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Analysis and pharmacokinetics of *N-l*-leucyldoxorubicin and metabolites in tissues of tumor-bearing BALB/c mice

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Summary. Leucyldoxorubicin (Leu-Dox) was developed as a prodrug of doxorubicin (Dox) with the aim of lowering the cardiotoxicity and improving the therapeutic index produced by Dox. To support the preclinical findings on its antitumor activity and cardiotoxicity, concentrations of Leu-Dox and its metabolites were determined in plasma, heart, and tumor after the administration of Leu-Dox to tumor-bearing mice. A liquid-liquid extraction procedure employing a chloroform/2-propanol/dimethylsulfoxide (DMSO) mixture was developed. By means of high-performance liquid chromatography (HPLC) with fluorescence detection, Leu-Dox and six of its metabolites could be assayed in the tissues with high sensitivity. Detection limits ranged from 0.01 nmol/g tissue for the aglycons to 0.06 nmol/g for Dox. Recoveries were in the range of 82%–110%, and calibration curves were linear over the concentration range tested (0.1–10 nmol/g tissue, $r \geq 0.998$). Concentration versus time curves were constructed for plasma, heart, and tumor over the first 72 h, and areas under the curves (AUCs) for the first 48 h were determined by the trapezoidal rule. Dox was rapidly formed from Leu-Dox, reaching peak levels in plasma within 5 min and in tissues within 1 h after i.v. administration of Leu-Dox (12 mg/kg). The elimination of Leu-Dox was also fast as illustrated by final half-lives of 1.1, 0.8, and 0.9 h in the plasma, heart, and tumor, respectively. For Dox, the final half-lives were 16.7 h in plasma, 15.3 h in heart tissues, and 27.4 h in tumor tissues. AUC values determined for Leu-Dox and Dox were 221 and 51 nmol ml^{-1} min, 443 and 4,262 nmol g^{-1} min, and 153 and 1,466 nmol g^{-1} min in the plasma, heart, and tumor, respectively. Comparison of these values with those obtained after an equimolar dose of Dox indicated 26%, 30%, and 16% of Leu-Dox appeared as Dox in the plasma, heart, and tumor, respectively. Thus, not only is the plasma compartment not representative for calculations of the conversion of Leu-Dox into Dox in tissue, but differences in its appearance also exist between the tissue compartments. The AUC values found for Dox in the heart may explain the reduced cardiotoxicity elicited by Leu-Dox as compared with Dox; however, the values obtained for Dox in the insensitive murine colon tumor cannot explain the enhanced antitumor activity exerted by Leu-Dox in the sensitive human tumor xenografts in nude mice.

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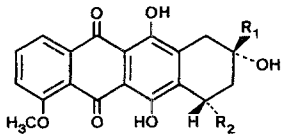
Introduction

Although doxorubicin (Dox) is successfully used against a variety of tumors, the cardiotoxicity associated with chronic treatment has provoked scientists to search for less cardiotoxic analogs. A promising direction may be the development of prodrugs from which the active anthracycline is (more or less) selectively released in the tumor. Among a range of amino acid derivatives of Dox, *N-l*-leucyldoxorubicin (Leu-Dox) has shown an efficient hydrolytic release of Dox by tissular peptidases [11]. In nude mice bearing human tumor xenografts, Leu-Dox displayed higher antitumor activity than Dox when both drugs were given at the maximum tolerated dose (E. Boven, unpublished data).

It was shown that the area under the concentration versus time curve (AUC) of Dox in the heart tissue of rabbits and mice was lower following the administration of the prodrug than after treatment with Dox itself when the two anthracyclines were given at equimyelotoxic doses [6, 9]; accordingly, diminished cardiotoxicity was observed in rabbits [9]. On the other hand, there is increasing evidence that metabolites of Dox, especially the 13-dihydro metabolite doxorubicinol (Dol), may play a role in the development of cardiotoxicity [13]. Therefore, it is also of importance to know the tissue concentrations of this and other Leu-Dox metabolites.

In contrast to Dox [1, 14, 15], no data are available on the concentration of Leu-Dox and its metabolites in tumor

Table 1. Molecular structures of the test compounds



Compound	Abbreviation	R_1	R_2	
N-l-leucyldoxorubicin	Leu-Dox	$COCH_2OH$ (A)		R_3 = *l*-leucyl = $-COCH(NH_2)CH_2CH(CH_3)_2$
N-l-leucyldoxorubicinol	Leu-Dol	$CH(OH)CH_2OH$ (B)		*l*-leucyl
Doxorubicin	Dox	A		H
Doxorubicinol	Dol	B		H
Doxorubicin aglycon	Doxon	A	OH	
Doxorubicinol aglycon	Dolon	B	OH	
7-Deoxydoxorubicin aglycon	7d-Doxon	A	H	
7-Deoxydoxorubicinol aglycon	7d-Dolon	B	H	

tissue. Therefore, the aim of the present study was to determine the pharmacokinetics of Leu-Dox in the same animal model used in prior investigations of Dox [15], thus enabling a comparison of heart and tumor exposure to both of these anthracyclines and their metabolites (see Table 1). To that end we analyzed Leu-Dox and its metabolites in plasma using our recently developed assay [4]. For tissues, we modified our existing procedures for the analysis of Dox, 4′-epidoxorubicin [10], and daunorubicin [2] as well as their metabolites.

Materials and methods

Materials. *N-l*-leucyldoxorubicin and *N-l*-leucyldoxorubicinol were kindly provided by Medgenix Group (Fleurus, Belgium); all other anthracyclines were supplied by Farmitalia Carlo Erba (Milan, Italy). Hexane sulfonic acid monosodium salt was obtained from Fluka (Buchs, Switzerland) and triethylamine from Pierce Chemical Co. (Rockford, Ill., USA). All reagents were of analytical grade. Bovine heart was used to prepare blank tissue homogenate, and heparinized plasma from human volunteers served as blank plasma. Separate stock solutions of Leu-Dox and its metabolites in methanol (100 μM) were combined to obtain an equimolar standard mixture (10 μM) of the compounds. From this mixture, further dilutions (25, 50, 100, and 250 nM; 1.25 and 2.5 μM) were prepared monthly in methanol and stored at −20° C. Polypropylene tubes and vials were used throughout the experiments to minimize the adsorption of anthracyclines.

Preparation of tissue homogenates. Tissue (heart or tumor) was cut into small pieces, weighed as a whole, and subsequently homogenized by dismembration (1 min, 77 K) using a Mikrodismembrator II (Braun, Melsungen, FRG). The resulting powder was suspended in 0.9% NaCl to obtain a concentration of 125 mg tissue (wet weight)/ml.

Sample processing. Calibration samples were prepared by successively pipetting 125 μl standard mixture in a 1.5-ml Eppendorf vial (Hamburg, FRG), evaporating the methanol by heating to 40° C under a stream of nitrogen, adding 250 μl blank tissue homogenate, and vortexing for 30 min. Thus, spiked tissue samples contained 0.1–10 nmol of each anthracycline per gram (wet weight) of tissue. Homogenates were extracted by the addition of 25 μl buffer (100 mM disodium hydrogen phosphate/30 mM hexylsulfonic acid, pH 8.5) and 1.0 ml extraction solvent (chloroform/2-propanol/DMSO, 60:30:1, by vol.) followed by vortexing for 30 min. After centrifugation (15 s; 12,000 *g*), the organic layer was transferred to another 1.5-ml vial and evaporated (nitrogen, 45° C). The aqueous phase was extracted a second time and the organic layer was added to the residue of the first extract. Following evaporation, the residue was redissolved in 125 μl mobile phase using ultrasound (15 min), and the solution was vortexed briefly. After centrifugation (5 min; 12,000 *g*), 50 μl of the sample was injected into the HPLC.

Plasma samples were analyzed using an on-line preconcentration/cleanup procedure as described elsewhere [4]. Briefly, samples were thawed and vortexed and the remaining precipitate was spun down (5 min; 12,000 *g*). In a 1.5-ml vial, 350 μl supernatant (or, when less was available, another known amount supplemented with blank human plasma) was mixed (5 s vortex) with 700 μl acidic phosphate buffer consisting of acetonitrile/1 M phosphoric acid in phosphate-buffered saline (PBS)/PBS (7.5:15:77.5, by vol.). In the case of calibration samples 700 μl buffer was added to 350 μl spiked human plasma prior to 5 s vortexing. The final pH of the samples was approximately 3. After centrifugation (5 min; 12,000 *g*), the vials were transferred to the autosampler and thermostatted at 4° C; 900 μl supernatant was preconcentrated and subsequently analyzed.

Chromatography. The HPLC system consisted of an ABI Spectroflow 400 pump (Separations Analytical Instruments, H. I. Ambacht, The Netherlands) and a Gilson 232-401 autosampler (Meyvis, Bergen op Zoom, The Netherlands). The injection valve was fitted with either a 100-μl injection loop or a Chromsep 40- to 50-μm C_{18} reversed-phase preconcentration column for plasma analysis (10 × 2.0 mm inside diameter; Chrompack, Middelburg, The Netherlands). A Merck-Hitachi F1000 fluorescence detector (Merck, Amsterdam, The Netherlands) set at λ_{ex} = 480 nm and λ_{em} = 580 nm was used for peak monitoring. Data processing was performed with a Jones Chromatography JCL 6000 data system (version 5.01, Meyvis). A Chromsep 3-μm Microspher C_{18} analytical column (Chrompack) of 20 cm × 4.6 mm (inside diameter; two 10-cm cartridges in series) was used together with a 10 × 2.0 mm (inside diameter) reversed-phase guard column (Chrompack) and thermostatted at 32° C. A 3-μm in-line filter was placed between the injection valve and the column. The mobile phase, 8 mM triethylamine in 28 mM sodium dihydrogen phosphate (pH 3.5)/acetonitrile (2:1, v/v), was passed over a 0.45-μm filter before its use and was delivered at a flow rate of 1.0 ml/min.

Pharmacokinetics. The pharmacokinetic study of Leu-Dox in tumor-bearing mice was performed as described elsewhere for Dox [15]. Female mice were inoculated s.c. in both flanks with colon-26 tumors. At 12 days after implantation, mice were injected in the tail vein with Leu-Dox (12 mg/kg); at 0, 5, 15, and 30 min and 1, 2, 4, 6, 24, 48, and 72 h after injection, blood as well as heart and tumor tissues were collected (two mice per time point). Concentrations of Leu-Dox and six of its metabolites were calculated by interpolation on the respective calibration curves. The concentration versus time curves (C-t curves)

were constructed for each anthracycline in plasma and tissue, and AUC values were determined for the first 48 h using the trapezoidal rule. Other pharmacokinetic parameters were calculated according to standard procedures [8]: $t_{1/2}\gamma = 0.693/\gamma$, mean residence time (MRT) = AUMC∞/AUC∞, where AUMC represents the area under the C-t curve. The AUC extrapolated to infinity (AUC∞) could be calculated from the $AUC_{48\,h}$ value together with the elimination half-life using the formula $AUC\infty = AUC_{48\,h} + C_{48\,h}/\gamma$, where $C_{48\,h}$ represents the plasma concentration at 48 h. AUMC∞ was calculated in analogy to AUC∞. The AUC∞ and AUMC∞ values were also used to calculate the total body clearance ($Cl_t = D/AUC\infty$) and the steady-state apparent volume of distribution, $V_{d,ss} = D \times AUMC\infty/(AUC\infty)^2$, where D represents the dose.

Results and discussion

Assay

Figure 1 A shows a chromatogram obtained after the injection of an extract from mouse heart tissue spiked with (1.0 nmol/g) Leu-Dox and six metabolites. It demonstrates the baseline separation of nearly all compounds. Under the isocratic conditions used, all compounds eluted within 12 min. The blank chromatogram, which was obtained after the injection of an extract of blank mouse heart, shows the presence of a minor interference co-eluting with 7d-Dolon. The applicability of the procedure to the tissue samples is illustrated in Fig. 1 B, which shows the chromatogram of an extract of heart tissue from a mouse, obtained at 2 h after the i. v. injection of 12 mg/kg Leu-Dox.

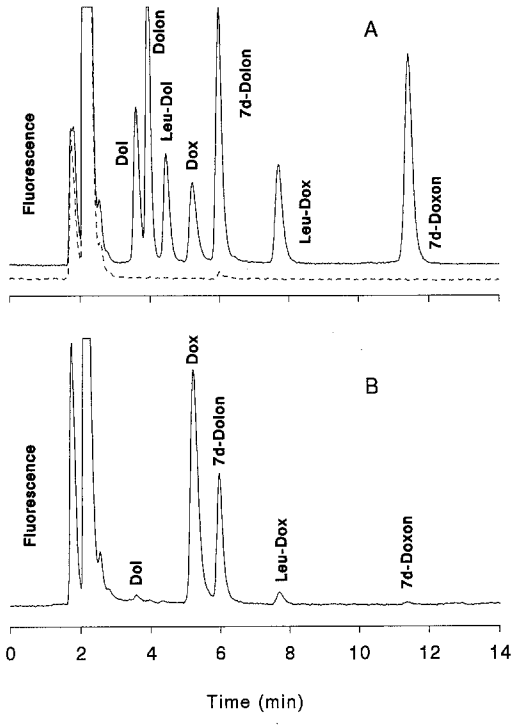


Fig. 1 A, B. Chromatograms obtained after extraction of **A** mouse heart tissue, either blank (– – – –) or spiked with 1.0 nmol of each anthracycline per gram wet weight (———), and **B** heart tissue obtained at 2 h after the administration of Leu-Dox (12 mg/kg) to tumor-bearing BALB/c mice. Column: Microspher (3 µm) C_{18} analytical column [20 (2 × 10) cm × 4.6 mm inside diameter] fitted with a Chromsep guard column (40–50 µm C_{18}, 10 × 2.0 mm inside diameter). Mobile phase: 8 mM triethylamine in 28 mM NaH_2PO_4 (pH 3.5)/acetonitrile (2:1, v/v). Flow rate: 1.0 ml/min. Detection: fluorescence with λ_{ex} = 480 nm and λ_{em} = 580 nm. For abbreviations, see Table 1

Our solid-phase extraction procedure for tissue was especially developed for the wide range of analyte polarities as present in the case of 4′-epidoxorubicin [10]. Since there are no indications for the formation of highly polar metabolites, such as glucuronides, from Leu-Dox [5], we reinvestigated the possibilities of a simple and inexpensive liquid-liquid extraction procedure. After studying several binary and ternary extraction mixtures, the optimal composition was determined to be chloroform/2-propanol/DMSO (60:30:1, by vol.). On the addition of the ion-pairing agent hexylsulfonic acid to the aqueous layer (buffered at pH 8.5) and a second extraction, recovery rates ranging from 82% for Dox to 110% for 7d-Doxon were achieved (Table 2). Especially for Dox and Dol, but not for the leucyl derivatives, the presence of DMSO and hexylsulfonic acid appeared to be crucial, as recovery rates dropped to 50% when the two additives were left out (results not shown). The rates of recovery achieved for Dox and Dol using this procedure compared favorably with those obtained using solid-phase extraction [10] and were comparable with those attained by liquid-liquid extraction methodologies [1, 7, 14]. However, most previous investigations have concerned only Dox and Dol, paying no attention to the aglycons.

To determine the within-day precision of the assay, we spiked three 4.0-ml portions of blank heart homogenate (125 mg wet weight/ml saline) with the anthracycline mixture at 0.1, 1.0, or 5.0 nmol/g. Ten 250 µl aliquots of each homogenate were extracted and analyzed within 1 day. The results are summarized in Table 2, which also lists the linearity and detection limits of all compounds. The coefficient of variation (CV) of the precision was lower than 6.7% ($n = 10$). The calibration lines for Leu-Dox, Leu-Dol, Dox, and Dol were linear over the entire concentration range of 0.1–10 nmol/g as indicated by r values of ≥ 0.998. In the case of the aglycons (Dolon, 7d-Doxon, and 7d-Dolon), however, the highest concentration was omitted from the calculation because of nonlinearity, probably due to aggregation of the rather apolar molecules. The detection limit ranged from 0.01 nmol/g wet weight for 7d-Dolon to 0.06 nmol/g for Dox (based on a signal-to-noise ratio of 3), which is comparable with that of the Sep-Pak method [10]. The between-day precision of the assay was determined over 6 days by the duplicate analysis of aliquots of blank heart homogenate spiked with the anthracycline mixture at 0.1, 1.0, or 5.0 nmol/g. The results were very satisfactory, with CV values ranging from 1.7% to 7.3% for 0.1 nmol/g, from 1.3% to 5.8% for 1 nmol/g, and from 2.2% to 7.8% for 5 nmol/g.

Pharmacokinetics

The C-t curves generated for Leu-Dox and its detectable metabolites in the plasma, heart, and tumor tissue of mice are shown in Fig. 2. The concentration of Leu-Dox decreased very quickly in all compartments, as indicated by the final elimination half-lives of 1.1, 0.8, and 0.9 h observed in the plasma, heart, and tumor, respectively (cal-

Table 2. Analytical performance of the assay of Leu-Dox and six metabolites in heart tissue

Anthracycline	Recovery	Within-day precision[a]				Detection limit (nmol/g)
	1.0[b] (%)	0.1[b] (%)	1.0[b]	5.0[b]	Linearity (r) 0.1–10[b]	
Leu-Dox	104	5.2	4.4	6.7	0.9975	0.04
Dox	82	6.1	2.3	5.3	0.9980	0.06
Dol	87	3.3	2.1	5.5	0.9998	0.03
Leu-Dol	96	4.8	1.9	4.9	0.9998	0.04
Dolon	104	3.4	1.9	4.8	0.9999	0.02
7d-Dolon	103	4.0	1.7	5.5	0.9999	0.01
7d-Doxon	110	4.0	1.8	6.0	0.9989	0.02

[a] CV, $n = 10$
[b] Concentration in nmol/g wet weight

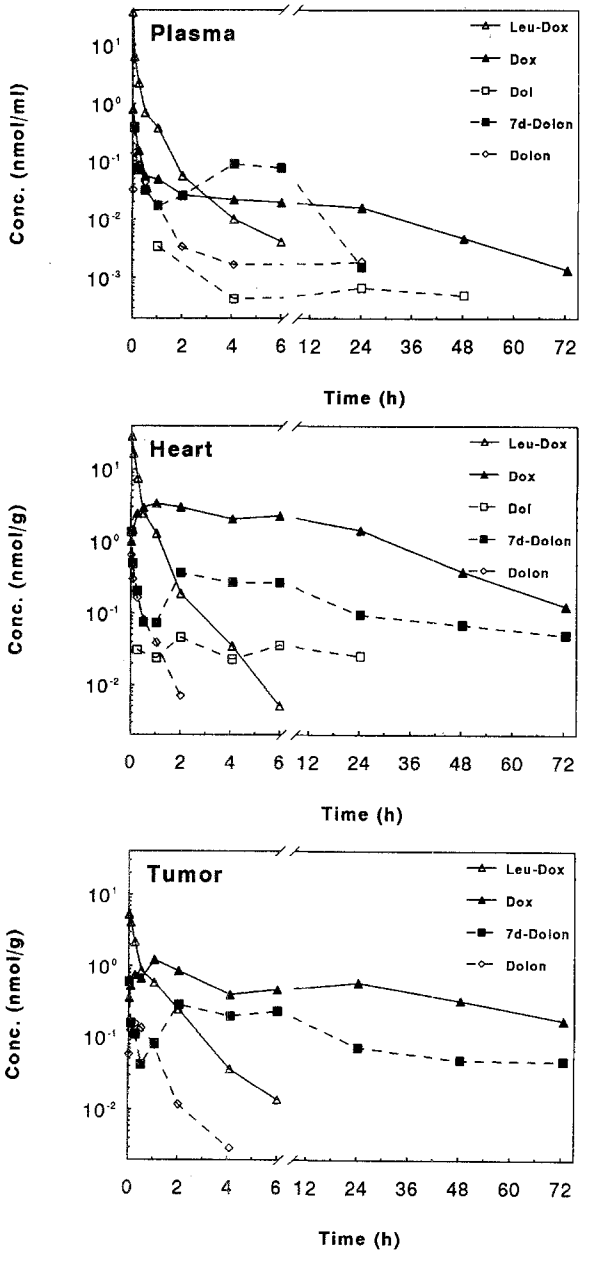


Fig. 2. Concentration versus time curves obtained for Leu-Dox and its metabolites in plasma, heart tissue, and tumor tissue of tumor-bearing mice during the 72-h period after i. v. administration of 12 mg/kg Leu-Dox. For abbreviations, see Table 1

Table 3. $AUC_{48\,h}$ values calculated for Leu-Dox, Dox, and their metabolites in plasma, heart, and tumor tissue after the i. v. administration of equimolar doses of Leu-Dox and Dox

Compound	$AUC_{48\,h}$		
	Plasma (nmol ml^{-1} min)	Heart (nmol g^{-1} min)	Tumor (nmol g^{-1} min)
Leu-Dox (12 mg/kg):			
Leu-Dox	221	443	153
Dox	51.0	4,262	1,466
Dol	1.9	63.7	0
Leu-Dol	0	10.8	0
Dolon	6.6	11.9	11.1
7d-Dolon	66.6	413	326
7d-Doxon	0.1	0	0.5
Dox (10 mg/kg)[a]:			
Dox	205	14,064	8,997
Dol	1.7	228	0
7d-Dolon	83.3	218	204
7d-Doxon	4.1	152	135

[a] AUC values for Dox and its metabolites were taken from [15]

culated from 2 h onward). This decay was accompanied by the rapid appearance of Dox. Peak levels of Dox were reached immediately after injection in plasma (0.8 nmol/ml) and after 1 h in heart (3.4 nmol/g) and tumor tissues (1.2 nmol/g). Dox was eliminated slowly, with final half-lives of 16.7 h in plasma, 15.3 h in heart tissue, and 27.4 h in tumor tissue being obtained (calculated from 24 h onward). These half-lives were comparable with the values observed after the administration of Dox [15]. Further derived pharmacokinetic parameters of Leu-Dox, i. e., total body clearance (Cl_t), steady-state volume of distribution ($V_{d,ss}$), and mean residence time (MRT), were 4.8 l h^{-1} kg^{-1}, 1.6 l/kg, and 19.3 min, respectively. These values are of the same order of magnitude as those calculated for humans [5].

Dox and 7d-Dolon were the most abundant metabolites as illustrated by the AUC values listed in Table 3. AUCs were calculated for the first 48 h (using the trapezoidal rule) to enable their comparison with the data available for Dox [15]. Neither of the 13-dihydro metabolites (Dol and Leu-Dol) was found in tumor tissue, which confirms previous findings after the administration of Dox. Small

amounts of both 13-dihydro compounds were found in the heart. The AUC value determined for Dol was 1.5% of that obtained for Dox, whereas the value calculated for Leu-Dol was 2.4% of that found for Leu-Dox. A comparable percentage (1.6%) was calculated for Dol after the administration of Dox [15]. In plasma, the Dol AUC values were 3.7% and 0.9% of the Dox value after the administration of Leu-Dox and Dox, respectively. For comparison, in human plasma these percentages were found to be 84% (after Leu-Dox [3, 5]) and 34% (after Dox [12]). The concentrations of the 13-dihydro compounds – including the aglycons – were much lower than those in man. Both in plasma and in tissue, Dol and Leu-Dol easily fell below the detection limit. However, the C-t curves and AUC values obtained for 7d-Dolon and, to a lesser extent, for Dolon clearly show that reduction of the C-13 keto function does take place. The C-t curve of 7d-Dolon shows a fast initial decline followed by an increase over the next 2–4 h after the injection of Leu-Dox. For instance, Cummings et al. [1] have shown that especially the liver contains considerable amounts of 7d-Dolon shortly after the administration of Dox to tumor-bearing AKR mice. Since the aglycons may be subject to enterohepatic circulation, subsequent redistribution to other organs such as the heart and the tumor may explain the C-t curves observed. Moreover, enterohepatic cycling of 7d-Dolon in humans has also been suggested after the administration of Dox [12].

The percentage of conversion of a compound into one of its metabolites can be determined by the ratio of the AUC values calculated for that metabolite after the i.v. administration of equimolar doses of the parent compound and the metabolite, respectively [8]. In this way, we calculated from the data shown in Table 3 that 26%, 30%, and 16% of the Leu-Dox appeared as Dox in the plasma, heart, and tumor, respectively. These data indicate that the occurrence in plasma does not reflect that in the tissues and that differences also exist between the tissue compartments. In heart tissue, the AUC value obtained for Dox is much lower following the administration of Leu-Dox than after treatment with Dox; this finding is in agreement with the results of another study in mice [6]. Extrapolation might even suggest that at equimyelotoxic doses (28 vs 8 mg/kg; E. Boven, unpublished data), the AUC value for Dox would be lower after the injection of Leu-Dox than after Dox administration, which would be in agreement with the previous finding of lower cardiotoxicity for Leu-Dox as compared with Dox [9]. In contrast to the correspondence of cardiotoxicity with the AUC for Dox, no correlation was found between the antitumor effect (E. Boven, unpublished data) and the AUC. One explanation may be that Leu-Dox also has an intrinsic antitumor effect. Another, and probably the most plausible, explanation may involve a substantial discrepancy in the conversion of Leu-Dox into Dox between different tumor types. This aspect will be studied more extensively in vitro as well as in vivo.

In conclusion, our newly developed liquid-liquid extraction assay is sensitive and specific enough to study the pharmacokinetics of Leu-Dox and its metabolites over several days after anthracycline administration. The reduced AUC value obtained for Dox in heart tissue after the injection of Leu-Dox as compared with Dox may explain the diminished cardiotoxicity of Leu-Dox. As the AUC for Dox was also reduced in tumor tissue, the favorable antitumor efficacy of Leu-Dox in nude mice cannot be explained. A better correlation between pharmacokinetics and pharmacodynamics might be expected from additional studies using the nude mouse model with sensitive tumors.

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