Doxorubicin Entrapped in Sterically Stabilized Liposomes: Effects on Bacterial Blood Clearance Capacity of the Mononuclear Phagocyte System

Gert Storm, Marian T. ten Kate, Peter K. Working, and Irma A. J. M. Bakker-Woudenberg

Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, and Groningen Utrecht Institute for Drug Exploration, 3508 TB Utrecht, the Netherlands [G. S.]; Institute of Clinical Microbiology and Antimicrobial Therapy, Erasmus University Rotterdam, 3000 DR Rotterdam, the Netherlands [M. T. t. K., I. A. J. M. B-W]; and SEQUUS Pharmaceuticals, Inc., Menlo Park, California 94025 [P. K. W.]

ABSTRACT

The introduction of long-circulating liposomes sterically stabilized by surface coating with polyethylene glycol has expanded the potential for drug targeting to tumors. In recent clinical studies, evidence of significant antitumor activity has been obtained with the industrially prepared formulation of long-circulating polyethylene glycol-coated liposomes containing doxorubicin, referred to as DOXIL. Previous studies performed in rats showed that doxorubicin-containing liposomes can exert major toxic effects on the liver macrophage population for a considerable period of time; a strong impairment of phagocytic function and even a substantial depletion of the liver macrophage populations were observed. In the present study, the phagocytic function of the mononuclear phagocyte system (MPS) after administration of DOXIL at a clinically relevant dosage schedule was evaluated in rats. Phagocytic function of the MPS was assessed by determining bacterial blood clearance capacity. The observations reported herein show that DOXIL is fairly well tolerated regarding bacterial blood clearance capacity of the MPS when administered in a regimen that resembles the clinical setting closely. This outcome has important implications with regard to the clinical utility of the liposomal drug, especially in the restricted context of immunocompromised cancer patients who easily develop systemic infections and should not be confronted with a therapy-induced reduction of the bacterial blood clearance capacity of the MPS.

INTRODUCTION

Attenuation of the characteristic dose-dependent cardiotoxicity has been demonstrated to be one of the most attractive features of the use of liposomes as carriers of DOX. Early research on DOX-liposome formulations revealed that liposomes direct the drug away from sites with tight continuous endothelium, such as the heart muscle, toward organs rich in phagocytic cells belonging to the MPS, such as the liver and spleen. However, despite the advantage of reduced (cardio)toxicity, awareness was growing that avid uptake by the MPS system may be disadvantageous by reducing the actual drug availability to the tumor (1, 2). Recent studies introduced a new type of liposome characterized by reduced uptake by the MPS and enhanced uptake by solid tumors (3-9). Liposomes containing a PEG-derivatized phospholipid, also referred to as "stealth" or "sterically stabilized" liposomes, can oppose rapid internalization by the MPS macrophages and therefore circulate in the blood for prolonged periods of time (10, 11). When such long-circulating liposomes are used as DOX carriers, an increased drug accumulation in tumor tissue in combination with superior antitumor activity and decreased toxicity when compared to the free drug has been found in a variety of animal tumor models. Clinical studies in patients with AIDS-related Kaposi's sarcoma and solid tumors are in progress or completed and have already yielded encouraging results (12-16). One such formulation (DOXIL) has been approved by the Food and Drug Administration for treatment of AIDS-associated Kaposi's sarcoma in patients who are refractory to conventional chemotherapy or cannot tolerate its toxicity, and by the European Agency for the Evaluation of Medicinal Products (as CAELYX) for the same indication as well as first-line therapy in these patients. Phase II studies in patients with solid tumors are under way.

The possibility that the pronounced tendency of particulates such as DOX liposomes to localize in macrophages with subsequent intracellular release of encapsulated drug could lead to serious damage of the MPS has been recognized for more than a decade (17, 18). However, experimental validation of this major concern was never given a high priority (19). A strong decrease in number of peritoneal macrophages was observed 24 h after i.p. injection of liposome-entrapped DOX (20). Critical toxicity to the livers of mice was not detected after i.v. administration of conventional (i.e., short-circulating) DOX liposomes (21). However, generally, liver toxicity is assessed by determining serum parameters reflecting toxicity at the level of hepatocytes rather than macrophages. Recently, a thorough in
vivo examination of potential toxic effects of conventional, short-circulating DOX liposomes on rat liver macrophages was presented (22). The results show that liposomal DOX can exert major toxic effects on the liver macrophage population for a considerable period of time. At the dosage schedule used, a strong impairment of phagocytic function and even a substantial depletion of the liver macrophage population was observed. After a single injection of DOX liposomes at a dose of 5 mg/kg, phagocytic uptake of subsequently administered radiolabeled “empty” liposomes by larger-sized liver macrophages was decreased by 70%; after two injections, by approximately 80%; and after three injections, by more than 90%. The decrease in phagocytic capacity of liver macrophages observed after two or three injections of DOX liposomes (5 mg/kg) was accompanied by a remarkable decrease in the total number of liver macrophages isolated (by 56 and 85%, respectively). Recovery from these toxic effects was seen only after 2 weeks. In a more recent article (23), the same authors also reported on the toxicity of DOX entrapped within long-circulating liposomes. It was shown that, similar to the effects observed after administration of DOX in short-circulating liposomes, long-circulating DOX liposomes affect liver macrophages, although the effects are slightly less acute and slightly less severe. In view of the key role of MPS macrophages in homeostasis and host defense, it is obvious that these results may have strong implications with regard to the clinical potential of DOX liposomes. In this respect, the selected dosage schedule is of high importance. However, from a clinical perspective, a meaningful interpretation of these findings is strongly hindered by the dosage schedule used (two doses of 5 mg/kg at a 3-day interval), which has low clinical relevance, taking into account clinical practice involving much lower absolute doses given every 3–4 weeks (12–16).

Possible implications of the administration of DOX liposomes regarding the bacterial blood clearance capacity of the MPS needs to be investigated thoroughly. Patients receiving cytostatic drugs are often immunocompromised and are susceptible to (opportunistic) infections. They often develop sepsisemia from a locally infected site. Malfunction of the MPS may easily result in generalization of the infection and hence, in increased mortality. The question arises as to whether the newly developed formulations of long-circulating DOX liposomes, which tend to avoid the MPS, are influencing the phagocytic activity of the MPS. Relevant in this respect is the industrially prepared formulation referred to as DOXIL (a registered trademark of SEQUUS Pharmaceuticals, Inc., Menlo Park, CA). This is a formulation of DOX in PEG-coated liposomes that has shown a prolonged circulation time in humans. Although these sterically stabilized liposomes are able to avoid large-scale uptake by the MPS, it should be realized that substantial MPS uptake is still observed. In the present study, the phagocytic function of the MPS after administration of DOXIL at a clinically relevant dosage schedule was evaluated in rats by determining bacterial blood clearance capacity.

**MATERIALS AND METHODS**

**Animals.** Female R-strain albino rats (20–25 weeks old, specified pathogen free, weighing 185–225 g) were obtained from Harlan Nederland (Austerlitz, The Netherlands).

**Liposomal DOX Formulation.** The formulation of long-circulating DOX liposomes (DOX in PEG-coated liposomes, or DOXIL) was provided by SEQUUS Pharmaceuticals, Inc. DOXIL has the following lipid composition expressed: hydrogenated soybean phosphatidylcholine cholesterol, and PEG (Mr 1900)–derivatized distearoylphosphatidylethanolamine in a molar ratio of 57:38:5. DOX (Farmitalia-Carlo Erba, Milan, Italy) is encapsulated in the internal aqueous phase via an ammonium sulfate gradient, at a drug:lipid ratio of approximately 125 μg DOX/mg lipid. Approximately 95% of the total drug present is in the encapsulated form. The particle size of the preparation is about 100 nm as determined by dynamic light scattering. DOXIL is formulated at a concentration of 2 mg DOX/ml and stored at 2–4°C in glass vials. Under these storage conditions, it is stable for more than 1 year with respect to drug potency, particle size, drug encapsulation, and phospholipid degradation.

**Measurement of Bacterial Blood Clearance Capacity.** Bacterial blood clearance was determined as described previously (24). At 24, 48, 72, and 144 h after the last injection of
buffer or DOXIL, Klebsiella pneumoniae organisms were injected i.v. at an inoculum of $3.2 \times 10^7$ colony-forming units/kg. After 60 and 120 min, blood samples were obtained by retro-orbital bleeding under CO2 anesthesia and cultured on tryptone soy agar (Mast International, Merseyside, UK) to determine the number of viable K. pneumoniae organisms.

**Experimental Design.** The dosage schedule used in the present study was carefully chosen to ensure its clinical relevance. Current clinical experience indicates that the maximum tolerated dose of DOXIL is 60 mg/m² (corresponding to about 1.5 mg/kg body weight) every 4 weeks (16). Distribution half-life of circulating DOXIL in humans is around 45-50 h (8). Plasma pharmacokinetic studies of DOXIL in rats have revealed a dose-independent distribution half-life of 27 h (25). Estimated from these data, a dosage of 1.5 mg/kg given to rats every 2 weeks would be roughly equivalent to the human maximum tolerated dose. For the present rat study, the time interval between the injections was selected to be 9 days, to be on the stringent side. Less than 1% of the injected dose would be present in the blood at 9 days after administration. In a second series of experiments, DOXIL was administered at a frequency of 4 days to study the effect of a higher frequency of administration.

**Statistical Analysis.** Statistically significant differences were analyzed using the Kruskal-Wallis test. To this aim, the mean number of bacteria per ml of blood was determined and expressed as log values. SDs were also determined from the log values. Ps < 0.01 were considered significant.

**RESULTS**

Rats were inoculated i.v. with $3.2 \times 10^7$ K. pneumoniae organisms per kg body weight. Blood clearance capacity was monitored by quantitation of bacterial numbers in the blood at 60 min and 120 min after i.v. inoculation. As shown in Fig. 1, substantial clearance of bacteria from the blood was obtained within 60 min after bacterial inoculation: bacterial numbers dropped from $4.6 \times 10^7$ per ml of blood to 150 per ml of blood.

The K. pneumoniae strain appeared not to be susceptible in vitro to DOX at concentrations up to 512 mg/liter.

To study the effect of DOXIL [DOX entrapped in sterically stabilized liposomes (DOX-SL)] on the phagocytic function of the MPS in terms of bacterial blood clearance capacity, rats received i.v. five dosages of DOXIL (1.5 mg/kg each) or buffer at 9-day intervals. At 24, 48, 72, and 144 h after administration of the last dosage, $3.4 \times 10^7$ K. pneumoniae organisms per kg body weight were inoculated i.v. Bacterial blood clearance capacity of the MPS was monitored at 60 min and at 120 min after bacterial inoculation. At the four intervals after administration of the last dosage of DOXIL, all groups of rats treated with DOXIL at a 9-day interval as well as the buffer-treated rats were able to clear >99% of the inoculated bacteria within 60 min (Fig. 2). Log bacterial numbers per ml of blood had dropped in 60 min from 5.7 to 2.3 in control rats treated with buffer.

DOXIL treatment did not have a significant effect on the bacterial clearance capacity of the MPS, as determined at the four time intervals after the last dosage of DOXIL. At 120 min after inoculation, bacterial numbers were even further decreased in both DOXIL- and buffer-treated rats (Fig. 3). Although efficient (>99%) clearance of bacteria from the blood was observed in all groups of rats, bacterial recoveries at 120 min after inoculation in the groups studied at 72 h after the last dosage of DOXIL were significantly ($P < 0.01$) higher compared to those in the buffer-treated control group. DOXIL treatment did not influence total body weight of the animals.

To investigate the consequence of a higher frequency of administration, the five dosages of DOXIL (1.5 mg/kg) were administered at 4-day intervals. Again, efficient clearance (>99%) of injected bacteria from the blood was observed within 60 min after inoculation (Fig. 4). However, bacterial recoveries from blood at all of the studied time intervals after the last dosage of DOXIL were significantly ($P < 0.01$) higher compared to those of buffer-treated rats. Still, a further decrease in bacterial numbers from 60 to 120 min after inoculation was observed in these rats, which was also seen in the case of the

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**Fig. 3** Numbers of $K. pneumoniae$ in the blood of rats 120 min after i.v. inoculation of $3.2 \times 10^7$ bacteria per kg body weight. Rats were pretreated i.v. with five dosages of DOXIL (1.5 mg/kg each) or buffer at 9-day intervals. Bacterial blood clearance capacity was monitored at 24, 48, 72, and 144 h after administration of the last dosage of DOXIL or buffer. Points, geometric means for five rats each; bars, SD. * $P < 0.01$.

**Fig. 4** Numbers of $K. pneumoniae$ in the blood of rats 60 min after i.v. inoculation of $3.2 \times 10^7$ bacteria per kg body weight. Rats were pretreated i.v. with five dosages of DOXIL (1.5 mg/kg each) or buffer at 4-day intervals. Bacterial blood clearance capacity was monitored at 24, 48, 72, and 144 h after administration of the last dosage of DOXIL or buffer. Points, geometric means for five rats each; bars, SD. * $P < 0.01$. 

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**Research.**

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Effects of DOX Liposomes on Phagocytic Function of MPS

1 Effects of DOX Liposomes on Phagocytic Function of MPS

liposome localization in the MPS has received only minimal note that the possibility of complications resulting from DOX administrations and metastatic outgrowth. Therefore, it is surprising to patients. because they are very liable to generalization of infection. clearance capacity of the MPS is important, especially in such administration of liposomal DOX, including AIDS-related Kaposi's sarcoma (12-14). The target patients for treatment schedule of DOXIL, total body weight of the rats did not decrease over the entire treatment period.

DISCUSSION

The introduction of formulations of sterically stabilized liposomes has expanded the potential for drug targeting to tumors. The current opinion is that the enhanced localization of sterically stabilized liposomes in tumors is the result of their long-circulating property enabling extravasation through the tumor microvasculature, which often shows an increased permeability (26-28). One of the most extensively tested formulations, DOXIL (PEG-coated DOX liposomes), has been shown to boost significantly the DOX levels in tumors and its antitumor activity, regardless of their type and anatomical location, in various mouse and human xenograft tumor models (29-34). Apparently, increased exposure of the tumor cells to higher intratumoral drug concentrations can be achieved with, consequently, a higher probability of tumor response. In recent clinical studies in patients with AIDS-related Kaposi's sarcoma (12-14) and solid tumors (15, 16), evidence of significant antitumor activity has been obtained with DOXIL. However, as with conventional drugs, toxic side effects need to be carefully studied and documented.

The importance of adequate MPS function, in particular the clearance capacity of Kupffer cells, in host defense against infections, systemic endotoxemia, and tumor growth and spread, has been well documented (35-38). The target patients for administration of liposomal DOX, including AIDS-related Kaposi's sarcoma patients and other cancer patients receiving chemotherapy, are often immunocompromised. Optimal blood clearance capacity of the MPS is important, especially in such patients, because they are very liable to generalization of infections and metastatic outgrowth. Therefore, it is surprising to note that the possibility of complications resulting from DOX liposome localization in the MPS has received only minimal attention (19). Some early reports indicated a low vulnerability of in vitro cultured macrophages to toxic effects of DOX (39, 40). However, appropriate consideration of this concern necessitates in vivo evaluation at a clinically relevant dosage schedule. The recent in vivo studies of Daemen et al. (22, 23) pointed to serious MPS impairment resulting from administration of DOX liposomes. However, these studies were not performed with a clinically relevant treatment protocol. In the present study, we used a clinically relevant dosage schedule to evaluate DOXIL, which tends to but does not completely avoid uptake by MPS macrophages of liver and spleen.

The results obtained show that administration of DOXIL (1.5 mg/kg) with a 9- or 4-day interval does not lead to serious impairment of bacterial blood clearance capacity. Even with the intensified schedule of injections given every 4 days, efficient bacterial clearance was observed. This observation is in strong contrast with the outcome of the experimental study performed in the same rat model reported by Daemen et al. (22). The latter study demonstrated major toxic effects of DOX entrapped in short-circulating liposomes on the phagocytic function of the MPS. The discrepancy between both studies can be explained as follows: (a) due to the MPS-avoiding property of the long-circulating DOX liposomes used in the present study, a reduced accumulation of the liposomal drug in the hepatosplenic macrophages is achieved that will be favorable in avoiding adverse effects on their phagocytic function, and (b) the dose and injection frequency of the DOX liposomes are important in this regard. Intensive treatment (2 injections of 5 mg/kg given at a 3-day interval) results in MPS toxicity (22), but such a treatment scheme is far from clinical reality. In our opinion, it is to be expected that the major toxic effects regarding MPS function reported in the previous study would not have been encountered or would have been minimized if dose and dosing frequency had been based on current clinical practice. The 3-fold lower dose is likely to induce less detrimental effects to the MPS cells, or at least the lower injection frequency will permit better recovery of the MPS cells between injections.

In summary, we conclude that DOXIL is fairly well tolerated regarding bacterial blood clearance capacity of the MPS when injected into rats at a clinically relevant dose and dosing frequency. This observation is of considerable clinical relevance, in particular in the case of the treatment of immunocompromised cancer and AIDS patients, who easily develop systemic infections. In these patients, optimal phagocytic function of the MPS is of vital importance.

REFERENCES


Fig. 5 Numbers of K. pneumoniae in the blood of rats 120 min after i.v. inoculation of 3.2 × 10^7 bacteria per kg body weight. Rats were pretreated i.v. with five dosages of DOXIL (1.5 mg/kg each) or buffer at 4-day intervals. Bacterial blood clearance capacity was monitored at 24, 48, 72, and 144 h after administration of the last dosage of DOXIL or buffer. Points, geometric means for five rats each; bars, SD. *, P < 0.01.


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