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A comparative study on the antitumor effect, cardiotoxicity and nephrotoxicity of doxorubicin given as a bolus, continuous infusion or entrapped in liposomes in the Lou/M Wsl rat

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Summary. Liposome encapsulation of doxorubicin (DXR) has been shown to increase the therapeutic index of the drug in several animal systems. The prevention of peak plasma concentrations of free drug might be a major factor contributing to the beneficial effects resulting from liposome encapsulation. If so, the administration of DXR as a continuous infusion should also lead to an improved therapeutic index. In the present paper, the administration of liposome-encapsulated DXR is compared with the infusion of DXR with regard to their potential to preserve antitumor activity, enhance survival and reduce cardiomyo- and nephropathy in IgM immunocytoma-bearing Lou/M Wsl rats. Plasma concentrations of DXR were determined to correlate the biological results with pharmacokinetic parameters. Liposomes containing phosphatidylcholine, phosphatidylserine and cholesterol (extrusion-multilamellar vesicles) were used. Bolus injections of free DXR (free DXR) and DXR liposomes (lip-DXR) in a multiple-dose regimen were compared with 24-h infusions of the same cumulative doses of DXR (inf-DXR). The antitumor activity of inf-DXR equalled that of free DXR as well as that of lip-DXR at doses of >0.25 mg/kg. The overall survival of tumor-bearing animals treated with 2.0 mg/kg lip-DXR was significantly prolonged ($P<0.01$) in comparison with that of animals treated with 2.0 mg/kg free DXR; however, treatment with 2.0 mg/kg inf-DXR did not induce a significant prolongation of survival. At a cumulative dose of 12 mg/kg, inf-DXR appeared to be as effective as lip-DXR in reducing the severity of cardiomyopathy induced by free DXR. However, for the reduction of nephropathy, only therapy with lip-DXR was effective. Inf-DXR induced high nephropathy scores comparable with those obtained with free DXR. For the first 24 h after an injection of 2.0 mg/kg or after the start of a continuous infusion of 2.0 mg/kg given over 24 h, similar areas under the plasma concentration-time curves (AUC) were calculated for free DXR and inf-DXR. However, for lip-DXR a much higher value was calculated. The higher plasma levels of lip-DXR did not result in higher cardiac levels. After five daily doses of 2.0 mg/kg, a much lower DXR concentration was found in cardiac tissue after the administration of lip-DXR than after the administration of free DXR or inf-DXR. This suggests that an important parameter to be determined and correlated with biological results is the free (i.e. not bound to liposomes) circulating fraction of DXR in lip-DXR-injected animals. In conclusion, in the IgM immunocytoma system the administration of DXR as a continuous infusion was as effective as DXR encapsulated in liposomes in reducing cardiotoxicity while preserving the antitumor effect; this indicates that the avoidance of peak plasma levels is an important component of the mode of action of DXR-containing liposomes. However, if both antitumor efficacy and nephrotoxicity are taken into account, lip-DXR could be considered to be superior to inf-DXR.

Introduction

Doxorubicin (DXR) is one of the major drugs in clinical oncology. The cumulative DXR dose is recommended not to exceed 550 mg/m^2, because the incidence of clinically overt heart failure has been found to rise sharply above that dose. To improve the therapeutic index, the formulation of the drug into liposomes is of considerable interest. In several animal systems, liposome encapsulation has been demonstrated to reduce in particular the incidence and grade of DXR-induced cardiomyopathy and nephropathy while fully retaining the drug's antitumor properties [10, 11, 13–15, 24–26, 30]. We recently provided evidence for the mechanism of drug delivery by DXR liposomes responsible for the improvement of therapeutic index [27]. Following i.v. administration, DXR-containing liposomes seem to act as a drug depot system, releasing DXR while circulating but also after being trapped in cells of the reticuloendothelial system (RES), in particular in macrophages located in liver and spleen. In the latter case, intralysosomal liposome degradation enables liberated DXR

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Abbreviations: DXR, doxorubicin; RES, reticuloendothelial system; MLV, multilamellar vesicle(s); PC, phosphatidylcholine; PS, phosphatidylserine; chol, cholesterol; free DXR, bolus injection of free DXR; lip-DXR, bolus injection of DXR liposomes; inf-DXR, 24-h infusion of free DXR

molecules to escape from the RES cells. As a result, the prevention of peak plasma concentrations of free drug seems to be a major factor contributing to the beneficial effects resulting from liposome encapsulation.

To confirm and extend our insights concerning the mode of action of DXR-containing liposomes, we studied in the IgM immunocytoma model the effects of giving DXR as a continuous infusion since this might simulate the slow-release effect introduced by delivering DXR in liposomal form. Using prolonged (10–96 h) continuous infusions [3, 17–20] as an alternative to the traditional bolus schedule has proved to be successful in increasing the therapeutic index of DXR. The prolonged administration of DXR via infusions has been reported to be associated with a low frequency of side effects, including less cardiac toxicity, nausea and vomiting [19] and the preservation of antineoplastic activity [16, 21].

The present paper compares liposome-encapsulated DXR with the infusion of DXR with regard to their potential to preserve antitumor activity, enhance survival and reduce cardio- and nephrotoxicity in Lou/M rats. Furthermore, plasma levels of DXR were determined to correlate the biological results with pharmacokinetic parameters.

Materials and methods

Animals. Breeding pairs of LOU/M Wsl rats and the transplantable IgM immunocytoma of LOU/C Wsl origin were kindly provided by Dr. H. Bazin (Catholic University of Louvain, Belgium) [2]. Animals were bred under specified pathogen-free conditions at the National Institute of Public Health and Environmental Hygiene (Bilthoven, The Netherlands). Male rats weighing 225–350 g and 10–18 weeks old were used. Animals were maintained according to accredited conditions in our facility and were in good health at the initiation of the studies.

Tumor model. LOU/M Wsl rats were inoculated s.c. on the left flank with 1×10^4 IgM immunocytoma cells in 0.5 ml plain RPMI 1640 medium (Grand Island Biological Co., Europe B. V.; Hoofddorp, The Netherlands). Details of the tumor model are described elsewhere [31]. Briefly, after 17–21 days inoculated animals developed a palpable tumor, which grew to a diameter of 25–35 mm within 6–8 days. At this time, the tumor had metastasized to the regional lymph nodes and micrometastases in the liver could be detected. Tumor size was measured with Vernier callipers and expressed as the mean value ±SEM of three perpendicular measurements.

Drug preparation. DXR (Adriablastine) was purchased from Laboratoire Roger Bellon S. A. (Neuilly sur Seine, France). For experiments with the free drug, the lyophilized powder was reconstituted with sterile saline (0.9% w/v).

Lipids and preparation of liposomes. Cholesterol and chromatographically pure egg phosphatidylcholine and bovine brain phosphatidylserine were purchased from Sigma Chemical Co. (St. Louis, Mo). DXR-containing multilamellar vesicles (extrusion-MLV) were prepared from phosphatidylcholine, phosphatidylserine and cholesterol (molar ratio, 10:1:4) as described elsewhere [27]. In short, DXR was added to a chloroform/methanol mixture (1/1 v/v) containing the lipids in the appropriate molar ratio. After hydration with a solution consisting of 145 m*M* NaCl and 10 m*M* TRIS-HCl (pH 4), the liposomes were extruded sequentially through Nucleopore membrane filters with pore sizes of 600 and 200 nm. Separation of free DXR from liposome-bound DXR (lip-DXR) was achieved by application of the cation exchange resin Dowex 50W-X4 (Serva, Heidelberg, FRG) [28]. The dispersion was mixed with Dowex (sodium form) and left for 5 min (minimally 1 g Dowex/ml dispersion). The resin was separated from the liposome-containing supernatant by filtration through 8.0-μm membrane filters (Uni-pore, Bio-Rad; Richmond, Calif). After separation, <10% of the total amount of DXR present was in the free form. The loading capacity always exceeded 45 mmol DXR/mol phospholipid; the encapsulation efficiency varied between 30% and 40%. Particle size was determined by dynamic light scattering (Nanosizer, Coulter Electronics, Ltd.; Luton, UK). The mean liposome diameter of the extruded liposomes was 0.27 ± 0.01 μm ($n = 11$). The polydispersity was low. The liposomes were negatively charged: from microelectrophoresis measurements (Rank Brothers Mark II; Bottisham, UK), a zeta-potential of −11 mV was calculated. As a rule, the liposome dispersions were stored protected from light at 4° C and used within 1 week after preparation. During this storage period, leakage of DXR from the liposomes was negligible.

Surgical procedures. Rats were anaesthesized with a 4:3 mixture of ketamine (100 mg/ml, Aescoket; Aesculaap, The Netherlands) and xylazine (2%, Rompun; Bayer, FRG) at 1 ml/kg. For the prevention of bacterial infection, a procain-benzyl-penicillin-streptomycin depot (Depomycin; Gist-Brocades, The Netherlands) was given (15,000 IU, 0.2 ml s.c.). The vena cava (inferior) was cannulated with silicone tubing via the iliac vein and the cannule was externalised by attaching it to a stainless steel curvature, which was fixed to the skull. The cannula was filled with polyvinylpyrrolidone (50% w/v) containing heparin (75 IU/ml). Infusions were done with Braun infusors (Unita I, FRG) and rotation of the external cannula was made possible by swivels.

Experimental design. In the part of the study dealing with the antitumor activity of different treatment modalities, all rats used were cannulated as soon as possible after tumor-cell inoculation. Groups of 5–6 animals each were formed at random. Treatment was started approximately 17 days after tumor-cell inoculation (day 0), when the tumor had reached a diameter of 15 mm or more. Administration was carried out i.v. (via the cannula) on 5 consecutive days and then weekly, as indicated in Fig. 1. The following doses were used: saline (NaCl 0.9%), 0.25, 0.5, 1.0 and 2.0 mg/kg DXR. Bolus injections of free DXR (free DXR) and DXR liposomes (lip-DXR) were compared with 24-h infusions of equal doses of DXR (inf-DXR). The animals were weighed twice weekly and tumor measurements were done at least twice weekly. Mortality was scored. Albuminuria was assessed at least twice weekly with Albustix (Ames, Division of Miles Nederland; Weesp, The Netherlands).

In the part of the study in which the severity of the cardio- and nephrotoxicity induced by the different treatment modalities was compared, non-tumor-bearing animals were used. After cannulation, groups consisting of six ani-

mals each were formed at random. DXR administration (via cannula) was carried out i.v. on 5 consecutive days (days 0–4) and then weekly until a cumulative dose of 12 mg/kg was reached. The following DXR dose was used: free DXR, 1 mg/kg; lip-DXR, 1 mg/kg; and inf-DXR, 1 mg/kg over 24 h. For free DXR, this regimen has been shown to induce damage to the structure of the myocardium and kidney [31]. For histological examination, animals were killed while under diethylether anaesthesia.

Non-tumor-bearing animals were also used to study DXR concentrations in plasma and cardiac tissue. After cannulation, six groups of six animals each were formed. DXR administration (via cannula) was carried out i.v. on 5 consecutive days. Two groups received 2 mg/kg free DXR, two were given 2 mg/kg lip-DXR and two were infused continuously with 2 mg/kg DXR over 24 h. At several time points distributed over the period of 5 days, blood was collected in heparinized polypropylene cups by orbital puncture; the samples were centrifuged as soon as possible. Plasma was transferred into polypropylene cups and stored at −25° C prior to analysis. At 120 h after the start of the experiment, rats were killed while under diethylether anaesthesia and the hearts were isolated and stored at −25° C until analysis.

Histopathology. Tissue of the heart and kidneys was fixed in 4% buffered formaldehyde and embedded in glycolmethacrylate. Sections measuring 1 μm were stained with Giemsa and examined with a light microscope. Cardiac lesions were graded according to the method proposed by Bertazzoli et al. [4]. Myocardial lesions were evaluated as follows:

A. Degrees of severity: sarcoplasmic microvacuolizations and/or inclusions and interstitial or cellular edema (1); same as 1 plus sarcoplasmic macrovacuolizations or atrophia, necrosis, fibrosis, endocardial lesions, and thrombi (2)
B. Degrees of extension: no lesions (0); <10 single, altered myocytes in the whole heart section (0.5); scattered single, altered myocytes (1); scattered small groups of altered myocytes (2); widely spread, small groups of altered myocytes (3); confluent groups of altered myocytes (4); most cells damaged (5).

The product of the severity and the extent of the damage observed in each rat gave a final, single score used for comparative purposes. Kidney lesions were scored on a severity scale with the following gradings as previously described [32]: no lesions (0), minor lesions (+), moderate lesions (++) and severe lesions (+++). The following items were assessed: alterations of glomerular basal membranes, changes in glomerular epithelial cells, the distribution of lesions through the glomerulus (segmental vs global) and the distribution of lesions through the renal cortex (focal vs diffuse).

Analysis of DXR in plasma and cardiac tissue. DXR was determined in plasma and in cardiac tissue by a high-performance liquid chromatographic method using fluorescence detection (excitation wavelength, 480 nm; emission wavelength, 560 nm) [7]. Daunorubicin was used as an internal standard. The drugs were extracted from the biological matrix by a solid-phase extraction (SPE) procedure using octadecylsilane SPE columns. Prior to extraction, tissue samples were digested by an enzymatic digestion procedure [6]. Separation of the drugs was done by a reversed-phase octadecylsilane column; by this method total DXR (free as well as liposome-entrapped DXR) was determined.

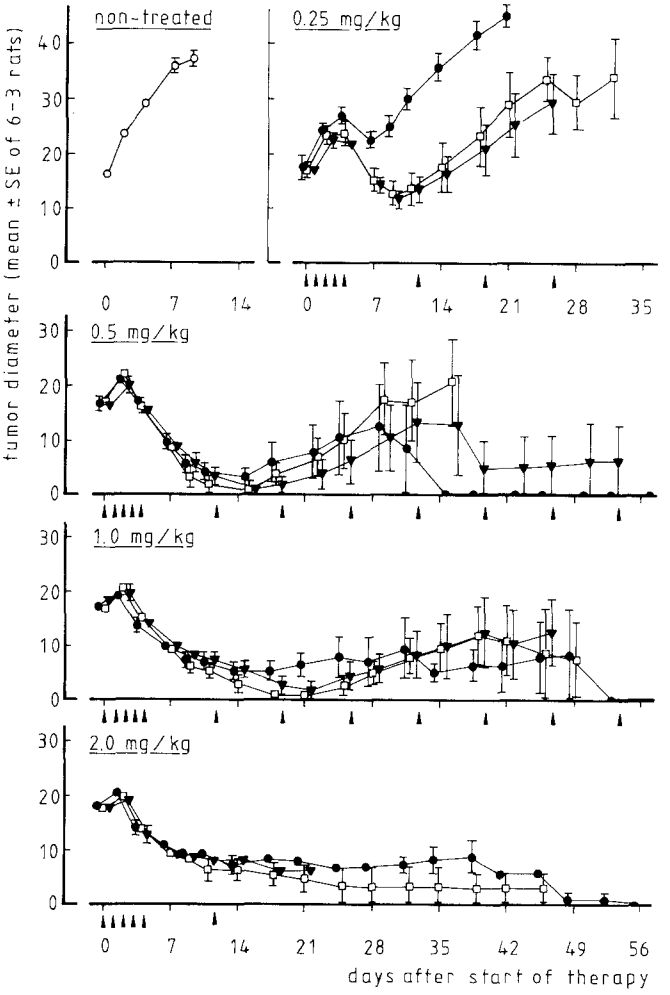

Fig. 1. Effect of free DXR, lip-DXR and inf-DXR on the growth of solid IgM immunocytoma in Lou/M Wsl rats. The mean value of three perpendicular measurements is presented. Bars, SEM. ▲, i.v. injection or 24-h infusion; ▼———▼, free DXR; ●———●, lip-DXR; □———□, inf-DXR

Statistics. Differences in group means were analysed by Student's *t*-test (two-sided). Survival data were analysed by the Wilcoxon non-parametric ranking test.

Results

Antitumor effect: dose-response relationship

To study the effects of DXR infusion, bolus injections of free DXR (free DXR) and DXR liposomes (lip-DXR) in a multiple dose regimen were compared with 24-h infusions of the same cumulative doses of DXR (inf-DXR) (Fig. 1). Control animals showed progressive tumor growth, leading to death of the animals within 10 days. Doses of 0.25 mg/kg free DXR, lip-DXR and inf-DXR retarded tumor growth. From day 7, the differences between 0.25 mg/kg lip-DXR vs both free DXR and inf-DXR were

Table 1. Effect of free DXR, lip-DXR and inf-DXR on urinary albumin concentrations in tumor-bearing animals

DXR dose (mg/kg)	Days after starting treatment	Urinary albumin concentration[a]		
		free DXR	lip-DXR	inf-DXR
0.25	0	+	+	+
	7	+	+	+
	14	+	+	+
	21	+	+	+
	25	+		+
0.5	0	+	+	+
	7	+	+	+
	14	+	+	+
	21	+	+	+
	28	++	+	+
	35	+++	+	+
	42	+++	+	
	49	+++	++	
1.0	0	+	+	+
	7	+	+	+
	14	+++	+	+
	21	+++	+	++
	28	+++	++	++++
	35	+++	+++	++++
	42	++++	++++	++++
2.0	0	+	+	+
	4	+	+	+
	7	+++	+	+
	9	++++	+	+++
	11	++++	++	++++
	14	++++	+++	++++
	18	++++	++++	++++

[a] Urinary albumin concentrations were assessed by Albustix: +, <1.0 g/l; ++, <3.0 g/l; +++, <20.0 g/l; ++++, >20.0 g/l

statistically significant ($P < 0.05$). Doses of 0.5 and 1.0 mg/kg free DXR, lip-DXR and inf-DXR resulted in regression of the tumor. No statistically significant differences in tumor-regression pattern between the various treatment modalities were observed, except on days 18 and 21 between the 1.0 mg/kg groups of lip-DXR and inf-DXR ($P < 0.05$ on both days). Regrowth of the tumor was seen in all treatment groups. All 2.0 mg/kg groups showed similar tumor regression. At this dose, regrowth of the tumor was only observed in one animal in the lip-DXR group. No adverse dermal toxicity due to extravasation was noted.

Body weight

In all groups, treatment with a 1.0 mg/kg dose initially resulted in a decrease in body weight of 7%. No statistically significant differences between treatment groups were observed. No reduction in body weight was observed in control animals. In both the lip-DXR and inf-DXR groups, from 7 days after the start of therapy, a rise in body weight was observed. On day 46 the body weight measured 21% (in the lip-DXR group) and 23% (in the inf-DXR group) above that recorded on the 1st day of treatment. This weight gain could not be ascribed to the development of ascites. In the free DXR group, only a gradual recovery from the initial weight loss was observed.

Body weight diminished by 27% in the 2.0 mg/kg free DXR group and 23% in the 2.0 mg/kg inf-DXR group within 7 days (difference not statistically significant). However, 11 days after the start of therapy, body weight in both groups started to rise sharply. Levels on day 18 amounted to 15% (in the free DXR group) and 10% (in the inf-DXR group) above the weight on day 0. This rise was paralleled by the development of ascites. In contrast, animals in the 2.0 mg/kg lip-DXR group lost 12% of their body weight, followed by a gradual recovery that could not be correlated with ascites.

Table 2. Comparative cardiotoxities in Lou/M Wsl rats treated with free DXR, lip-DXR and inf-DXR[a]

Treatment modality	Scores							Animals (*n*)
	0	1	2	3	4	5	6	
free DXR	–	–	1	–	2	–	2	5
lip-DXR	–	4	1	–	1	–	–	6
inf-DXR	–	–	4	–	1	–	–	5

[a] A DXR dose of 1 mg/kg was injected i.v. daily for 5 days and then weekly up to a cumulative dose of 12 mg/kg

Table 3. Comparative nephrotoxicities in Lou/M rats treated with free DXR, lip-DXR and inf-DXR[a]

Treatment modality	Scores				Animals (*n*)
	0	+	++	+++	
free DXR			3	2	5
lip-DXR	2	4			6
inf-DXR			4	1	5

[a] A DXR dose of 1 mg/kg was injected i.v. daily for 5 days and then weekly up to a cumulative dose of 12 mg/kg

Ascites

None of the 1.0 mg/kg treatment groups developed ascites. On day 18, signs of ascites were observed in three of six animals belonging to the 2.0 mg/kg free DXR group and in one of five belonging to the 2.0 mg/kg inf-DXR group. No ascites was observed in the 2.0 mg/kg lip-DXR group.

Albuminuria

Lou/M rats have spontaneous albuminuria in the range of 0.3–1.0 g/l. At dose levels of 1.0 and 2.0 mg/kg, albuminuria developed most rapidly during treatment with free DXR (Table 1). Treatment with inf-DXR induced albuminuria more rapidly than treatment with lip-DXR.

Cardiomyo- and nephropathy

Low and comparable cardiomyopathy scores were observed for lip-DXR and inf-DXR (Table 2). In contrast, free DXR induced relatively high cardiomyopathy scores. The increase in histology scores was due to an increase in the extent of the lesions, as all DXR-related lesions fulfilled the criteria of grade 2 severity as defined in *Materials and methods*. Therapy with lip-DXR did not result in the severity of nephropathy induced by free DXR (Table 3); inf-DXR induced high scores comparable with those obtained with free DXR. The cardiac and renal lesions were in accordance with those previously described by us [30–32].

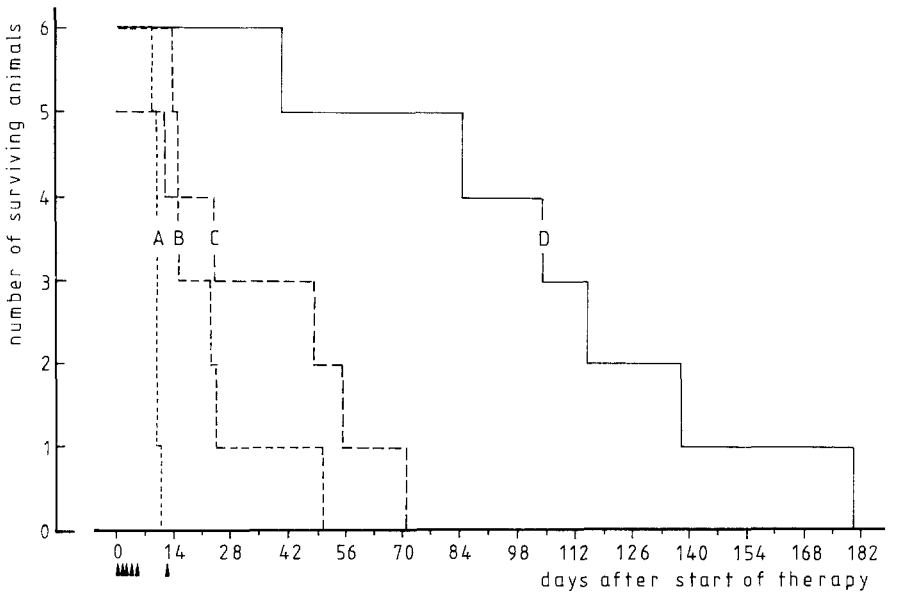

Fig. 2. Survival of Lou/M Wsl rats bearing solid IgM immunocytoma. *A*, non-treated; *B*, free DXR; *C*, inf-DXR; *D*, lip-DXR; ▲, i.v. injection of 2 mg/kg or continuous infusion of 2 mg/kg over 24 h (cumulative dose, 12 mg/kg). Statistical analysis (Wilcoxon): *B* vs *A*, $P < 0.01$; *C* vs *B*, not significant; *D* vs *B*, $P < 0.01$

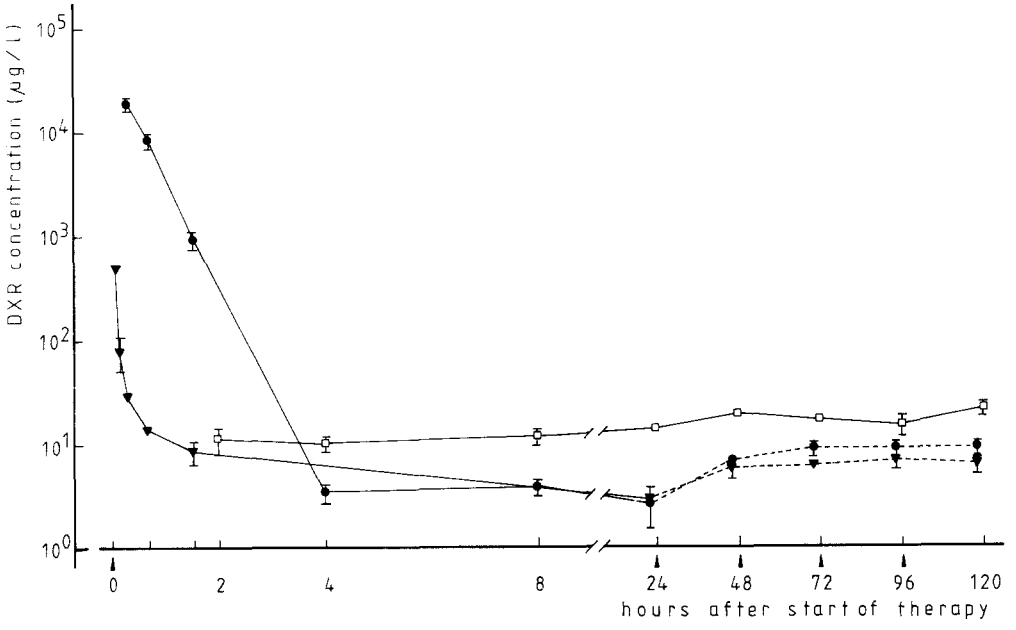

Fig. 3. Plasma DXR concentrations determined up to 120 h after the start of treatment with free DXR (▼——▼), lip-DXR (●——●) or inf-DXR (□——□). ▲, i.v. injection of 2 mg/kg or continuous infusion of 2 mg/kg over 24 h (cumulative dose, 10 mg/kg). Each point represents the mean ±SD for six animals

Survival

Tumor regrowth frequently occurred in animals treated at the 0.25, 0.5 and 1.0 mg/kg dose levels (Fig. 2); this was not observed at a dose of 2.0 mg/kg, except in one animal in the lip-DXR group. Therefore, because of the low incidence of tumor recurrence in the 2.0 mg/kg groups, survival is the best reflection of systemic toxicity accompanying the therapy. Control tumor-bearing animals showed a median survival of 10 days (range, 9–11 days) after the start of treatment. The 2.0 mg/kg free DXR-treated animals survived for 19 days (range, 13–50 days) after the initiation of therapy. The 2.0 mg/kg lip-DXR group survived considerably longer ($P < 0.01$): a median of 110 days (range, 41–180 days). The median survival of the 2.0 mg/kg inf-DXR group was 48 days (range, 12–71 days). The median survival of the inf-DXR group was not significantly different from that of the free DXR group.

Plasma and cardiac levels of DXR

After the first injection of the dose scheme used (2.0 mg/kg daily for 5 days), in the free DXR group the drug disappeared very rapidly from plasma (Fig. 3). A mean concentration of 2.8 ± 0.7 µg/l (mean ± SD) was found 23 h after administration. Somewhat higher values (mean concentrations between 5.1 and 6.5 µg/l) were found 23 h after each subsequent injection. After lip-DXR, the plasma drug concentration decreased more slowly than after free DXR to a mean concentration of 2.4 ± 0.6 µg/l, with values between 6.1 and 8.1 µg/l 23 h after each subsequent injection. When DXR was given as a continuous infusion (2.0 mg/kg over 24 h), a steady-state concentration of between 13.6 and 18.2 µg/l was obtained after 24 h. For the first 24 h after the initial injection and after the start of continuous infusion, respectively, similar AUCs were calculated (trapezoidal rule) for free DXR (226 $\mu g \cdot h \cdot l^{-1}$) and

Table 4. DXR concentrations in cardiac tissue

Treatment modality	DXR concentration[a] (mg/kg)
free DXR	2.02 ± 0.30
lip-DXR	1.25 ± 0.26
inf-DXR	2.34 ± 0.33

[a] DXR concentrations in cardiac tissue were determined 24 h after 5 consecutive daily doses of 2 mg/kg free DXR or lip-DXR and after a 120-h continuous infusion of 2 mg/kg over 24-h (cumulative dose, 10 mg/kg). Data represent the mean ± SD of 11 – 12 animals

inf-DXR (264 $\mu g \cdot h \cdot l^{-1}$). For lip-DXR a value of 18.2×10^3 $\mu g \cdot h \cdot l^{-1}$ was calculated; however, it should be noted that the latter figure includes liposome-entrapped DXR as well as drug not bound to liposomes. The major part of the AUC after lip-DXR administration can obviously be attributed to liposome-entrapped DXR. Evidence for this assumption is provided by the DXR concentrations in cardiac tissue. A 120-h continuous infusion of DXR (2.0 mg/kg over 24 h) induced the highest DXR level in cardiac tissue (Table 4). At 24 h after the fifth injection of 2.0 mg/kg free DXR (120 h after the start of treatment), a somewhat lower level was found ($P < 0.01$ vs inf-DXR). A considerably lower DXR level was detected 24 h after the fifth injection of 2.0 mg/kg lip-DXR ($P < 0.01$ vs inf-DXR).

Discussion

The present findings show that in the IgM immunocytoma system the administration of DXR as a continuous infusion is as effective as DXR encapsulated in liposomes in reducing cardiotoxicity with preservation of the antitumor effect. Mayhew and Rustum [22] have reported on a comparison made between lip-DXR and inf-DXR with respect to their toxicity and therapeutic efficacy; however, these authors did not score cardiotoxicity. They found that the LD_{50} determined from deaths of mice treated with lip-DXR was much higher than that resulting from inf-DXR treatment. Furthermore, at equitoxic doses (maximum tolerated dose) lip-DXR improved therapeutic efficacy against L1210 mouse leukemia, whereas inf-DXR was no more therapeutically efficacious than i.v. bolus administration. Discrepancies between their results and ours are difficult to evaluate due to a lack of detailed information and might be related to differences in experimental design (e.g. the type of tumor system and liposomes used).

When the administration of free DXR or lip-DXR via bolus injections was replaced in our therapy protocol by 24-h infusions resulting in the same cumulative doses, the antitumor activity of inf-DXR equalled that of free DXR as well as that of lip-DXR at doses above 0.25 mg/kg. The cytostatic effect of DXR in vitro and in vivo has been correlated with the AUC [5, 8]. Calculations based on the data presented in Fig. 3 indicate that the administration of DXR either as a continuous infusion or as a bolus injection resulted in similar AUC values, which may account for the preserved antitumor effect. However, the calculated AUC value for lip-DXR is 70- to 80-fold higher than those for either free drug or inf-DXR. We recently provided strong evidence that liposomal delivery of DXR follows a slow-release pattern [27]. Only non-liposomal DXR liberated from the liposome structures during in vivo destabilization processes is supposed to be the active species. Therefore, for a correct interpretation of the pharmacodynamics of DXR liposomes with respect to their antitumor effects in relation to the plasma kinetics of DXR, it is necessary to differentiate between liposomal and non-liposomal DXR. Presently, a method is under development in our laboratory to determine specifically plasma concentrations of free circulating DXR in the presence of liposome-entrapped DXR.

The reduction in cardiotoxicity induced by either continuous infusion or liposomal encapsulation (Table 2) is most likely related to alterations in the plasma profile of DXR. The observation that a reduction in initial peak concentrations obtained by the administration of DXR as a continuous infusion (Fig. 3) was accompanied by a reduction in cardiotoxicity suggests that the avoidance of peak plasma levels is an important component of the mode of action of DXR-containing liposomes. The importance of peak levels for the development of cardiotoxicity is underlined by the observation that inf-DXR induced the highest DXR level in cardiac tissue (Table 4), whereas the severity of cardiomyopathy was low and comparable with that induced by lip-DXR (Table 2). After lip-DXR administration, the initial peak plasma DXR concentration apparently was not reduced. However, since the total DXR concentration in plasma is mainly determined by liposome-entrapped drug, the peak concentration of circulating free DXR in plasma was definitely reduced. In addition, cardiac tissue has only a very low endocytotic capacity for the uptake of liposomes [29]. Therefore, with the administration of DXR liposomes, only the non-liposomal drug levels seem to be relevant to the development of cardiotixicity. Further evidence that non-liposomal DXR is the toxic species was provided by the DXR concentration in cardiac tissue (Table 4), which was relatively low in the case of lip-DXR, whereas the AUC obtained for lip-DXR was very high compared with that for free DXR and inf-DXR.

With respect to the induction of nephropathy, the replacement of DXR liposome injections (1 mg/kg) by 24-h infusions of drug resulted in greater toxicity. Scoring of nephropathy was of interest, since in rats the chronic toxicity of DXR includes not only cardiomyopathy but also nephropathy [9, 30, 31]. The nephropathy causes albuminuria (Table 1) and hypoalbuminemia, finally resulting in nephrotic syndrome. Recent unpublished observations suggest that nephropathy represents an important cause of death in Lou/M rats chronically treated with DXR. The lack of preservation of renal integrity might, then, contribute to the fact that infusion does not result in prolonged survival compared with the bolus administration of free DXR (Fig. 2). A recent report [32] has demonstrated that the cardiomyopathy observed in Lou/M Wsl rats is a phenomenon independent of nephropathy. The present results obtained for inf-DXR, showing a reduction in cardiotoxicity without an accompanying reduction in nephrotoxicity, also point out the independence of both phenomena. In addition, the development of nephropathy might be related to prolonged exposure to DXR rather than to the occurrence of peak plasma levels of drug. Although DXR-

induced nephropathy is not observed in humans, the possibility that prolonged exposure to infused plasma concentrations of DXR initiates damage to other organs cannot be excluded.

In conclusion, this study shows that under the present experimental, neoplastic conditions both liposome encapsulation and slow drug delivery by infusion offer a considerable degree of protection against the cardiotoxicity of DXR while enabling its antitumor activity to be fully expressed. The prevention of high peak concentrations of free DXR seems to be the common factor underlying this. The question arises as to which of either form of drug delivery is preferable in chemotherapy. In clinical practice, the administration of DXR liposomes as a bolus injection at a given dose fits best for an in- and outpatient department. In addition, extravasation of DXR during prolonged infusion is a serious clinical problem, despite the care with which the drug is infused [1]. The incorporation of DXR into liposomes has been reported to provide significant protection against the vesicant activity normally associated with extravasation at the local infusion site [12, 23]. Finally, the marked prolonged survival (Fig. 2), relatively small loss in body weight, low grade and incidence of renal lesions (Table 3), absence of ascites and slower development of albuminuria (Table 1) observed during the treatment of IgM immunocytoma with DXR liposomes may indicate a better tolerance of liposomes than of infusion.

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