DNR (= 12 mg/kg = two-thirds of the LD_{50}), one can see that 4-dDNR drug equivalents were lower than those of DNR in all the organs tested at 1 and 6 h after treatment, except for small intestine and stomach of mice treated PO.

The Cxt values for each organ investigated up to 48 and 72 h are reported in Table 1. When groups treated with equal doses (12 mg DNR and 4-dDNR/kg IV) were compared, significantly higher values were found in all the organs except heart of 4-dDNR-treated animals, both at 48 and at 72 h. After administration of equitoxic doses of DNR given IV (12 mg/kg), 4-dDNR given IV (3 mg/kg), and 4-dDNR administered PO (11 mg/kg), the values were significantly higher in all the organs of DNR-treated mice except stomach and small intestine of mice treated PO with 4-dDNR. The areas obtained after IV and PO administration of equitoxic doses of 4-dDNR are comparable in all the organs tested except kidney at 72 h and stomach and small intestine at 48 and 72 h, where the drug reached higher concentrations when given PO.

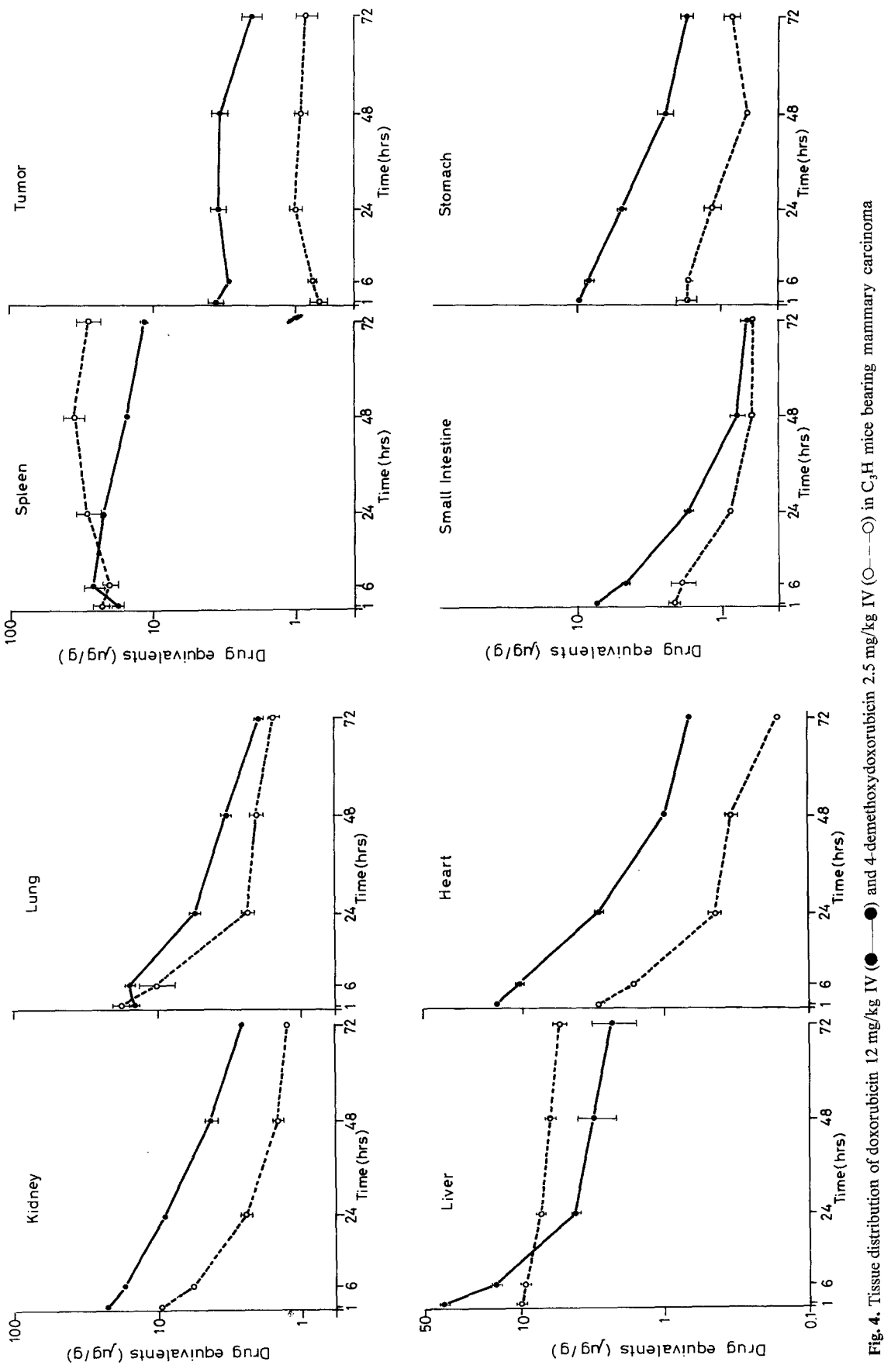

Fig. 4. Tissue distribution of doxorubicin 12 mg/kg IV (●—●) and 4-demethoxydoxorubicin 2.5 mg/kg IV (○—○) in C_3H mice bearing mammary carcinoma

Plasma Drug Concentrations. Figure 3 shows the drug concentrations found in plasma of DNR- and 4-dDNR-treated mice. When administered IV, both DNR and 4-dDNR were cleared from plasma very quickly during the first hour. Five minutes after treatment, drug levels in the plasma of mice treated IV with 4-dDNR were lower than those found in mice treated with equal doses of DNR. After 24 h DNR was no longer measurable, while 4-dDNR was still detectable. In the plasma of animals treated orally with 4-dDNR, the drug concentration increased during the first 3 h and drug levels were still measurable 24 h after treatment.

4-Demethoxydoxorubicin

Tissue Drug Levels. The tissue distribution of 4-dDX was compared with that of DX in mice bearing a transplanted mammary carcinoma. The animals were treated IV with the maximum tolerated doses of the two compounds (2.5 mg/kg for 4-dDX and 12 mg/kg for DX), as determined by percentage mortality at 3 months after treatment. The fluorescence concentrations found in several organs at different times are reported in Fig. 4. Higher levels of DX than of 4-dDX were found in kidney, heart, tumor, small intestine, and stomach at any time tested, and in lung from 24 h onward.

In the spleen of DX-treated mice, drug equivalents decreased from 24 to 72 h after treatment; in spleen of 4-dDX-treated mice no decrease of drug concentration was observed, so that at 48 and 72 h the drug levels were significantly higher than in DX-treated mice. In the liver, 4-dDX concentrations remained almost constant throughout the investigation, possibly because of recirculation from the spleen, while DX concentrations decreased. In tumor, drug equivalents of neither DX nor 4-dDX decreased during the investigated period. In kidney, lung, and heart, the release of the two drugs was similar. In small intestine and stomach, the rate of disappearance of 4-dDX was slower than that of DX.

Plasma Drug Concentrations. The plasma clearances of the two compounds are reported in Fig. 5. After a rapid decrease of both drugs during the first 30 min, the rate of 4-dDX disappearance was slightly slower than that of DX.

Metabolism

Chromatographic analysis was performed on the *n*-butyl alcohol extracts of liver, heart, spleen, and stomach of all experimental groups at 6 h after treatment. Owing to the high fluorescence due to tissue pigments, no quantitative analysis was possible. Polar metabolites and aglycones were found in liver, heart, and spleen of all the treated groups. No qualitative differences were seen between animals treated with the parent drugs and those that received their respective demethoxyderivatives.

In the stomach of animals treated orally with 4-dDNR, traces of polar metabolites and four aglycones were found, but most of the fluorescence was due to the parent drug.

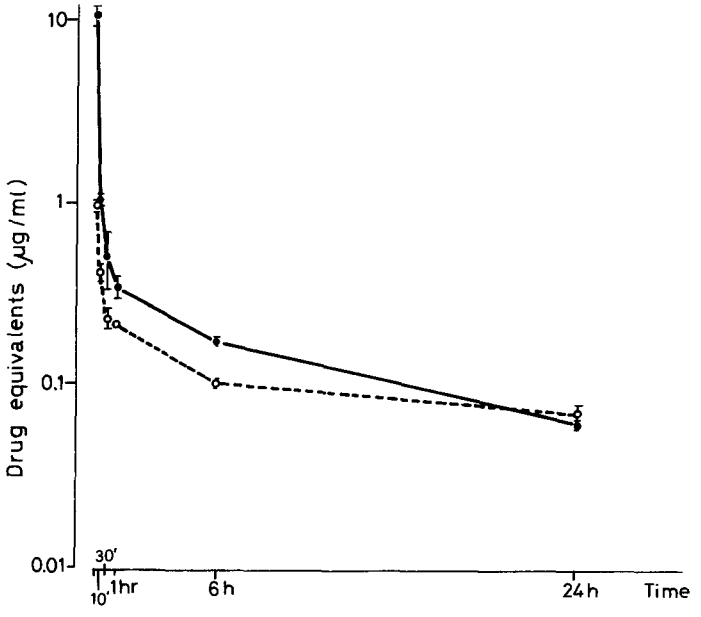

Fig. 5. Plasma clearance of doxorubicin 12 mg/kg IV (●——●) and 4-demethoxydoxorubicin 2.5 mg/kg IV (O– –O) in C_3H mice bearing mammary carcinoma

Discussion

Even taking into account that the assay we used does not distinguish metabolites from initial drugs, the results reported here show that the substitution of the methoxyl group in the C-4 position with a hydrogen atom in the molecules of DNR and DX can bring about profound differences in pharmacological behavior. In mice treated with equal doses of DNR and 4-dDNR the Cxt values of 4-dDNR equivalents were significantly higher than those found in DNR-treated mice in all the organs tested except the heart. Since the initial 4-dDNR equivalent levels were similar to those of DNR in all the organs tested except the lungs, the higher concentrations of 4-dDNR maintained with time can be attributed to the fact that 4-dDNR is released more slowly than DNR from organs. This is in accordance with the higher plasma levels of 4-dDNR than of DNR 24 h after treatment. The longer persistence of 4-dDNR than DNR in organs might be relevant for comparison of their activities in continuous and intermittent schedules of treatment [4].

In mice treated with equitoxic doses of DNR and 4-dDNR IV, the Cxt values for 4-dDNR were constantly lower than those for DNR. However, owing to the longer persistence of 4-dDNR than of DNR, at 72 h after treatment tissue levels of the two drugs were similar. These data show that the higher toxicity and potency of 4-dDNR than DNR is not due to a higher uptake in the tissues, but rather to a higher retention or to a higher activity of the drug at the cell level. It should also be considered that the uptake of the drugs by the organ in toto gives only partial information, since it has been shown that DX and DNR differ not only in cellular retention but also in intracellular distribution [14]. There may be similar differences between 4-demethoxyderivatives and their parent compounds, which would also be worthwhile investigating. A different rate of metabolism and the possibility that 4-dDNR can give rise to metabolites having different cytotoxic activity should also be taken into consideration.

At 72 h after treatment the Cxt values of the two drugs in tumor and heart were, respectively, 8.82 and 11.95 mg/g × min in DNR-treated mice, and 5.43 and 4.18 mg/g × min in 4-dDNR-treated mice. The ratios of tumor to heart Cxt values were therefore 0.74 for DNR and 1.30 for 4-dDNR. Whether or not this more favorable ratio is relevant for cardiac toxicity remains to be proved. In fact it has not been established whether the cardiac toxicity of anthracyclines is related to drug concentration in the heart. It might also be that the cytotoxicity of similar drug concentrations is different in different tissues.

Up to now only a few anthracyclines have been reported to be active when given orally: 4-dDNR and 4-dDX [8, 10], studied here, carminomycin [13], and aclacinomycin A, which has been reported to be well absorbed after oral administration [12]. The high lipid-water partition coefficient of 4-dDNR probably favors its rapid absorption across the membranes of the gastrointestinal tract. This rapid uptake can prevent the drug inactivation to aglycones due to the low pH of the gastric juices and to enzymatic cleavage [3]. In this study, the levels of 4-dDNR equivalents found in organs of mice treated PO were similar to those found in mice treated IV with equitoxic doses, and this can account for the antitumor activity exerted by oral 4-dDNR treatment. In stomach and small intestine of orally treated mice, drug concentrations shortly after treatment were higher than in IV treated mice. Whether the drug levels found were due to intracellular drug or to drug absorbed to the gastrointestinal surfaces cannot be established by the methods used in these experiments.

As observed with 4-dDNR, in mice treated IV with 4-dDX tissue concentrations were lower than those found in kidney, lung, tumor, heart, small intestine, and stomach of mice treated with an equitoxic dose of DX. Again, one could say that the higher toxicity of 4-dDX than DX is not due to a higher uptake, but to higher retention or activity of the drug at the cell level.

A peculiar behavior was observed in spleen and liver, which deserves some comment. The exceptionally high spleen levels of 4-dDX indicate that studies on the immunodepressive activity of this compound should be carried out, as previously suggested [9].

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