

Determination of Doxorubicin Levels in Whole Tumor and Tumor Nuclei in Murine Breast Cancer Tumors

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Abstract Purpose: Pharmacokinetic studies on liposomal drugs have previously measured total drug levels in tumors, which include nonbioavailable drug. However, drugs must be released from liposomes to have activity. We have developed a method for measuring levels of bioavailable (released) doxorubicin *in vivo* in tumors that will allow therapeutic activity to be correlated with bioavailable drug levels.

Experimental Design: Mice orthotopically implanted with mammary carcinoma (4T1) were injected i.v. 10 days after implantation with free doxorubicin or formulations of liposomal doxorubicin with different drug release rates. Tumors were excised at various times after injection, and total tumor doxorubicin levels were determined by acidified isopropanol extraction of whole tumor homogenates. Bioavailable doxorubicin levels were determined by extraction of doxorubicin from isolated tumor nuclei.

Results: Free doxorubicin had high levels of bioavailability in tumor tissue; 95% of the total doxorubicin in tumors was bound to nuclear DNA by 24 hours after injection. Administration of Doxil, a slow release liposomal formulation of doxorubicin, gave an area under the time-versus-concentration curve (AUC) for total doxorubicin 7 days after injection that was 87-fold higher than that obtained for free doxorubicin, and 49% of the liposomal doxorubicin was bioavailable. For liposomes with a more rapid doxorubicin release rate, by 7 days after injection, the AUC_{0-7 days} for total doxorubicin was only 14-fold higher than that for free doxorubicin and only 27% of liposomal doxorubicin was bioavailable.

Conclusions: This technique allows correlations to be made between drug bioavailability and therapeutic activity and will help in the rational design of drug carriers.

Long-circulating pegylated liposomal formulations of doxorubicin, Doxil, have been shown to result in increased accumulation of drug in solid tumors and reduced dose-limiting toxicities such as myelosuppression and cardiotoxicity. This is due to alterations in the pharmacokinetics and biodistribution of the encapsulated drug (1–3). Doxil is currently approved for use in AIDS-related Kaposi sarcoma, refractory ovarian cancer, and metastatic breast cancer (4–8).

Doxorubicin-loaded liposomes have enhanced efficacy in some solid tumors compared with free doxorubicin, because they passively target solid tumors through the enhanced permeability and retention effect (9, 10), resulting in increased drug payloads delivered to tumors. The enhanced permeability

and retention effect is a result of defective vascular endothelial linings of growing tumors, resulting in gaps in the endothelium up to ~800 nm in diameter, which are large enough to permit the extravasation of liposomes with diameters in the range of 100 nm (11). In addition, growing tumors have defective lymphatic drainage, which contributes to the extended residence time of extravasated liposomes in the interstitial space of the tumor. Liposomes residing in the interstitial space gradually release their entrapped drug, exerting antitumor effects.

Many studies have measured total drug levels in solid tumors following administration of liposomal drugs (12–17). These studies do not distinguish between entrapped (nonbioavailable) drug and released (bioavailable) drug in the tumor. However, only released drug has biological activity, and the therapeutic effects of liposomal drugs will (we propose) be correlated with the levels of bioavailable drug in tumor as a function of time and not the levels of total drug in the tumor. Therefore, knowledge of the levels of bioavailable drug in tumor tissue and the rate of bioavailability will help in the design of improved liposomal formulations of anticancer drugs.

An important site of cytotoxic action of the anticancer drug doxorubicin is the nucleus, where doxorubicin intercalates into DNA, forming DNA adducts and inhibiting topoisomerase II (18). When free doxorubicin reaches the tumor site, doxorubicin that is released from liposomes within the tumor interstitial space is capable of diffusing widely within the tumor. Doxorubicin can diffuse into surrounding cells, become

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membrane or protein associated, or diffuse into subcellular compartments such as mitochondria and nuclei. Confocal studies show that a large proportion of doxorubicin that diffuses into the cell accumulates in the cell nucleus (19), where it binds strongly to nuclear DNA. Nuclear DNA functions as a sink for the drug, including drug initially present in other subcellular organelles (20, 21). Therefore, we hypothesize that measurement of doxorubicin bound to nuclear DNA will provide a good estimate of levels of bioavailable doxorubicin in tumor tissue *in vivo*. Levels of doxorubicin in total tumor and tumor nuclei were measured in mice orthotopically implanted with 4T1 murine mammary carcinoma. Tumor-bearing mice were injected i.v. with either free doxorubicin, Doxil (slow drug release), or with a faster drug release formulation with a fluid bilayer composed of unsaturated dioleoylphosphatidylcholine and cholesterol. Total tumor doxorubicin and nuclear doxorubicin were determined as a function of time after injection.

Materials and Methods

Chemicals and reagents. Doxorubicin hydrochloride, Doxil [pegylated liposomal doxorubicin (PLD), also called Stealth liposomal doxorubicin and Caelyx] and methoxy polyethylene glycol (M_n 2,000)-distearoylphosphatidylethanolamine (mPEG₂₀₀₀-DSPE) were provided by ALZA Corp. (Mountain View, CA). Cholesterol and dioleoylphosphatidylcholine were from Avanti Polar Lipids (Alabaster, AL). MEM, DNase 1, and digitonin were from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum, penicillin, streptomycin, and L-glutamine were from Invitrogen (Burlington, Ontario, Canada). Sephadex-G50 and Sepharose CL-4B were from Amersham-Pharmacia Biotech (Baie d'Urfe, Quebec, Canada). Halothane was from MTC Pharmaceuticals (Cambridge, Ontario, Canada). The DNeasy Tissue Kit was from Qiagen (Mississauga, Ontario, Canada). Sterile, pyrogen-free saline was from Baxter (Toronto, Ontario, Canada). Dextrose U.S. Pharmacopeia (D5W), 5% w/v in water was from Baxter Corp. (Mississauga, Ontario, Canada). Chol-[1,2-³H-(N)]hexadecyl ether ([³H]CHE, 1.48-2.22 TBq/mmol) was from Perkin-Elmer Life Sciences (Woodbridge, Ontario, Canada). All other chemicals were of the highest grade possible.

Animals, cell line, and tumor implantation. Female BALB/c mice (6-8 weeks) were purchased from Health Sciences Laboratory Animal Services, University of Alberta. 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 animals were treated in accordance with the Guide to the Care and Use of Experimental Animals set forth by the Canadian Council on Animal Care.

The 4T1 mouse mammary carcinoma cell line is a metastatic, thioguanine-resistant cell line, donated by Dr. Fred Miller (Barbara Ann Karmanos Cancer Institute, Detroit, MI). The cell line was maintained in MEM supplemented with 10% fetal bovine serum, penicillin (100 units/mL), streptomycin (100 µg/mL), and L-glutamine (0.292 mg/mL) at 37°C in a humidified incubator with a 5% CO₂ atmosphere (22). Cells were released from their substrate by rinsing with PBS-containing EDTA [0.54 mmol/L EDTA, 137 mmol/L NaCl, 3 mmol/L KCl, 8 mmol/L Na₂HPO₄, 1.5 mmol/L KH₂PO₄ (pH 7.4)] followed by trypsin-EDTA (0.05% trypsin in 137 mmol/L NaCl, 5.4 mmol/L KCl, 7 mmol/L NaHCO₃, 0.34 mmol/L EDTA). Cells were kept in exponential growth.

Tumors were orthotopically implanted in female BALB/c mice as previously described (23). Briefly, a small incision was made in the lower right abdominal region of anesthetized mice, and 1×10^5 4T1 cells, in full medium, were injected into the right 4 mammary fat pad. A surgical wound clip was used to close the incision and was removed 7 days later.

Preparation of liposomes. Doxil was used as obtained from the manufacturer. Liposomes with faster release doxorubicin rates were

composed of dioleoylphosphatidylcholine/cholesterol/mPEG₂₀₀₀-DSPE at a 2:1:0.1 molar ratio (dioleoylphosphatidylcholine-PLD). For these liposomes, [³H]CHE was added as a nonexchangeable, non-metabolized lipid tracer. A lipid mixture of chloroform stocks was prepared and dried to form a thin film using a rotovaporator; this was then placed in a vacuum overnight. Doxorubicin was remote loaded into liposomes using the ammonium sulfate gradient method previously described (24). The resulting liposomes contained 0.2:1 (w/w) drug/lipid ratios, and the diameter of liposomes was determined to be 100 ± 20 nm, using a Brookhaven BI-90 particle sizer (Brookhaven Instruments, Holtsville, NY). Doxorubicin concentrations were determined in methanol extracts of doxorubicin-loaded liposomes from the absorption of the drug at 480 nm compared with a standard curve; phospholipid concentrations were determined by counting the [³H]CHE on a Beckman LS-6800 scintillation counter.

The *in vitro* half-life values for release of doxorubicin from either Doxil or from dioleoylphosphatidylcholine-PLD were previously determined in 50% (v/v) adult bovine plasma in fetal bovine serum (pH 7.4), to be ~118 and 12 hours, respectively (25).

Measurement of doxorubicin. To determine if the tumor homogenization process disrupted liposomes, resulting in premature doxorubicin release, Doxil, or dioleoylphosphatidylcholine-PLD in nuclear lysis buffer [0.25 mol/L sucrose, 5 mmol/L Tris-HCl, 1 mmol/L MgSO₄, 1 mmol/L CaCl₂ (pH 7.6)] was homogenized in an ice-cold Potter-S homogenizer. After each homogenization stroke, aliquots of liposomal doxorubicin were taken. Doxorubicin fluorescence ($\lambda_{ex} = 470$ nm, $\lambda_{em} = 590$ nm) was measured before and after the addition of 10% (v/v) Triton X-100, which lyses the liposomes and releases 100% of the entrapped doxorubicin. Total tumor doxorubicin and doxorubicin in tumor nuclei were quantified using a method similar to Mayer et al. (26). At various times after injection, 10% w/v tumor homogenates from mice receiving free doxorubicin, Doxil, or dioleoylphosphatidylcholine-PLD were prepared in nuclear lysis buffer. Samples of the homogenate (200 µL) were placed in 2-mL microcentrifuge tubes, 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 vortexed to ensure complete mixing, and doxorubicin was extracted overnight at -20°C. The next day, the microcentrifuge tubes were warmed to room temperature, vortexed for 5 minutes, centrifuged at $15,000 \times g$ for 20 minutes, and stored at -80°C until analysis. Doxorubicin was quantified fluorometrically ($\lambda_{ex} = 470$ nm, $\lambda_{em} = 590$ nm). To correct for nonspecific background fluorescence, the samples were compared with a standard curve made from the fluorescence emission of known amounts of doxorubicin added to acidified isopropanol extracts of homogenized tumor tissue from untreated mice. The data are the mean \pm SD of triplicate aliquots from tumor homogenates from three to five individual mice, expressed as microequivalents of doxorubicin/g tissue, because this assay does not discriminate between doxorubicin and fluorescent doxorubicin metabolites, if any.

Tumor cell nuclei were isolated from total tumor homogenates by differential centrifugation through a 1.8 mol/L sucrose gradient at $1,000 \times g$ for 10 minutes. The efficiency of the nuclear doxorubicin extraction was first compared by two different methods: an acidified isopropanol extraction, described above and a DNase 1 digestion method, previously described (27). Because the extraction efficiency of the acidified isopropanol method was superior to that of the DNase 1 method, all further nuclear doxorubicin accumulation assessments were done using the acidified isopropanol extraction method.

In addition, the effect of tumor homogenization on liposome redistribution was investigated. Doxil or dioleoylphosphatidylcholine-PLD in concentrations comparable with what was observed in tumors was homogenized together with tumors in nuclear lysis buffer. After homogenization, total tumor doxorubicin and nuclei-associated doxorubicin were determined by the methods described above.

In vivo tumor bioavailability experiments. BALB/c mice were orthotopically implanted with 4T1 tumor cells, and 10 days after implantation, the mice were given i.v. injections via the lateral tail

vein (200 μ L) with 9 mg/kg of doxorubicin either as free doxorubicin, Doxil, or dioleoylphosphatidylcholine-PLD. To see if the method scaled with dose, some mice received 16 mg/kg doxorubicin from Doxil. Control mice were injected with 200 μ L sterile saline. Mice ($n = 3$ -5 per time point) were euthanized, and tumors were excised at various time points up to 7 days after injection or until doxorubicin levels were below detectable levels (0.005 microequivalents doxorubicin/mL). Total and nuclear doxorubicin in 4T1 tumors were determined via the acidified isopropanol extraction method. The % bioavailability of doxorubicin in tumor tissue was determined from the area under the time-versus-concentration curve (AUC) of nuclear drug relative to the AUC of drug in whole tumors over the time course of the experiment.

Statistics. The pharmacokinetic variables, tumor $AUC_{0-24 \text{ hours}}$ (free doxorubicin) or tumor AUC_{0-7d} (liposomal doxorubicin), maximum drug concentration (C_{max}), and time to maximum concentration (T_{max}), were calculated for total and nuclear doxorubicin using WinNonlin 4.1 software (Pharsight Corp., Mountain View, CA). Statistical differences between groups were determined by ANOVA with a Tukey-Kramer post-test using GraphPad InStat version 3.01 (GraphPad Software, Inc., San Diego, CA).

Results and Discussion

In determining the amount of bioavailable drug in tumors, it is important that the liposomal formulations do not release their entrapped drug prematurely during the homogenization procedure. The prematurely released drug could diffuse rapidly to nuclei in the homogenate thus artificially increasing the amount of tumor bioavailable drug. Drug release from liposomes can be measured by the doxorubicin-dequenching assay. When doxorubicin is entrapped at high concentrations in liposomes, its fluorescence signal is quenched, and release of the drug increases the fluorescence signal (28). For example, addition of 10% Triton X-100 (v/v) to our liposomal doxorubicin samples, mimicking 100% drug release, caused the relative fluorescence signal to increase by ~10-fold (data not shown). However, up to 10 strokes of the Potter-S homogenizer caused no increase in the fluorescence signal; thus, we conclude that no doxorubicin was released from either Doxil or dioleoylphosphatidylcholine-PLD during homogenization. The effect of homogenization of tumor in the presence of liposomal drug was also investigated. If the presence of tumor tissue during homogenization affects the release of drug and its redistribute to the nucleus, this could also result in an artificial increase in the amount of bioavailable drug in tumor. Both liposomal doxorubicin formulations used during *in vivo* experiments were tested with 10 strokes of the Potter-S homogenizer in the presence of tumor and it was determined that between 3% and 10% of liposomal drug distributes to the nucleus during this procedure (data not shown).

The extraction efficiency for doxorubicin using the acidified isopropanol extraction method for both total tumor and tumor cell nuclei was >90%; this is consistent with previous findings by us and others that >90% of doxorubicin can be extracted by this method (25, 29). There was a linear relationship between the amount of doxorubicin added to control samples and the amount of doxorubicin recovered from either total tumor homogenates or tumor nuclei purified from the homogenates (data not shown). We used nuclear doxorubicin accumulation as an indicator of bioavailable liposomal drug, because the nucleus acts like a "sink" for doxorubicin. Once doxorubicin leaks out of liposomes in the interstitial space of solid tumors, the drug rapidly diffuses into cells and can traffic to cell nuclei,

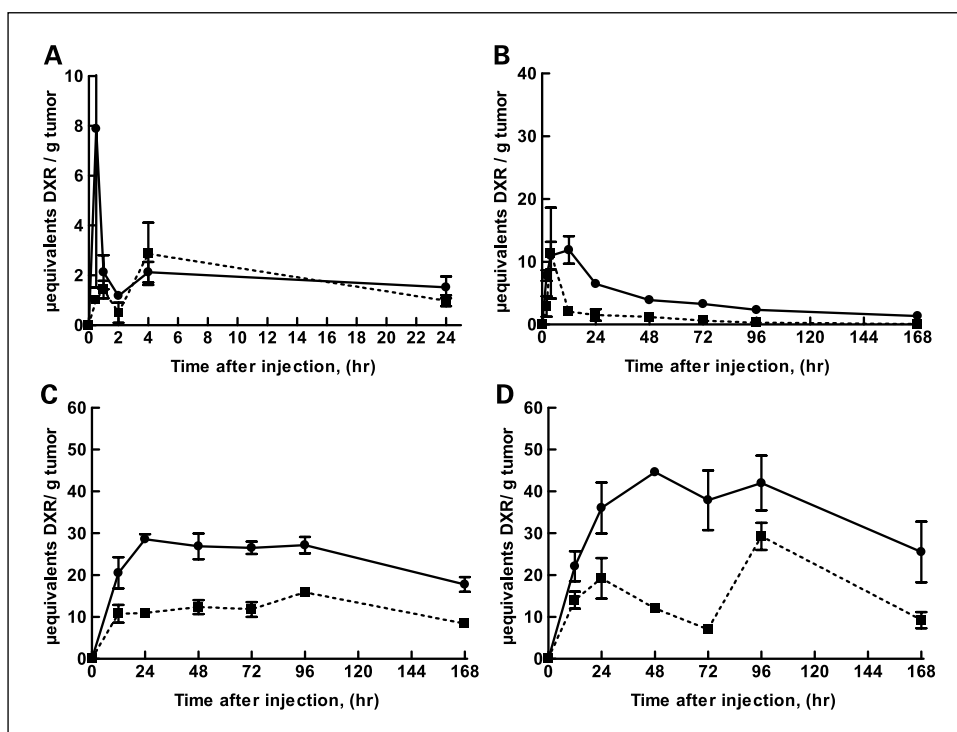
where it intercalates with DNA, rendering the drug immobile. The nucleus is not the only site of action of doxorubicin; for example, subcellular organelles, such as mitochondria, are important sites of drug toxicity (30, 31), but it is the only site that functions as a sink for the drug. Hence, nuclear doxorubicin, we postulate, provides a reasonable first approximation to bioavailable doxorubicin in cells.

Figure 1A presents total doxorubicin levels in mouse mammary tumors and doxorubicin levels in tumor nuclei after treatment of the mice with 9 mg/kg of free doxorubicin. As expected, total doxorubicin levels in tumors increased quickly, then dropped rapidly, and fell to below-detectable levels by 48 hours after injection. The free drug was rapidly bioavailable; the tumor $AUC_{0-24 \text{ hours}}$ for nuclear drug was 95% of that of the $AUC_{0-24 \text{ hours}}$ of total drug (Table 1). The maximum total doxorubicin concentration in tumor (C_{max}) was 8 microequivalents doxorubicin/g tumor and occurred at 30 minutes after administration. Nuclear doxorubicin levels did not reach C_{max} (3 microequivalents doxorubicin/g tumor) until 4 hours after injection. Concentrations of doxorubicin that were determined in these experiments were close to the detectable limits of the drug; thus, quantification of both total and nuclear doxorubicin resulted in large SDs.

Figure 1C and D shows levels of total tumor doxorubicin and doxorubicin levels in tumor nuclei for Doxil at doses of 9 and 16 mg/kg. At a dose of 9 mg/kg, this liposomal formulation of doxorubicin had a very different tumor distribution profile than free doxorubicin, with significantly higher tumor $AUC_{0-7 \text{ days}}$ for total doxorubicin levels (87-fold higher) and nuclear doxorubicin levels (45-fold higher) in the tumors ($P < 0.001$) compared with free doxorubicin (Table 1). The $AUC_{0-7 \text{ days}}$ seemed to scale with dose, with the tumor AUC in mice receiving 16 mg/kg being 1.5-fold higher than that in mice receiving 9 mg/kg. Total doxorubicin levels in tumor for either Doxil dose reached C_{max} much later than free doxorubicin (24-48 hours after injection versus 0.5 hours). These differences reflect the different mechanism of accumulation of liposomal versus free drugs in tumor tissue, both of which are distributed to tumors via the blood stream. Liposomes, however, are particles of ~100 nm in diameter and must extravasate through gaps between the endothelial cells in the permeable blood vessels within solid tumors to accumulate in the interstitial space of tumors (11). This is a slow process. Therefore, the delay in time to C_{max} and the slow rate of clearance reflect the time that it takes for liposomal drugs to extravasate into tumor tissue by the enhanced permeability and retention effect (10). These data are similar to the delayed time to C_{max} and prolonged residency times for liposomal drugs that have been observed in other tumor models (32, 33). Nuclear accumulation of liposomal doxorubicin also showed a different profile than free doxorubicin (Fig. 1C versus A). For doses of either 9 or 16 mg/kg, C_{max} for nuclear accumulation of doxorubicin in murine mammary tumors occurred at 2 to 3 days after treatment versus 4 hours for free doxorubicin. This reflects the slow rate of release of doxorubicin from the carrier, which must occur before the drug can traffic to the nucleus. During the 7-day time course of these experiments, 40% to 50% of the liposomal drug was bioavailable (Table 1).

Liposomes with more rapid release rates than Doxil have pharmacokinetic profiles in tumor that are intermediate between Doxil and free doxorubicin (Fig. 1B versus A and C).

Fig. 1. Doxorubicin (DXR) concentrations in 4T1 tumors in mice receiving free doxorubicin, dioleoylphosphatidylcholine-PLD, or Doxil. Female BALB/c mice were orthotopically implanted with 4T1 murine mammary carcinoma and were injected 10 days after implantation (time 0) with either free doxorubicin at a dose of 9 mg/kg, dioleoylphosphatidylcholine-PLD at a dose of 9 mg/kg, or Doxil at a dose of 9 or 16 mg/kg. Total (solid line) and nuclear (dashed line) doxorubicin levels were determined following acidified isopropanol extraction. Points, means of triplicate aliquots from three to five mice per time point; bars, \pm SD. *A*, free doxorubicin, 9 mg/kg; *B*, DOPC-PLD, 9 mg/kg; *C*, Doxil, 9 mg/kg; *D*, Doxil, 16 mg/kg.



Dioleoylphosphatidylcholine-PLD liposomes have a more fluid bilayer due to the presence of the unsaturated fatty acyl chains in dioleoylphosphatidylcholine. This leads to more rapid rates of drug release from dioleoylphosphatidylcholine-PLD than from Doxil, which contains long-chain saturated fatty acyl chains. Using dual-labeled liposomes (radiolabeled doxorubicin and radiolabeled lipid), the half-life for release of doxorubicin from liposomes *in vivo* has recently been reported to be 1.9 hours for dioleoylphosphatidylcholine-PLD versus 315 hours for Doxil (34). In clinical trials, it has been shown that <10% of the encapsulated doxorubicin is released from Doxil by 24 hours after i.v. administration (35). However, because dioleoylphosphatidylcholine-PLD has a substantially greater rate of drug release, a considerable portion of the drug will be

released before much tumor accumulation occurs. In other words, drug from dioleoylphosphatidylcholine-PLD will reach tumors as a combination of residual doxorubicin still entrapped in liposomes and free doxorubicin that has been released from liposomes into the blood compartment and has redistributed to tissues including tumor tissue.

The levels of total doxorubicin from dioleoylphosphatidylcholine-PLD in tumors were only 14-fold higher than those from free doxorubicin versus 87-fold higher for Doxil. The C_{max} for total tumor doxorubicin from dioleoylphosphatidylcholine-PLD occurred 12 hours after injection (i.e., between the 0.5 hours for free doxorubicin and the 24 to 48 hours for Doxil). The tumor $AUC_{0-7 \text{ days}}$ of total drug for dioleoylphosphatidylcholine-PLD was over 6-fold lower than that seen for

Table 1. Comparison of tumor pharmacokinetics of free doxorubicin versus liposomal formulations of doxorubicin

Formulation	Doxil (16 mg/kg)		Doxil (9 mg/kg)		DOPC-PLD (9 mg/kg)		Free doxorubicin (9 mg/kg)	
	Total	Nuclear	Total	Nuclear	Total	Nuclear	Total	Nuclear
AUC ($\mu\text{eq h/g tumor}$)	5,918	2,444	3,988	1,970	641	172	45.8	43.3
% Bioavailable	41.3%		49.4%		26.8%		95.1%	
C_{max} ($\mu\text{eq doxorubicin/g tumor}$)	48.8	25	28.6	15.9	11.9	11.4	7.9	2.9
T_{max} (h)	48	96	24	96	12	4	0.5	4
$t_{1/2}$ (h)	135	ND	151	ND	78	ND	25	ND
MRT (h)	81	ND	81	ND	52	ND	10	ND
Clearance (g/h/kg)	1.5	ND	1.1	ND	11.3	ND	89	ND

NOTE: Tumor levels of total doxorubicin and nuclear doxorubicin were determined in mice bearing orthotopic 4T1 mammary carcinomas following administration of 9 mg/kg doxorubicin as Doxil, DOPC-PLD, or free doxorubicin. Some mice also received 16 mg/kg of Doxil. $AUC_{0-24 \text{ h}}$ for free doxorubicin and $AUC_{0-7 \text{ d}}$ for liposomal doxorubicin formulations. All variables were determined using WinNonLin software.

Abbreviations: ND, no value determinable by the pharmacokinetic program; DOPC-PLD, dioleoylphosphatidylcholine/CHOL/mPEG₂₀₀₀-DSPE; MRT, mean residence time; μeq , microequivalents.

Doxil at the same dose, and the $AUC_{0-7 \text{ days}}$ of bioavailable drug (i.e., bound to nuclei) was over 11-fold lower. When drug-depleted liposomes accumulate in the tumor, only limited amounts of the drug are available for release, cellular uptake, and trafficking to the nuclei. Nuclear doxorubicin concentrations reached maximum levels by 4 hours after injection, similar to free doxorubicin and much more rapidly than Doxil. This suggests that a considerable amount of the drug was reaching tumor nuclei as released drug. Doxorubicin bioavailability from the dioleoylphosphatidylcholine-PLD formulation was 26.8%, and there was 4-fold more bioavailable doxorubicin delivered to tumors than for free doxorubicin (Table 1). Nuclear levels of drug were at or below measurable limits for many of the later time points, possibly resulting in an underestimate of the amount of bioavailable drug. Also provided in Table 1 are the $t_{1/2}$ for doxorubicin in tumor tissue, mean residence time, and tumor clearance of doxorubicin, which reflect a similar rank order as those seen for other pharmacokinetic variables.

Our results for bioavailable drug levels in tumor tissue for the two liposomal formulations correlate well with the pharmacokinetics and therapeutic effects for the same formulations in the same animal model, reported recently by our group (25). In this pharmacokinetic study, we reported that the plasma half-life for doxorubicin was 18.2 hours when the slow release formulation was given to tumor-bearing mice, and the plasma $AUC_{0-\infty}$ was 3,020 $\mu\text{g h/mL}$. In contrast, the plasma half-life for doxorubicin in dioleoylphosphatidylcholine-PLD was only 2.1 hours, and the plasma $AUC_{0-\infty}$ was 430 $\mu\text{g h/mL}$. The plasma $AUC_{0-\infty}$ for the lipid component of the liposomes was very similar for the two formulations (10,600 $\mu\text{g h/mL}$ for DSPC versus 12,700 $\mu\text{g h/mL}$ for dioleoylphosphatidylcholine), indicating that drug-depleted liposomes were circulating in plasma (25). In distribution experiments using fast versus slow release liposomal doxorubicin, doxorubicin in the slow release liposomes (similar formulation to Doxil) accumulated to a significantly higher extent in 4T1 tumors compared with liposomes with fast release rate (dioleoylphosphatidylcholine-PLD; ref. 33). In therapeutic experiments, the slow release formulation had superior therapeutic activity to the fast release formulation (25), which correlates with the observations on doxorubicin bioavailability in tumor tissue report in the article.

Currently, several strategies to optimize liposomal drug delivery are being evaluated by us and others, and a bioavailability assay for doxorubicin in tumor tissue will be useful in designing optimal formulations. One approach is to use targeting moieties against malignancies that uniquely express or over-express cell surface antigens. Targeting drug-loaded liposomes with monoclonal antibodies such as anti-HER2 or anti-CD19, for example, has resulted in improved efficacy *in vivo* in murine xenograft models over nontargeted liposomes or free drug

(36, 37). We have begun studies to examine how targeting internalizing or noninternalizing epitopes at the surface of cancer cells affects the rate and extent of bioavailability of the entrapped drugs. Another strategy is to design triggered release formulations of liposomes that release their contents in a burst when triggered by temperature or pH after the liposomes have localized to tumors (38, 39). Bioavailable measurements will also be useful in the design of these types of formulations.

The fluorescent properties of doxorubicin and its interchelation into DNA made it relatively easy to make bioavailability measurements for this drug in tumor tissue. For other drugs, more complex techniques may be required. For example, it has been hypothesized that lack of bioavailability of liposomal cisplatin may account for the relatively poor clinical results found for liposomal formulations of cisplatin (40). Recently, the release of active platinum from liposomes was determined in murine melanoma tumors by the use of microdialysis probes and atomic absorption spectrophotometry (41). Although more total liposomal cisplatin distributed to tumors than free cisplatin in this study, less Pt was released into the tumor extracellular fluid, suggesting that tumor bioavailability of active Pt was suboptimal. In an alternative approach, liposome drug concentrations and levels of released drugs were measured by magnetic resonance imaging using liposomes loaded with doxorubicin and $\text{Mn}(\text{SO}_4)$ and measuring the relaxivity $T(1)$ of Mn^{2+} . When the drug is inside the liposomes, $T(1)$ was relatively unaffected; release of the drug from a flank fibrosarcoma tumor, mediated by hyperthermia, resulted in a significant shortening of $T(1)$ (42).

The ability to quantitate bioavailable drug at the site of drug action may assist in developing new anticancer drug carriers, such as the ones mentioned above, that may have improved therapeutic effects and reduced side effects compared with conventional drug treatments. We believe the results presented in this article are a good first approximation of the rate and extent of bioavailable drug in tumor tissues for liposomal carriers; refinement of the methodology may be necessary to achieve definitive results. For example, studies on the rate and extent of tumor tissue bioavailability of liposomal doxorubicin, done in combination with apoptotic assays, can provide valuable information on the minimum concentrations of bioavailable doxorubicin required for cell kill and on the exposure times needed for driving the cells into apoptosis.

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