Multiple Injections of Pegylated Liposomal Doxorubicin: Pharmacokinetics and Therapeutic Activity

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Received April 24, 2003; accepted June 5, 2003

ABSTRACT

Effects of multiple injections of liposomal doxorubicin on pharmacokinetics, therapeutic outcome, and toxicity were studied in mice using different dosing schedules and dose intensities. Biodistribution of doxorubicin to the cutaneous tissues of mice (skin and paws) and to orthotopically implanted mammary tumors (4T1) was examined. Weekly intravenous administration of pegylated (STEALTH) liposomal doxorubicin (SL-DXR) at a dose of 9 mg/kg (every week × 4 doses) resulted in accumulation of doxorubicin in cutaneous tissues of mice and development of lesions resembling palmar-plantar erythrodysesthesia (PPE). Lengthening the dose interval to every 2 weeks × 4 doses reduced the accumulation of doxorubicin and lowered the incidence of PPE-like lesions. A dose interval of every 4 weeks × 4 resulted in complete clearance of doxorubicin from tissues between subsequent doses and a negligible incidence of PPE-like lesions. Doses of 9 mg/kg SL-DXR given at every week × 2 or every 2 weeks × 2 had similar therapeutic activities, whereas prolonging the dose interval to every 4 weeks × 2 reduced therapeutic activity. Pharmacokinetics, biodistribution, and therapeutic activity were studied in tumor-bearing mice for three dose schedules having the same dose intensity (4.5 mg/kg every 3 days × 4, 9 mg/kg every week × 2, or 18 mg/kg every 2 weeks × 1). For these schedules, larger doses administered less often tended to be superior therapeutically to smaller doses given more often. These data provide the first pharmacokinetic measurements of doxorubicin concentrations in cutaneous tissues and tumors with repeat administration of liposomal formulations, and they provide a useful model for the study of factors leading to PPE in humans.

Pegylated (STEALTH) liposomal doxorubicin (Doxil/Caelyx) (SL-DXR) is a long-circulating formulation of liposomal doxorubicin that is currently approved for use in AIDS-related Kaposi's sarcoma and refractory ovarian cancer. It has also shown activity in other tumors, including metastatic breast cancer (Northfelt et al., 1997; Ranson et al., 1997; Gordon et al., 2000). As reviewed by Allen et al., STEALTH liposomes have dose-independent, log-linear pharmacokinetics (Allen et al., 1995). Encapsulating doxorubicin within these liposomes alters its pharmacokinetics and biodistribution, and results in a decrease in doxorubicin-associated toxicities, including its dose-limiting cardiomyopathy and myelosuppression (Berry et al., 1998; Safra et al., 2000). The dose-limiting toxicities of SL-DXR are mucocutaneous reactions such as palmar-plantar erythrodysesthesia (PPE) and mucositis/stomatitis (Gordon et al., 1995; Uziely et al., 1995; Lotem et al., 2000; Hamilton et al., 2002).

Palmar-plantar erythrodysesthesia primarily affects the palms of the hands and the soles of the feet. Patients who develop PPE experience erythema and edema that can lead to blistering and desquamation if the next dose is not delayed or reduced. The current hypothesis for the development of PPE is that the small size (100-nm diameter) and long circulation time (t1/2 is approximately 48 h in humans) of SL-DXR allows liposomes to accumulate in the skin. The basal layers of the skin are damaged with prolonged exposure to doxorubicin as the liposomes slowly release their contents. The accumulation of liposomes is thought to mimic the anatomical distribution of lesions and to be greatest in regions of skin that are subjected to pressure or irritation, such as the flexure creases of the hands, soles of the feet, or belt lines (Gordon et al., 1995; Lotem et al., 2000).

This hypothesis is supported by current experimental and clinical data. Liposomes with long circulation times accumulate in the skin of experimental animals to a greater extent than liposomes with shorter circulation times (Allen et al., 1991; Papahadjopoulos et al., 1991). In mice, this accumulation is dependent on liposome size; furthermore, mouse paws (homologous to human hands and feet) accumulate more liposomes than skin, supporting the idea of the pressure-
developing PPE is related to the dose intensity of SL-DXR after multiple doses of SL-DXR. In addition, the likelihood of myelosuppression affects the therapeutic activity of SL-DXR.

Clinical data suggest that PPE is more likely to develop after multiple doses of SL-DXR. In addition, the likelihood of developing PPE is related to the dose intensity of SL-DXR therapy, with patients receiving greater than 10 to 12 mg/m²/week more likely to develop symptoms (Gabizon et al., 1994b; Ranson et al., 1997). When PPE develops, clinical interventions include lengthening the dose interval and/or decreasing the dose intensity. Either of these interventions may compromise the therapeutic outcome (Hensley et al., 2001).

Despite the widespread clinical use of SL-DXR, few studies have looked at the pharmacokinetics and biodistribution of repeat injections in experimental models, and no studies have quantified the cutaneous localization of doxorubicin from SL-DXR with repeat administration (Amantea et al., 1999). Therefore, a small-animal model for the pharmacokinetics and biodistribution of doxorubicin (from SL-DXR) in plasma, tumor, and cutaneous tissues will be beneficial in understanding the relationship between the dose schedule and dose intensity of SL-DXR therapy and its therapeutic activity and toxicity. We performed experiments studying the plasma pharmacokinetics and biodistribution of doxorubicin to the skin and paws of mice as a function of time using either the same dose of SL-DXR and different dose intervals (i.e., different dose intensities) or different dose schedules with the same dose intensity. The latter experiments included tumor tissue (4T1 murine mammary carcinoma) and were performed to test the hypothesis that, for a given dose intensity, it is therapeutically beneficial to administer larger infrequent doses than smaller more frequent doses (Gabizon, 2001). Therapeutic experiments were also performed using the 4T1 murine mammary carcinoma model to determine whether altering either the dose schedule or the dose intensity affects the therapeutic activity of SL-DXR.

Materials and Methods

Chemicals and Reagents. SL-DXR (STEALTH liposomal doxorubicin, Doxil/Caelyx) was a generous gift from ALZA Corporation (Mountain View, CA). Dextrose USP (D5W), 5% w/v in water (Baxter Toronto, ON, Canada), was purchased from the pharmacy at the University of Alberta Hospitals. Minimal essential medium was from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum, penicillin, and streptomycin were from Invitrogen (Burlington, ON, Canada). All other chemicals were of the highest grade available.

Animals and Tumor Model. Female BALB/c mice (6–8 weeks) were purchased from the breeding colony at the Health Sciences Laboratory Animal Services (University of Alberta, Alberta, Canada). Mice were housed under standard conditions and had access to food and water ad libitum. All animal protocols were approved by the Health Sciences Animal Policy and Welfare Committee (University of Alberta) and are in accordance with the Guide to the Care and Use of Experimental Animals set forth by the Canadian Council on Animal Care.

Pharmacokinetics and Biodistribution. Pharmacokinetic and biodistribution studies were carried out in either tumor-free mice or in mice bearing murine mammary carcinoma (see below). The SL-DXR was diluted in D5W, and 200 μl was injected intravenously (i.v.) via the lateral tail vein. In tumor-free mice, 4 doses of 9 mg/kg (27 mg/m²) SL-DXR were administered either weekly (q1 week × 4), every 2 weeks (q2 weeks × 4), or every 4 weeks (q4 weeks × 4) for a total dose of 36 mg/kg. The dose intensities for these schedules were 9 mg/kg/week (27 mg/m²/week), 4.5 mg/kg/week (13.5 mg/m²/week), and 2.25 mg/kg/week (6.75 mg/m²/week), respectively (Freireich et al., 1966). In experiments at the same dose intensity, tumor-bearing mice received 9 mg/kg/week (27 mg/m²/week) of SL-DXR using either 4 doses of 4.5 mg/kg (q3 day), 2 doses of 9 mg/kg (q1 week), or 1 dose of 18 mg/kg (q2 weeks). At various time points after each injection, mice were euthanized (n = 4–5), blood was collected with a heparinized syringe, and plasma was isolated by centrifugation (3,000 ′′ for 5 min). Organs were removed and doxorubicin quantified as described below.

Pharmacokinetic parameters were calculated for total doxorubicin. The area under the concentration versus time curve (AUC) was calculated using the trapezoidal rule. Plasma half-lives (t1/2) were calculated using the formula: t1/2 = 0.693/k, where k is the elimination constant derived from the plasma concentration versus time curve. Tissue t1/2 was calculated in a similar manner, using the terminal slope of the tissue concentration versus time curve; t1/2 was not calculated for q3-day or q1-week dosing, as there were not sufficient time points in the terminal portion of the curves. For experiments using different dose intensities, the average steady state concentration (Cₘₕ) was calculated by taking the fourth dose AUC (taken as steady state) as determined by the trapezoidal rule and dividing by the dose interval in hours.

Quantification of Doxorubicin. Total tissue doxorubicin was quantified using a method similar to that of Mayer et al. (1997). Briefly, tissue homogenates of 10% w/v were prepared in water. Skin and paws were frozen in liquid nitrogen and crushed with a mortar and pestle before homogenization with a Polytron homogenizer (Brinkmann Instruments, Inc., Mississauga, ON, Canada). Homogenates or 25% plasma (200 μl) were placed in a 2-ml micro-centrifuge tube, and 100 μl of 10% (v/v) Triton X-100, 200 μl of water, and 1,500 μl of acidified isopropanol (0.75 N HCl) were added. The tubes were mixed thoroughly, and the doxorubicin and doxorubicin metabolites (if any) were extracted overnight at −25°C. The next day, the tubes were warmed to room temperature, vortexed for 5 min, centrifuged at 15,000g for 20 min, and stored at −80°C until analysis. Doxorubicin was quantified fluorometrically (λₐₖₚₜ₄ₒₒ 470 nm and λₑᵲᵢₐᵲₐₕₜₐ 590 nm). To correct for nonspecific background fluorescence, the samples were analyzed using a standard curve containing tissue extracts derived from drug-free mice. The data represent the mean ± S.D. of triplicate aliquots from four to five mice and are expressed as doxorubicin microequivalents per milliliter of plasma or per gram of tissue, as this assay does not discriminate between doxorubicin and any fluorescent metabolites that may have similar excitation and emission profiles.

Tumor Implantation/Therapeutic Experiments. The 4T1 murine mammary carcinoma was a generous gift from Dr. Fred Miller (Barbara Ann Karmanos Cancer Institute, Detroit, MI) and was maintained in minimal essential medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified incubator with a 5% CO₂ atmosphere (Aslakson and Miller, 1992). Tumors were orthotopically implanted as previously described (Moase et al., 2001). Briefly, a small incision was made in the lower abdomen of anesthetized mice, and 10⁵ 4T1 cells in 10 μl of supplemented medium were implanted in the right no. 4 mammary fat pads. The incision was closed with a surgical wound clip, which was removed 1 week later. For tissue distribution studies in tumor-bearing mice, mice were injected with the chosen dose of SL-DXR 10 days after tumor implantation when...
tumors were large enough to excise. Studies were then performed as described above.

For therapeutic experiments, mice were treated 4 days after tumor implantation. Mice treated with different dose intensities received 9 mg/kg SL-DXR either q1 week × 2, q2 weeks × 2, or q4 weeks × 2. When the dose intensity was kept constant, mice received one dose of 9 mg/kg (54 mg/m²), 9 mg/kg (27 mg/m²) q1 week, or 4.5 mg/kg (13.5 mg/m²) q3 days for a total drug dose of 18 mg/kg. Tumor growth was monitored by measuring tumor diameters with calipers, and tumor volume was calculated using the formula: \( V = 0.4ab^2 \), where \( a \) and \( b \) represent perpendicular diameters and \( a > b \). The experiment was repeated once, and the data represent the mean ± S.D. from 5 to 10 mice except for the group receiving 18 mg/kg, where \( n = 4 \) to 5 (see Toxicity).

Statistical Analysis. Statistical comparisons were performed using a one-way ANOVA with a Tukey-Kramer post test or Student’s \( t \) test (as appropriate) with Graph Pad InStat Version 3.01 for Windows 95/NT (GraphPad Software, Inc., San Diego CA).

Results

Pharmacokinetics for Different Dose Schedules. Figure 1 presents the plasma, skin, and paw doxorubicin profiles for mice receiving weekly i.v. doses of 9 mg/kg (27 mg/m², q1 week × 4). Results shown in Fig. 1A indicate that the drug was not completely cleared from the plasma before administration of subsequent doses. Plasma \( t_{1/2} \) values were on the order of 40 h, and plasma concentrations for each dose peaked at approximately the same values. Plasma AUC values plateaued after the second dose, suggesting that steady state was reached (Table 1).

Skin and paw drug concentrations for a dose schedule of q1 week × 4 are seen in Fig. 1, B and C, respectively. Similar to plasma, doxorubicin was not completely cleared from either tissue between doses. For the first three doses of SL-DXR, skin \( C_{\text{max}} \) was reached 72 h postinjection (\( p < 0.001–0.05 \)) and at 24 h after injection for the fourth dose. The nadir occurred at increasing drug levels with each subsequent dose. Skin AUCs increased 3-fold between the first and third doses and then appeared to reach steady state (Table 1).

The \( C_{\text{max}} \) for total doxorubicin was reached in paws 72 h after the first dose but was earlier for subsequent doses (\( p < 0.01–0.001 \)). Paws achieved higher drug concentrations than skin for the first two doses, as reflected in their higher AUC levels, but were similar to skin for the next two doses (Fig. 1, Table 1). The nadir drug levels for paws remained high throughout the study, and paw levels appeared to reach steady state after the first dose (the AUCs for paws did not change with subsequent doses). The higher drug levels in paws than in skin may be due to the pressure-dependent extravasation of liposomes as the mice walk around the cage, groom, feed, etc.

Doxorubicin levels in plasma, skin, and paws of mice receiving i.v. SL-DXR at a dose of 9 mg/kg q2 weeks are presented in Fig. 2. Extending the dose interval allowed plasma drug levels to fall to below detectable limits before the next dose of SL-DXR was given. As with the q1-week dosing schedule, plasma AUC values plateaued after the second dose (Table 1).

Skin and paws reached \( C_{\text{max}} \) for total doxorubicin for the q2 weeks × 3 schedule at approximately 72 h postinjection. Prolonging the dose interval allowed more drug to be cleared from the skin and paws, and the nadir drug levels were significantly lower than those reached for the q1-week dose schedule (\( p < 0.001 \) for skin and \( p < 0.01–0.001 \) for paws). Again, paw concentrations of doxorubicin were initially higher than those in skin. With subsequent doses, however, the skin \( C_{\text{max}} \) increased (\( p < 0.05 \) for dose 1 versus doses 3 and 4), while unexpectedly the paw \( C_{\text{max}} \) decreased significantly between the first and second doses (\( p < 0.05 \)) and between the second and third doses (\( p < 0.01 \)) (Table 1). These changes are also reflected in their respective AUC values (Table 1).

Figure 3 presents results for an i.v. dose schedule of 9 mg/kg SL-DXR q4 weeks × 4. Peak plasma levels were the same as for the previous two dosing schedules, and as was seen in mice receiving the q2 weeks × 4 dosing schedule, the
longer dose interval resulted in plasma doxorubicin concentrations that were below detectable limits between doses. The $t_{1/2}$ and AUC values were also similar to those for previous dosing schedules (Table 1).

Skin and paw doxorubicin concentrations for this dose schedule are presented in Fig. 3, B and C, respectively. Again, the $C_{max}$ for total doxorubicin was achieved at approximately 72 h postinjection. For this dose schedule, the drug concentrations in both skin and paws fell to low levels before each successive injection. Skin $C_{max}$ and AUC values increased with each dose ($C_{max}$ dose 1 versus dose 4, $p < 0.05$), whereas those for paws decreased, particularly between the first and subsequent doses ($C_{max}$, $p < 0.001$ dose 1 versus dose 2) (Table 1).

The skin and paw clearance $t_{1/2}$ values for doxorubicin are given in Table 1. Skin and paw $t_{1/2}$ values could not be calculated for the q1-week dosing schedule. Modest increases in plasma $t_{1/2}$ were observed for all dosing schedules from the first to fourth dose. Mice receiving 9 mg/kg q2 weeks or q4 weeks had greatly increased clearance $t_{1/2}$ values for skin from the first to fourth dose. Mice receiving SL-DXR with a q4 weeks schedule had an increase in clearance $t_{1/2}$ for paws, while skin $t_{1/2}$ did not change appreciably. The average steady state drug concentration ($C_{ss}$) for each dose schedule was calculated by dividing the AUCss (fourth dose) by the dose interval in hours (Table 1). As expected, doubling the dose interval resulted in a halving of the $C_{ss}$ values for all tissues.

**Toxicity.** PPE-like lesions were more frequent in mice receiving the 9 mg/kg q1 week $\times$ 4 dose schedule (Table 2). The lesions included hair loss on the mouse’s muzzle (area exposed to pressure while the mouse feeds) and red inflamed paws with mild swelling (the presence of lesions did not, however, have an important effect on the weight of the paws; data not shown). This is consistent with current clinical and laboratory data demonstrating that PPE is more likely to occur with higher Doxil dose intensities (Ranson et al., 1997; Amantea et al., 1999; Lotem et al., 2000).

During these experiments, some additional drug toxicity was observed, particularly for the weekly dose schedule. Four mice from the 9 mg/kg q1 week schedule were euthanized due to severe weight loss (three mice, no cause determined; 1 mouse, heart failure). Three mice from the 9 mg/kg q2 weeks schedule were euthanized (2 mice, no cause determined; 1 mouse, mild subacute cardiac and hepatic degeneration). In the 9 mg/kg q4 weeks group, 1 mouse was euthanized due to severe weight loss (no cause determined). The staff veterinarian at the University of Alberta’s Health Sciences Laboratory Animal Services performed all postmortem exams.

The total cumulative SL-DXR dose for these animals was high (36 mg/kg, 108 mg/m2). Since toxicity was encountered, mice in the therapeutic experiments received only two doses of SL-DXR (18 mg/kg, 54 mg/m2 total drug).

**Pharmacokinetics for the Same Dose Intensity.** To determine the effect of different dose schedules at the same dose intensity, we performed pharmacokinetic and biodistribution experiments in mice bearing the 4T1 murine mammary carcinoma and measured doxorubicin levels in plasma, tumor, skin, and paws. At 10 days postimplantation, when the tumors were well developed, mice were injected i.v. with a total dose of 18 mg/kg SL-DXR (54 mg/m2) given as either 4.5 mg/kg q3 days, 9 mg/kg q1 week $\times$ 2, or 18 mg/kg q2 weeks $\times$ 1. Tissue concentrations and pharmacokinetic parameters are given in Fig. 4 and Table 3.

The results for plasma doxorubicin concentrations are presented in Fig. 4A. For mice receiving 4.5 mg/kg q3 days, there was a significant increase in plasma $C_{max}$ from the first dose to the second and subsequent doses ($p < 0.001$). In addition, plasma levels appeared to reach steady state after the second dose, as evidenced by the AUCs (Table 3). As with naive mice, in tumor-bearing mice receiving 9 mg/kg q1 week, there was detectable drug in the plasma at 7 days after injection (Fig. 1A versus 4A). Interestingly, the plasma $t_{1/2}$ and AUC values were lower in tumor-bearing mice than for naive mice receiving 9 mg/kg q1 week (Table 3 versus Fig. 1A). Distribution to
the tumor may account for the lower $t_{1/2}$ and tissue AUC values, which is consistent with results from studies using the C26 colon carcinoma tumor model in BALB/c mice (Hong et al., 1999).

A single dose of 18 mg/kg resulted in a plasma $C_{\text{max}}$ approximately twice that of the first dose of the 9 mg/kg dose schedule and approximately 4 times that of the first dose of the 4.5 mg/kg dose schedules (Table 3). For each schedule, there is also a linear relationship between the AUC of the first injection and the dose ($r^2 = 0.9937$). These observations are in line with the dose independence of the plasma pharmacokinetics for single doses of SL-DXR in this dose range (Allen and Hansen, 1991; Gabizon et al., 1994b).

Tumor levels of doxorubicin are given in Fig. 4B. For mice receiving 18 mg/kg and for the first dose at the 4.5 or 9 mg/kg dose schedules, tumor doxorubicin reached $C_{\text{max}}$ at 24 h, which was earlier than skin and paw levels reached $C_{\text{max}}$ for the two higher doses. The $C_{\text{max}}$ for the dose schedule was approximately double that of the 9 mg/kg q1-week dose schedule and approximately 4-fold higher than the $C_{\text{max}}$ for
the 4.5 mg/kg q3-day dose schedule (Fig. 4B), i.e., the $C_{\text{max}}$ increased proportionately with dose. The tumor AUC($\tau_{\omega}$), values were similar for all dose schedules (Table 3).

Skin drug levels from each of the dose schedules are seen in Fig. 4C. Results for mice receiving 9 mg/kg were similar to those for nontumor-bearing mice in that the $C_{\text{max}}$ for skin doxorubicin in tumor-bearing mice peaked at 72 h postinjection. In addition, the $C_{\text{max}}$ and AUCs for the second dose were higher than the first ($p < 0.0008, t$ test) (Table 1A versus 3; Fig. 1B versus 4C). As with tumor, the doxorubicin $C_{\text{max}}$ in skin increased proportionately with dose (Fig. 4C). The total AUC($\tau_{\omega}$), values for the 18 mg/kg and 9 mg/kg dose schedules were similar, and higher than that seen for the 4.5 mg/kg dose schedule (Table 3). These results demonstrated that skin, like tumor, was exposed to sustained levels of doxorubicin for all three dose schedules, although the 9 and 18 mg/kg schedules resulted in exposure to higher drug concentrations.

As in naive mice, the paw concentrations of doxorubicin were higher than skin concentrations in tumor-bearing mice.

### Table 3

Pharmacokinetic parameters for mice receiving SL-DXR at a dose intensity of 9 mg/kg/week

Mice received i.v. either four doses at 4.5 mg/kg q3 days, two doses at 9 mg/kg q1 week, or one dose at 18 mg/kg q2 weeks. The AUC values were calculated using the trapezoidal rule. Plasma $t_{1/2}$ values were calculated using the formula $t_{1/2} = \frac{0.693}{k_{\text{elm}}}$, where $k_{\text{elm}}$ is the elimination constant derived from the plasma concentration versus time curve.

<table>
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<th>Dose Schedule</th>
<th>Plasma $t_{1/2}$</th>
<th>AUC doxorubicin $\mu$Eq $\times$ h/ml</th>
<th>Skin AUC $\mu$Eq $\times$ h/g</th>
<th>Paws AUC $\mu$Eq $\times$ h/g</th>
<th>Tumor AUC $\mu$Eq $\times$ h/g</th>
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<tr>
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<tr>
<td>Dose 4 AUC ($0-72$ h)</td>
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<tr>
<td>Total AUC ($0-\omega$)</td>
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Fig. 4. Tissue concentrations of doxorubicin in mice given SL-DXR at the same dose intensity. The BALB/c mice were implanted in the no. 4 mammary fat pad with the 4T1 tumor and injected i.v. with SL-DXR 10 days later. Data represent the mean ± S.D. of triplicate aliquots from five mice and are expressed as doxorubicin microequivalents: A, plasma; B, tumor; C, skin; D, paws. ■, 18 mg/kg q2 weeks (1 dose); ▲, 9 mg/kg q1 week (2 doses); ●, 4.5 mg/kg q3 days (4 doses).
(Fig. 4D versus 1C). For mice receiving 4.5 mg/kg (Fig. 4D), the $C_{\text{max}}$ in paws continued to increase for 7 days after initiation of therapy. The $C_{\text{max}}$ in paws also increased proportionately with dose. The AUC$_{(0-\infty)}$ for the 18 mg/kg dose was higher than that for the 9 mg/kg $\times$ 2 dose schedule, which in turn was higher than the AUC for the 4.5 mg/kg $\times$ 4 dose schedule (Table 3). The increased paw AUC at higher doses may indicate a greater likelihood of developing skin toxicities such as PPE at these doses. For mice receiving 18 mg/kg, the tumor, skin, and paw clearance $t_{1/2}$ values were 117, 90, and 110 h, respectively. It is notable that the tissue $t_{1/2}$ values were considerably higher than those for plasma $t_{1/2}$ (Table 3). The values for skin and paws are consistent with results from naïve mice receiving SL-DXR with different dose schedules.

**Therapeutic Experiments.** The results of therapeutic experiments in tumor-bearing mice receiving 9 mg/kg q1 week, q2 weeks, or q4 weeks by the i.v. route are presented in Fig. 5. Tumor volume can be difficult to measure when tumors exceed 400 mm$^3$; however, tumor growth in control mice receiving sterile D5W was similar for all dose schedules. The therapeutic activities of SL-DXR were equivalent for mice receiving the drug for either a q1 week $\times$ 2 or a q2 weeks $\times$ 2 dose schedule. SL-DXR administered using a q4 weeks $\times$ 2 dose schedule appeared to have reduced therapeutic activity compared with the other two dose regimes. In other words, if the dose interval was too long, antitumor activity was affected adversely. This may have therapeutic implications, as clinical interventions for PPE include lengthening the dose interval or reducing the dose (i.e., reducing the dose intensity) to decrease the incidence and/or severity of PPE.

The results for mice receiving the same dose intensity (9 mg/kg/week, 27 mg/m$^2$/week) at different dosing schedules are presented in Fig. 6. All three schedules delayed tumor growth considerably. Nevertheless, the two dosing schedules with larger doses given less frequently (9 mg/kg q1 week $\times$ 2 or 18 mg/kg) appeared to delay tumor growth to a greater extent than smaller doses given more frequently. When this experiment was repeated, five mice had to be euthanized due to toxicity in the group receiving 18 mg/kg. A gross postmortem examination by the University of Alberta’s Health Sciences Laboratory staff pathologist found evidence of cardiac toxicity. This dose is well below the reported LD$_{50}$ of 38 mg/kg reported for a bolus injection of SL-DXR in CD-1 mice (Working and Dayan, 1996). Whether this difference was because of strain-specific differences in sensitivity to doxorubicin or was tumor-related was not examined further. No further experiments were carried out with this dose.

**Discussion**

The results from these murine experiments suggest that this species is a reasonable animal model for studying factors influencing the development of Doxil-associated PPE. We demonstrated that repeat administration of SL-DXR using short dose intervals (q1 week) resulted in an accumulation of doxorubicin in the cutaneous tissues of mice. Multiple doses were shown to increase the incidence of mice developing PPE-like lesions. We also demonstrated that lengthening the dose interval allows for more accumulated drug to be cleared from these tissues, resulting in fewer PPE-like lesions in mice. These experimental results confirm clinical observations that longer dose intervals in humans reduced the incidence and severity of PPE (Uziely et al., 1995; Ranson et al., 1997). If our murine results can be extrapolated to humans, then dose delay appears to be useful in controlling PPE because it allows time for drug to be cleared from the skin and for existing lesions to heal. As shown here, however, the advantages of dose delay may be offset by reduced therapeutic activity.

A recent review of toxicities associated with Doxil in patients with metastatic breast cancer provides support for our model (Lyass et al., 2000). The recommended dose intensity for these patients was $\sim$12 mg/m$^2$/week (e.g., 40–50 mg/m$^2$ q4 weeks), and the average plasma $t_{1/2}$ was 79.4 h, corre-
responding to 8.5 times the plasma $t_{1/2}$ for SL-DXR administered every 4 weeks. Our data mimic these clinical data in that a dose interval of q2 weeks (13.5 mg/m²/week) corresponds to 8.5 plasma $t_{1/2}$ (the average plasma $t_{1/2}$ in naive mice was 39.4 h). Interestingly, our experiments show that a dose of 13.5 mg/m²/week resulted in good therapeutic efficacy combined with low levels of PPE-like symptoms.

It is also important to note that the plasma $t_{1/2}$ did not change substantially for multiple doses of SL-DXR, although there was a modest increase in $t_{1/2}$ after the first dose for each schedule. This is significant because the development of PPE has been correlated to the plasma half-life of SL-DXR (Lyass et al., 2000). If SL-DXR is cytotoxic to cells of the mononuclear phagocyte system (MPS), which is responsible for clearing liposomes, then multiple dose regimes could result in extended $t_{1/2}$ as a result of impaired clearance mechanisms (Daemen et al., 1995). We conclude that the dose schedules used in this study did not impair MPS function to a degree that affected the pharmacokinetics of SL-DXR. This lack of substantial MPS toxicity with SL-DXR is consistent with studies from other laboratories (Storm et al., 1998).

The observation that skin and paw pharmacokinetic parameters were different from those for plasma is interesting. Plasma drug levels fell to low values between doses for even a q1-week dose schedule, while skin and paws drug levels remained elevated for several days. Plasma levels in mice have been important for determining the dosing schedule for liposomal drugs in efficacy studies, and a q1 week schedule is often chosen. This schedule is based on clearance of inert liposomal markers such as $^{125}$I-tyraminylinulin in naive mice ($t_{1/2}$ of 18–24 h in liposomes of similar composition to those used in these studies) (Allen et al., 1993). Hence, within 1 week (>8 half-lives), this marker would be cleared almost completely from the plasma of mice. Nevertheless, the clearance rate of doxorubicin is approximately 2-fold longer than the clearance rate of $^{125}$I-tyraminylinulin (an average 39 h in naive mice), and 8 half-lives, in this case, corresponds to one dose every 2 weeks. The difference between the $t_{1/2}$ of doxorubicin and $^{125}$I-tyraminylinulin liposomes reflects differences in release rates and volumes of distribution of the two compounds. Furthermore, loading doxorubicin into liposomes has been shown to increase their circulation times in other models (Bally et al., 1989). Regardless of the model, these data demonstrate that pharmacokinetic studies that do not follow the pharmacologically active agent should be interpreted with caution.

The half-life of SL-DXR was shorter in tumor-bearing mice than in naive mice. This is consistent with work by Hong et al. (1999), who found that the $t_{1/2}$ for SL-DXR was shorter in mice bearing subcutaneous implants of the C26 colon carcinoma (19.1 h) than in naive mice (25.1 h). This can partially be explained by significant distribution of drug-loaded liposomes to tumors.

Our results show that half-lives for elimination of drug from skin, paws, and tumors were longer than that for plasma. A longer $t_{1/2}$ will lead to retention of drug in tumors and, arguably, improved antitumor effects, but longer $t_{1/2}$ values in cutaneous tissues will lead to unwanted side effects such as PPE. The challenge is to find the proper balance between minimizing PPE and maintaining therapeutic activity. Our studies show that increasing the dose interval to q2 weeks did not significantly affect the therapeutic outcome in our tumor model; however, extending the dose interval to q4 weeks compromised the therapeutic activity.

Skin concentrations of doxorubicin and their respective AUCs continued to increase with each successive dose (Table 1). This may be a consequence of skin cytotoxicity accompanied by inflammation. It is well known that inflamed tissue, like tumor tissue, has increased capillary permeability and can accumulate liposomes via the enhanced permeability and retention effect (Matsumura and Maeda, 1986; Maeda et al., 2000). This will increase localization of liposomes into skin with subsequent injections in a vicious cycle. Alternatively, since our dorsal skin samples were not subject to pressure or
irritation, the increased localization of liposomes into skin may reflect an increase to steady-state levels, which normally occurs within three to five doses. For drug clearance, an interval of five half-lives results in approximately 3% of the total dose remaining in tissues. For skin, five half-lives would be approximately 23 days, roughly corresponding to the q4-week dosing interval that produced the lowest incidence of PPE-like lesions.

One unexpected observation was the decrease in the $C_{\text{max}}$ for paws using the q2- and q4-week dose schedules. This decrease was not due to the alterations in the plasma pharmacokinetics (i.e., $t_{1/2}$ values did not decrease). Therefore, fewer liposomes localized in paws. This may be a result of doxorubicin-associated tissue damage causing tissue remodeling or scarring, which would reduce the ability of subsequent doses to accumulate. Alternatively, it could be due to a reduction in the pressure-dependent extravasation of liposomes if mice developed "sore paws" (PPE-like lesions) and moved around their cages less, although this was not specifically measured.

The cytotoxicity of doxorubicin is not cell cycle-dependent; therefore, one can speculate that the antitumor activity of doxorubicin might be dependent upon tumor $C_{\text{max}}$. Our therapeutic studies demonstrated that SL-DXR doses of 9 mg/kg q1 week or 18 mg/kg doxorubicin, which result in higher peak concentrations of total drug, had better therapeutic activity than smaller doses (4.5 mg/kg) given more frequently.

As previously observed (Charrois and Allen, 2003) and as verified in these experiments, the 4T1 tumor accumulates liposomes at a faster rate than either skin or paws. Therefore, it may be possible to reduce the incidence of SL-DXR-associated cutaneous toxicities by engineering a liposomal drug delivery system, e.g., a triggered release system, that accumulates in tumors and releases its contents before maximal liposome accumulation in skin or paws. This hypothesis is supported by work by Needham et al. (2000), who demonstrated improved therapeutic outcomes in tumor-bearing mice when doxorubicin release was triggered by hyperthermia in single tumors (i.e., not metastatic disease).

Our study measured total doxorubicin, which includes both liposome-encapsulated and released drug. An important consideration in pharmacokinetic, biodistribution, and therapeutic studies with liposomes is the bioavailability of the drug. As long as the drug, e.g., doxorubicin, remains encapsulated within the liposomes, it is not bioavailable and will have no biological activity, including no antitumor effect. It is possible to have high tissue AUCs for liposomal drugs, but low levels of efficacy, if the drug is released very slowly since minimal therapeutic levels of drug in tissues may not be reached. At the opposite end of the spectrum, if the drug is released too rapidly, before the liposomes localize in target tissues, the therapeutic effects may not be different from the administration of nonencapsulated drug. To determine optimum drug release rates, it will be necessary to develop methods for measuring bioavailable drug in the target tissues and in the tissues that are subject to toxic side effects; this has been a relatively neglected area of liposome research (Krishna et al., 2001). Nevertheless, several laboratories are developing methods to trigger the release of liposomal contents once the liposomes have accumulated in target tissues such as tumors (Adlakha-Hutchison et al., 1999; Kong et al., 2000; Goldberg et al., 2002).

In summary, these studies using a murine model reinforce the importance of dose schedule and dose intensity on the therapeutic activity and cutaneous toxicity of SL-DXR and provide the first experimental data on the pharmacokinetics and biodistribution of liposomal doxorubicin in tumor and cutaneous tissue for multiple dosing schedules. They also provide experimental evidence supporting the utility of a mouse model for predicting side effects and therapeutic activity in the clinic.

Acknowledgments

We gratefully acknowledge Dr. Dion Brooks (Faculty of Pharmacy, University of Alberta) for helpful discussions. The technical assistance for tumor implantation of Elaine Mose, Janny Zhang, and the University of Alberta Health Sciences Laboratory Animal Services is also gratefully acknowledged, as well as Dr. Richard Uwiera for performing gross pathological examinations.

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