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Original Paper

Superior Therapeutic Efficacy of *N*-L-leucyl-doxorubicin Versus Doxorubicin in Human Melanoma Xenografts Correlates with Higher Tumour Concentrations of Free Drug

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***N*-L-leucyl-doxorubicin (Leu-DOX), a prodrug of doxorubicin (DOX), has previously shown antitumour activity against human ovarian, breast and lung carcinomas in nude mice. In the present study, the efficacy of Leu-DOX was compared with free DOX in inhibiting the growth of four DOX-sensitive and -resistant malignant melanoma xenografts. In an attempt to elucidate mechanisms underlying any differential effect, a sensitive high-performance liquid chromatography (HPLC) method was established for measuring plasma and tumour concentrations of the two drugs and their main metabolites. Leu-DOX was more effective than free DOX in inhibiting xenograft growth. At equitoxic intravenous doses of Leu-DOX (28 mg/kg) and DOX (8 mg/kg) administered to tumour-bearing nude mice, comparable levels of DOX were found in plasma, whereas differences were seen in tumour tissue concentrations. Thus, in animals carrying highly sensitive (LOX) and resistant (THX) melanomas, higher tumour concentrations of free DOX were observed in the Leu-DOX treated group from 24 up to 240 h after drug injection. Notably, the difference in drug-induced tumour growth inhibition correlated with differences in tumour exposure to free DOX, assessed as area under the curve (AUC) calculated over the first 48 h. In conclusion, the results confirm the prodrug nature of Leu-DOX and provide a possible explanation for its increased antitumour efficacy.** © **1999 Published by Elsevier Science Ltd. All rights reserved.**

Key words: doxorubicin, *N*-L-leucyl-doxorubicin, anthracyclines, melanoma, nude mice, human tumour xenograft, antitumour activity, HPLC, pharmacokinetics, AUC
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INTRODUCTION

N-L-LEUCYL-DOXORUBICIN (Figure 1) was developed as a prodrug of doxorubicin (DOX), and it has been supposed that Leu-DOX is converted into active DOX intracellularly or in the pericellular space by hydrolytic enzymes [1–3]. Tissue peptidases such as cathepsins, shown to have high expression levels in several tumour tissues including melanomas [4–7], are candidates for such activation, which may lead to higher concentration of free DOX close to and within the tumour, both at primary sites and metastases. At equitoxic doses, Leu-DOX has shown stronger activity compared with DOX against human tumour xenografts in nude mice [8, 9]. Moreover, at equimolar doses of the drug, toxicity studies in mice, rats and rabbits have indicated 3–4-fold lower overall toxicity [10–13], notably lower heart damage. Together these data indicate that the prodrug may have a wider therapeutic window than DOX. To investigate this further, we developed a sensitive HPLC method and studied the pharmacokinetics of DOX, Leu-DOX and its main metabolites in an experimental therapeutic setting involving subcutaneous (s.c.) human malignant melanoma xenografts. The aim was to examine whether pharmacokinetic parameters studied in blood and tissue samples could elucidate mechanisms underlying the differential efficacy of the two compounds.

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MATERIALS AND METHODS

Animals

Balb/c (nu/nu) nude mice were bred in our nude rodent facilities, maintained under specific pathogen-free conditions, with food and water supplied *ad libitum*. Housing and all procedures involving animals were performed according to protocols approved by the animal care and use committee at the Norwegian Radium Hospital, in compliance with National Ethical Committee's guidelines on animal welfare.

Tumour implantation was performed in 5–8 week old mice. When the tumours had reached a volume of approximately 100 mm^3, DOX or Leu-DOX were injected intravenously (i.v.) at equitoxic doses of 8 or 28 mg/kg [9], respectively. At 0, 5, 15, 30 min and 1, 2, 4, 8, 24, 48, 96 and 240 h after treatment, blood samples were taken by heart puncture from groups of 3 animals anesthetised with 10–20% halothan (Halothan, Zeneca, Cheshire, U.K.) in a mixture of N_2O and O_2 (flow: 3500 and 500 ml/min, respectively). Immediately thereafter, the mice were sacrificed by vertebral dislocation and the tumours were removed. Blood was centrifuged for 5 min at 3000*g*, and plasma and pieces of tumour tissue were frozen in liquid nitrogen and stored at $-20°C$ until analysed.

Tumour lines and construction of growth curves

Four human malignant melanoma xenograft lines LOX, THX, SESX and HHMSX [14–19] were used for studying antitumour drug effects. Fragments of xenografted tumour tissue were implanted s.c. in both flanks of nude mice, and in the therapy experiments the tumour diameters were measured twice weekly until the individual xenograft reached a maximum diameter of 20 mm. Tumour volumes and relative tumour volumes (RTV) were calculated as previously described [9], and median RTV values were used to construct growth curves and for calculating treatment efficacy. The tumour volume doubling time (TD) of test and control groups was defined as the period required to reach a median RTV value of 200 (TD_{200}) or 400 (TD_{400}).

Drugs, doses and calculation of antitumour effects

DOX, Leu-DOX and metabolites (Figure 1) were supplied by Medgenix Group (now; Coulter Pharma, Fleurus, Belgium). The drugs were dissolved in sterile water to obtain stock solutions of 12.5 mg DOX/ml and 20 mg Leu-DOX/ml, respectively. The stock solutions were kept protected from light at 4°C, and were further diluted with 0.9% NaCl immediately before use. Prior to the start of the study, dose-finding experiments for Leu-DOX and DOX in mice were carried out in order to confirm the previously determined maximum tolerated doses (MTDs). The MTD is defined as the dose causing a maximum median body weight loss of approximately 10–15% of the initial weight within 2 weeks after the first injection. In therapy experiments, both drugs were administered i.v. weekly for two weeks at the MTD [9].

Treatment efficacy was assessed by the evaluation criteria of specific growth delay (SGD) and optimal growth inhibition (T/C%). The SGD was calculated both for one and two doubling times ($SGD = (TD_{treated} - TD_{control})/TD_{control}$), from which the highest value obtained was chosen. Optimal growth inhibition on a particular day was calculated from the median RTV values of treated versus control ($T/C\% = RTV_{treated}/RTV_{control} \times 100\%$).

A T/C value of $<50\%$ and a SGD value of >1.0 were defined as minimum criteria for antitumour activity [19, 20].

Tissue preparation and HPLC analysis

Tissues were homogenised by sonication for 1 min after mechanical disintegration, using a Vibra-Cell VC50 50W sonicator (Sonics & Materials, Danbury, Connecticut, U.S.A.). The suspending liquid, composed of glucose:glucaric acid 1,4-lactone (in the ratio 0.5 g:3 g, Sigma Chemical Co, St Louis, Missouri, U.S.A.) in 1 L H_2O [21], was added to each sample tube (consisting of selected tumour samples weighing about 50–100 mg) to a final concentration of 0.1 g/ml. Of the homogenate, 0.2 ml was used for further analysis allowing a volume of 50 μL to be injected in triplicate into the HPLC system.

Plasma and tissue homogenates were extracted and purified using Sep-Pak C_{18} cartridges (SEP-PAK, Waters Associates, Inc., Milford, Massachusetts, U.S.A.) as described by the manufacturer and by others [21–23]. Reverse phase HPLC analysis was performed using a Shimadzu LC-6A pump and RF-535 fluorescense detector, combined with Nucleosil C_{18} column (KS Nucleosil 100-5 C_{18}, 250×4 mm, Machery-Nagel, Düren, Germany) and Nucleosil 120-5 C_{18}, 30×4 mm pre-

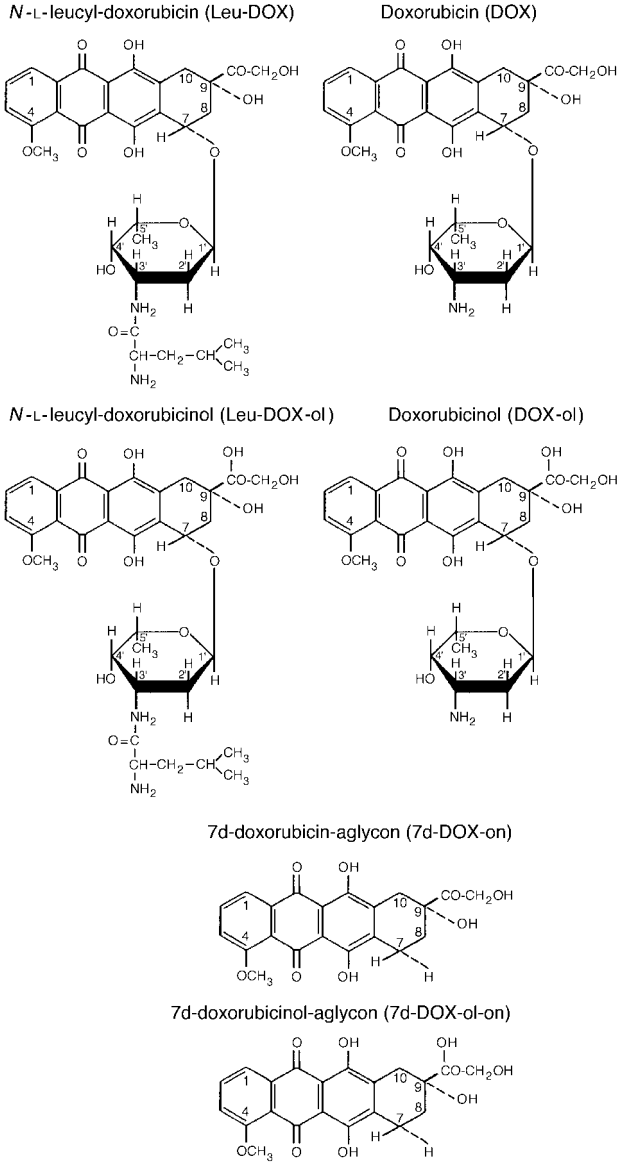

Figure 1. Molecular formula of DOX, Leu-DOX and metabolites.

column. The detector was operated at 480 nm excitation and 550 nm emission wavelengths, respectively. Elution of Leu-DOX, DOX and metabolites was completed within 12 min, using a mixture of 32% acetonitrile in sodium dihydrogen phosphate buffer (0.02 M, pH = 3.5) at a flow rate of 1 ml/min. The injection volume into the column was kept at 50 μL throughout the analyses. Calibration curves for DOX from spiked standard samples showed linearity in the concentration range of interest (0.01–100 nmol/ml, $r^2 \geq 0.99$). The limit of detection of Leu-DOX, DOX, DOX-ol and Leu-DOX-ol were <0.01 nmol/g for plasma and tumour tissue. Assay variability for Leu-DOX, DOX, DOX-ol and Leu-DOX-ol, calculated as coefficient of variation (CV) ranged from 1.7 to 2.9 within-run, and 3.3 to 8.2 between-run (data not shown).

RESULTS

In vivo *antitumour effects*

MTD's of 8 and 28 mg/kg had previously been determined for DOX and Leu-DOX [8, 9]. In the present study, the same level of body weight reduction, 3 to 10% for DOX and 3 to 8% for Leu-DOX (Table 1) was observed at these doses, confirming the MTDs.

Both Leu-DOX and DOX showed activity against the LOX and SESX melanomas, whereas HHMSX according to the activity criteria was sensitive only to Leu-DOX (Table 1). In mice with LOX xenografts, Leu-DOX caused a significant reduction in median tumour volume, whereas DOX resulted in only growth retardation (Figure 2a). The initial reduction in tumour size observed with Leu-DOX, administered on days 0 and 7, continued until day 14, at which time the tumours started to regrow at the same rate as those in the untreated control group. The SGD_{200} and T/C% values for Leu-DOX were 7.8 and 3.9%, respectively, compared with 0.8 and 13% for DOX (Table 1). With THX both drugs failed to fulfil the criteria for activity, but marginal and similar growth-retarding effects were observed, with SGD and T/C% values of ≪1 and 36.6% for Leu-DOX and ≪1 and 33.6% for DOX (Figure 2b). Both compounds retarded the growth of SESX xenografts, with slightly better effects for the prodrug, reflected in SGD and T/C% values of 1.4 and 32% for Leu-DOX and 0.6 and 39.7% for DOX (Figure 2c). The HHMSX tumour showed differences in response to the two drugs, with SGD and T/C% values of 0.5 and 32% for Leu-DOX and ≪1 and 50.6% for DOX (Figure 2d). Based on these data, the LOX and THX tumours were selected for pharmacokinetic studies because of differences in sensitivity to the two drugs but with similarity in growth rates.

Plasma concentrations

For both compounds, the plasma concentrations were similar, with slightly higher drug concentration levels in mice carrying LOX compared with THX melanomas. This difference was seen during the first 24 h, both for DOX metabolised from Leu-DOX (DOX met.), and for DOX (Figure 3). In mice with LOX tumours the plasma levels slowly declined from 0.32 (DOX met.) and 0.55 (DOX) nmol/ml at 2 h to 0.03 nmol/ml at 48 h in both cases. The comparable levels in mice with THX xenografts were, respectively, 0.36 and 0.22 nmol/ml at 2 h, declining to 0.06 and 0.05 nmol/ml at 48 h. Independent of tumour line, the plasma levels of unmetabolised Leu-DOX decreased rapidly during the first 8 h, from 4.2 and 2.0 nmol/ml at 1 h down to a threshold level of 0.03 nmol/ml after 8 h in LOX and 0.01 nmol/ml at 8 h in THX bearing animals.

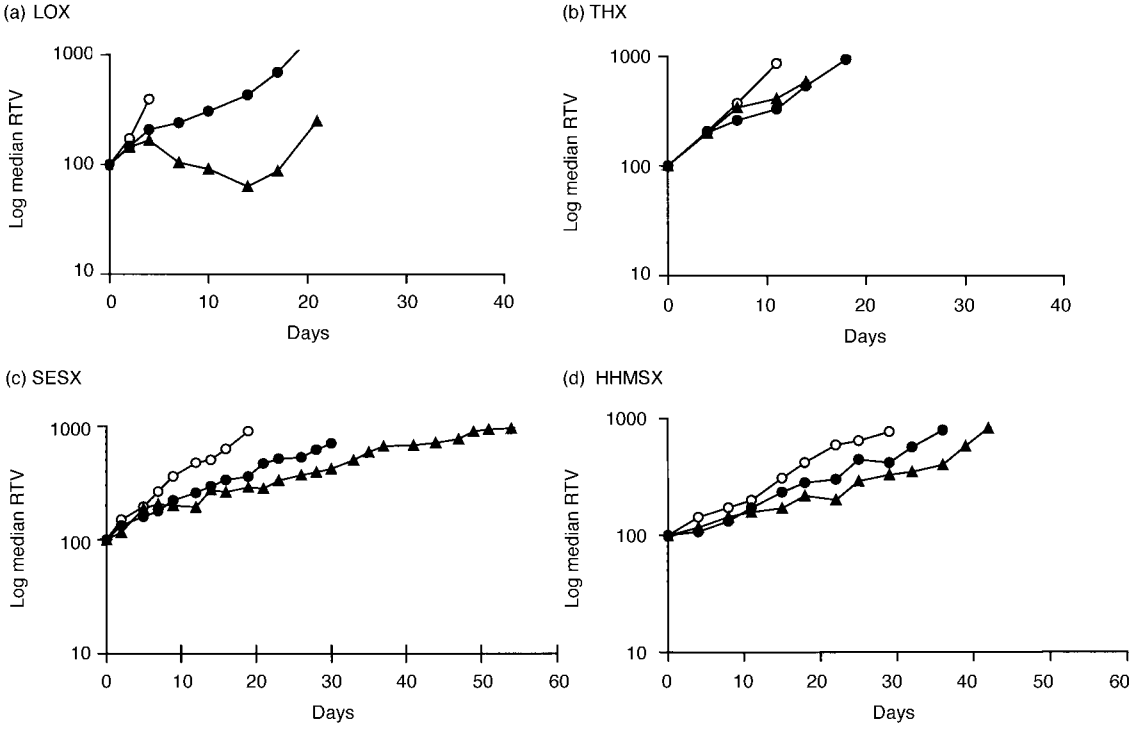

Figure 2. Growth curves of four different human malignant melanoma xenografts in nude mice: Groups: saline control (○–○), DOX (●–●) and Leu-DOX (▲–▲) given intravenously (i.v.) days 0 and 7. The difference at TD_{400} between both drug groups and control was statistically significant ($P \leq 0.005$) in all experiments except with THX. Leu-DOX treatment was significantly better than DOX in the LOX ($P \leq 0.005$), SESX ($P \leq 0.01$) and HHMSX ($P \leq 0.025$) experiments.

Table 1. *Antitumour effects of DOX (*8 *mg/kg) and Leu-DOX (*28 *mg/kg) in human malignant melanoma xenografts, intravenously days* 0 *and* 7

Tumour	Drug	Dose mg/kg	Number of tumours	Body weight loss in %	Toxicity	TD_{200}	TD_{400}	T/C%	SGD_{200}	SGD_{400}	Efficiacy*
	Control	–	16	—	0/8	2.2	4.0	100	–	–	
LOX	DOX	8	16	8	0/8	3.7	13.0	13.0	0.8	2.3	+++
	Leu-DOX	28	16	7	0/8	19.7	22.5	3.9	7.8	4.5	++++
	Control	–	12	–	0/8	3.7	7.2	100	–	–	
THX	DOX	8	9	3	0/7	4.0	10.2	33.6	0.03	0.7	(+)
	Leu-DOX	28	10	3	0/8	4.0	12.0	36.6	0.04	0.4	(+)
	Control	–	10	–	0/7	5.2	10.0	100	–	–	
SESX	DOX	8	10	3	0/7	8.0	19.7	39.7	0.6	1.0	+
	Leu-DOX	28	8	3	1/7†	12.2	28.7	32.0	1.4	1.9	++
	Control	–	8	–	0/7	11.0	17.5	100	–	–	
HHMSX	DOX	8	9	10	0/7	12.7	24.0	50.6	0.2	0.4	–
	Leu-DOX	28	9	8	0/7	16.7	35.5	32.0	0.5	1.1	+

*Efficacy as defined in [19]. †Sacrificed at day 7 due to poor health condition. (+), T/C <50% or SGD >1.0; +, T/C <50% and SGD >1.0; ++, T/C <40% and SGD >1.5; +++, T/C <25% and SGD >2.0; ++++, T/C <10% and SGD >3.0. TD, tumour doubling time; T/C, treatment versus control; SGD, specific growth delay; DOX, doxorubicin; Leu-DOX, *N*-L-leucyl-doxorubicin.

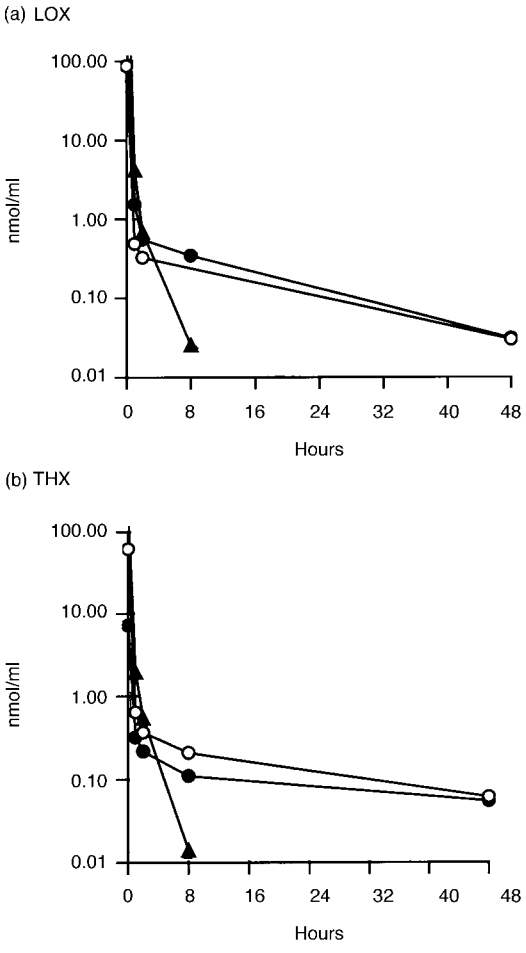

Figure 3. Drug plasma levels in nude mice carrying subcutaneously (s.c.) LOX (a) or THX (b) melanoma xenografts, after treatment with Leu-DOX (28 mg/kg) or DOX (8 mg/kg). Each figure shows the plasma concentration over a time period of 48 h, for Leu-DOX (▲–▲), its main metabolite (DOX met.) (●–●), and for DOX (○–○).

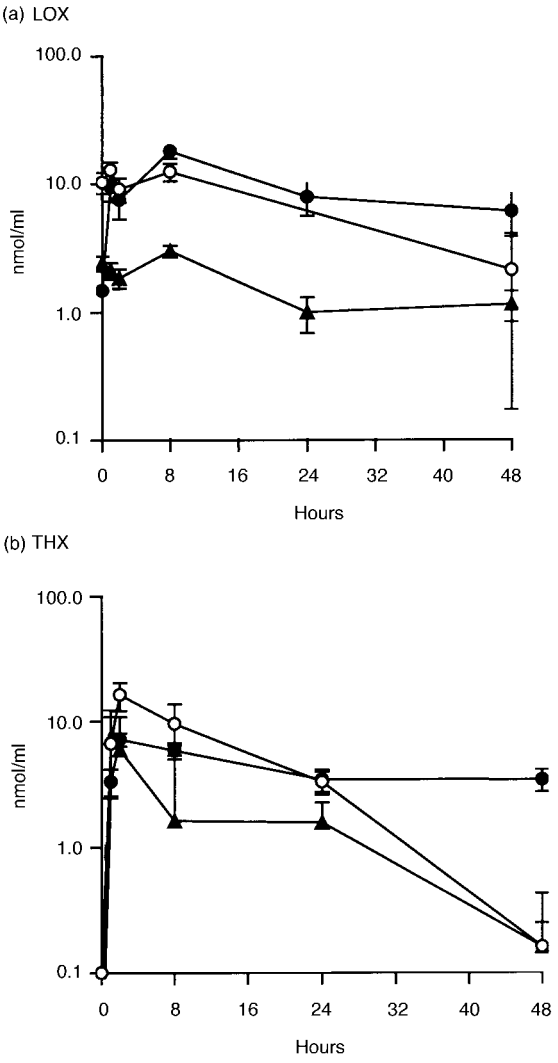

Figure 4. Drug tumour levels in subcutaneous (s.c.) melanoma xenografts after treatment with Leu-DOX (28 mg/kg) or DOX (8 mg/kg). Each figure shows tumour tissue concentration over a time period of 48 h, for Leu-DOX (▲–▲) and its main metabolite (DOX met.) (●–●), and for DOX (○–○).

Table 2. AUC values in nmol/g/min of drug and their metabolites in LOX and THX human tumour xenografts, after injection of one intravenous bolus injection of DOX (8 mg/kg) and Leu-DOX (28 mg/kg)

	LOX tumour AUC_{48h}		THX tumour AUC_{48h}		THX tumour AUC_{240h}	
Compound	Leu-DOX 28 mg/kg	DOX 8 mg/kg	Leu-DOX 28 mg/kg	DOX 8 mg/kg	Leu-DOX 28 mg/kg	DOX 8 mg/kg
Leu-DOX	6021	–	5323	–	5323	–
Leu-DOX-ol	849	–	134	–	134	–
DOX	34182	21058	12838	13540	21291	15880*
DOX-ol	1230	24	340	0	340	0
7d-DOX-on	806	0	948	0	948	0
7d-DOX-ol-on	0	3	0	0	0	0

*AUC at 96 h, since DOX was not detectable after 240 h. AUC, area under the curve; Leu-DOX, *N*-L-leucyl-doxorubicin; DOX, doxorubicin.

Tumour concentrations and AUC

In drug-sensitive LOX tumours, high levels of Leu-DOX, DOX met. and DOX were observed compared with THX xenograft tissue. The concentration versus time curves (Figure 4a) showed initially a slightly higher level of DOX (peak 13 nmol/g after 1 h), compared with DOX met. However, at 4 h the curves crossed each other with DOX met. reaching its peak level of 18 nmol/g after 8 h. Thereafter, the concentration levels decreased to 6.0 and 2.1 nmol/g at 48 h for DOX met. and DOX, respectively. The curve for non-metabolised Leu-DOX followed that of DOX met. but at much lower levels (peak 3 nmol/g after 8 h).

In animals carrying relatively resistant THX xenografts, the drugs were cleared more rapidly from the tumour tissue than what was observed with LOX (Figure 4b). The peak levels were 16.2 nmol/g for DOX (after 2 h), 8.3 nmol/g (after 4 h) for DOX met., and 7.5 nmol/g after 1 h for Leu-DOX. Notably, whereas the tumour concentrations for both DOX and Leu-DOX decreased to 0.16 nmol/g after 48 h, the DOX met. level remained constant, ending at 3.44 nmol/g after 48 h. Because of this unexpected high late level of DOX met., drug levels in THX tumours were followed for longer. DOX met. levels were measurable for up to 240 h (not shown). At 96 h, the level of DOX met. was 0.7 nmol/g (compared with 0.3 nmol/g, for DOX), and 0.1 nmol/g at 240 h. Leu-DOX was undetectable at 96 h and DOX at 240 h.

The time points 1, 2, 8, 24 and 48 h were used for calculation of the area under the curve (AUC) values after the trapezium rule. After treatment of LOX tumours with Leu-DOX, the AUC values were 6021 and 34182 nmol/g/min for Leu-DOX and DOX met., respectively (Table 2). Low levels of the metabolites Leu-DOX-ol (849 nmol/g/min) and DOX-ol (1230 nmol/g/min) were also found. After treatment with equitoxic dose of DOX (8 mg/kg), the calculated AUC for DOX was 21058 nmol/g/min whereas DOX-ol was detected in one sample with AUC calculated to 24 nmol/g/min.

In THX tumours, AUC values were 5323 and 12838 nmol/g/min for Leu-DOX and DOX met, respectively (Table 2), with Leu-DOX-ol and DOX-ol levels of 134 and 340 nmol/g/min, respectively. In DOX treated mice, the AUC of DOX was 13540 nmol/g/min, a value similar to that for DOX met., in contrast to the difference found in Leu-DOX-sensitive LOX tumours.

Of the aglycons 7d-DOX-on and 7d-DOX-ol-on (Figure 1), only the first was detected, and only after treatment with Leu-DOX. The AUC levels were 806 and 948 nmol/g/min in LOX and THX tumours, respectively.

DISCUSSION

Differential effects and an overall higher growth inhibitory efficacy was observed for Leu-DOX, compared with equitoxic doses of the parent compound DOX, in 3 out of 4 s.c. xenografted human malignant melanomas in nude mice. Pharmacokinetic distribution data of the drugs and their main metabolites in two of the tumour xenografts provide a possible explanation for the observed differences in antitumour effects. Thus, differences in calculated AUC values for the active compound correlated with differences between Leu-DOX and DOX in antitumour activity.

The stronger inhibition of s.c. melanoma growth observed with Leu-DOX is in agreement with previous studies with the two compounds in xenografts of other tumour types. In a study on breast and lung tumours 10/16 (63%) of the lines were sensitive to Leu-DOX, whilst only 7/16 (44%) responded to DOX [9]. In another study, growth inhibition >50% was observed for Leu-DOX in three out of four (75%) xenografted human ovarian lines, as compared with two (50%) for DOX [8].

The present pharmacokinetic findings show that metabolisation of Leu-DOX into DOX can cause prolonged higher concentrations of free DOX in tumour tissue, and that the resulting increased AUC of DOX in the tumour xenografts correlates with improved antitumour efficacy. The results support the hypothesis of a prodrug nature for the biotransformation of Leu-DOX. The increased tumour, but not plasma, concentration of the active metabolite DOX, might be explained by an enzymatic cleavage of the prodrug, taking place either on the surface of or inside the tumour cells [1]. The melanoma lines LOX and THX have shown high expression of the proteolytic enzyme cathepsin B (data not shown), a candidate enzyme for such activation. The magnitude of prodrug activation in the tumours assessed as AUC_{48h} of free DOX differed, however, significantly between LOX and THX, in agreement with the differences between the two tumour lines in their response to Leu-DOX.

Indications that a relationship could exist between the tumour tissue pharmacokinetics and the antiproliferative effects of four anthracyclines, including DOX and Leu-DOX and their metabolites, was found in a previous study involving ovarian xenografts [24]. However, another study in which equimolar doses of Leu-DOX and DOX were given to mice carrying a resistant murine colon tumour line, failed to find such correlations [11].

In our study, the doses chosen for the pharmacological and therapeutic experiments were the same. In animals treated with equitoxic doses of Leu-DOX or DOX (28 mg/kg or

8 mg/kg), the plasma concentration versus time profiles of active DOX were similar in mice carrying LOX and THX xenografts. These findings indicate that the two compounds may have a similar acute toxicity, when given at equitoxic doses. Together with the generally superior antitumour efficacy (Table 1) [8, 9] this should give Leu-DOX an improved therapeutic window compared with DOX. In addition, lower initial peak concentrations of free DOX (Figure 3) may result in a reduced risk of cardiotoxicity, including acute myocarditis and pericarditis that is related to peak plasma concentration of DOX [25–28].

Previous toxicity studies have, by relating levels of DOX to the toxicity observed, suggested a prodrug nature of Leu-DOX. The maximum tolerated dose of Leu-DOX was approximately 3-fold higher than that used for standard treatment with DOX, in animal species like mice [10, 11], rats [12] and rabbits [13], as well as in two clinical phase I studies [29, 30]. Questions arose about whether the reduction in MTD was due to low cellular uptake or to a limited biotransformation of the prodrug to the active species doxorubicin, causing an equivalent reduction in antitumour efficacy. Bennis and colleagues [31] found an association between *in vitro* cytotoxicity and cellular incorporation of DOX and Leu-DOX in two cell lines (MCF-7 breast cancer and K562 leukaemia) and their DOX-resistant counterparts, not related to differences in biotransformation of Leu-DOX to DOX. DOX appeared as the likely cytotoxic species after Leu-DOX administration, and intracellular transformation of Leu-DOX to DOX did not appear as a limiting step in their study.

The pharmacokinetic data presented here show a rapid biotransformation of Leu-DOX into DOX in the plasma compartment and a possible correlation between the plasma level and toxicity to normal haematological cells, since the drugs were given at equitoxic doses. The drug concentration in plasma did not, however, reflect the levels in tumour tissue and a difference has also been shown to exist between different tissue compartments [11]. In rabbits, a reduced concentration level in heart tissue, and a resulting lower cumulative cardiotoxicity, has been demonstrated for the related compound leucyl-daunorubicin compared with what was found with equitoxic doses of daunorubicin. A study in Balb/c mice showed a 10-fold reduction in the AUC level in the heart tissue for DOX after Leu-DOX compared with DOX, when the drugs were given at equimolar doses [11].

The prolonged high tumour tissue levels of DOX after treatment with Leu-DOX compared with DOX seen in our study, is likely to be caused by both uptake of metabolised Leu-DOX in the plasma, and by biotransformation of Leu-DOX into DOX in the tumour tissue. The data emphasise the importance of parallel pharmacokinetic and therapeutic *in vivo* studies of prodrugs such as Leu-DOX.

The increased antitumour effects observed here with malignant melanoma xenografts, comparable to the effects obtained in previous studies involving breast, lung and ovarian cancers, suggest a potential for Leu-DOX in the treatment of patients with a number of different tumour types. Compared with DOX, the possibility for lower toxicity together with improved antitumour efficacy, should encourage further clinical evaluation of Leu-DOX.

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