ABSTRACT

Coadministration of anticancer drugs and multidrug resistance modulators directed against P-glycoprotein overexpressed in tumors also results in nonspecific blockade of this drug efflux pump in excretory tissues such as the liver and kidneys. These interactions often result in impaired renal and biliary clearance for anticancer agents such as doxorubicin (DOX). In the present investigation, we characterized the excretory processes associated with liposomal DOX administration to elucidate how liposome encapsulation may bypass adverse pharmacokinetic interactions between DOX and (3'-keto-Bmt1)-(Val2)-cyclosporin (Valspodar). Renal and biliary clearance properties of liposome-encapsulated DOX were compared with those for nonencapsulated DOX in the presence and absence of Valspodar using an instrumented rat model with implanted jugular vein and bile duct catheters for continuous sampling. Two types of liposomal DOX formulations were used, a drug-permeable egg phosphatidyl choline/cholesterol system and a sterically stabilized polyethylene glycol/1,2 diestearoyl-sn-glycero-3-phosphocholine/cholesterol system to establish the relative roles of liposome-encapsulated and released drug on the pharmacokinetic and excretion alterations induced by Valspodar. DOX and its primary metabolites were quantitated using high-performance liquid chromatography. When Valspodar was coadministered with nonencapsulated DOX, 3.5- and 37.5-fold reductions in renal clearance (CLr) and biliary clearance (CLb), respectively, were observed, which resulted in increased plasma DOX concentrations and total exposure. However, Valspodar-induced alterations in CLr and CLb were less profound with egg phosphatidyl choline/cholesterol DOX (1.7- and 2.0-fold reductions, respectively) and negligible with the long-circulating polyethylene glycol-containing liposomal formulation. These results indicate that liposomes may circumvent Valspodar-induced DOX pharmacokinetic changes by reducing the rate of drug excretion in liver and kidney tissue to a level that is within the renal and biliary excretion capacity in the presence of P-glycoprotein blockade.

INTRODUCTION

The hepatobiliary system plays an important role in the elimination of many drugs from the body (1, 2). Multiple elimination mechanisms, depending on size and the chemical nature of drugs, control the excretion of xenobiotics in bile and urine (3–5). The role of PGP4 in the biliary and renal excretion processes has important implications for drug-drug interactions that can arise when multiple agents are coadministered to reverse MDR (1, 2). Chemosensitization approaches to circumvent PGP-mediated MDR typically combine anticancer drug treatment with a PGP inhibitor to cause enhanced intracellular drug accumulation in MDR tumors. However, because of the lack of tumor tissue specificity displayed by present MDR reversing agents, PGP blockade may also occur in healthy excretory tissues, where such transport pumps are expressed. The implication of PGP blockade at extratumoral sites is that excretion of anticancer drugs may be impaired when coadministered with MDR modulators that directly block PGP function. Indications of such adverse interactions have been observed frequently in clinical trials using PGP inhibitors (6–8) and have

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1 Supported by the National Cancer Institute of Canada with funds from the Canadian Cancer Society. R. K. is a British Columbia Medical Services Foundation Predoctoral fellow.
2 Present address: Department of Metabolism and Pharmacokinetics, Pharmaceutical Research Institute, Bristol-Myers Squibb Company, P O Box 4000, Princeton, NJ 08543.
3 To whom requests for reprints should be addressed, at Department of Advanced Therapeutics, British Columbia Cancer Agency, 600 West 10th Avenue, Vancouver, British Columbia, V5Z-4E6 Canada. Fax: (604) 877-6011; E-mail: lmayer@bccancer.bc.ca.

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4 The abbreviations used are: PGP, P-glycoprotein; MDR, multidrug resistance; Valspodar, SDZ PSC 833 [(3'-keto-Bmt1)-(Val2)-cyclosporin]; DOX, doxorubicin; PEG2000-DSPE, polyethylene glycol 2000-diestearoylglycerophosphatidyl ethanolamine; EPC, egg phosphatidyl choline; DSPC, 1,2 distearoyl-sn-glycero-3-phosphocholine; Chol, cholesterol; HPLC, high-performance liquid chromatography; JV, jugular vein; AUC, area under the concentration-time curve.
introduced additional complications in both the design and analysis of these clinical studies.

In general, MDR modulator-induced pharmacokinetic alterations may occur at the hepatic, intestinal, renal, or blood-brain barrier levels of elimination (9–11). In support of this, several reports have demonstrated that PGP inhibitors can cause inhibition of anticancer drug biliary excretion (12–17). DOX is readily eliminated in urine and bile via a PGP-dependent process as a result of the expression of this drug efflux pump in the luminal side of the kidney proximal tubule and biliary canaliculi. In humans, biliary excretion is a major route of drug elimination, where DOX appears in bile within 5 min after an i.v. bolus administration (18, 19). Intact DOX is the major chemical entity in bile and urine, with lower levels of DOXol and aglycone metabolites (18). In humans, biliary excretion represents 41% of injected drug excreted in bile compared with 14% in urine (19). Similar relationships between total biliary and urinary excretion have been observed in preclinical models (20, 21). Consequently, it may be expected that coadministration of an MDR modulator with DOX may lead to inhibitions of DOX renal and biliary excretion because of PGP blockade.

Both cyclosporin A and its nonimmunosuppressive analogue, Valspodar (previously referred to as PSC 833), have been shown to block biliary and renal excretion of anticancer agents (14, 15, 22). Dose-dependent excretion inhibitory effects have been observed with both cyclosporin A (14) and Valspodar (15). We have shown previously that liposomes can protect encapsulated DOX from drug–drug interactions with Valspodar, thereby avoiding the anticancer drug dose reductions typically associated with this combination therapy. In addition, combining Valspodar with liposomal DOX led to significant improvements in antitumor activity compared with that achievable with nonencapsulated DOX and the MDR modulator (23, 24). However, the mechanism(s) underlying the ability of liposomal DOX to avoid Valspodar-induced PK alterations is not understood. This is highlighted by the fact that after liposomal DOX injection, DOX levels in liver tissue are actually elevated compared with nonencapsulated DOX administration, without any significant toxicological consequences when combined with Valspodar.

In this report, we have evaluated the DOX renal and biliary clearance following i.v. injection of nonencapsulated and liposome-entrapped DOX in an instrumented rat model. Two liposomal formulations were compared, i.e., EPC/Chol DOX (a system where >50% of the drug is released in the first hour [24]), and a sterically stabilized PEG$_{2000}$-DSPE/DSPC/Chol DOX formulation that retains entrapped drug over 24 h. Selection of these two formulations was based on: (a) an ability to assess the importance of DOX released from liposomes in the circulation on Valspodar-associated drug excretion alterations; and (b) the fact that these formulations reflect the two liposomal doxorubicin products that are either approved (sterically stabilized) or are pending approval (EPC/Chol) for widespread clinical use. Consequently, determining the influence of Valspodar on their DOX excretion properties would be of considerable clinical interest. In addition, characterizing the relative effects of Valspodar on the plasma and excretion pharmacokinetic properties of nonencapsulated and liposomal DOX formulations may assist in identifying important pharmacodynamic relationships that are associated with toxicity and/or therapeutic activity.

**MATERIALS AND METHODS**

**Materials.** DOX hydrochloride for injection, U. S. P., was purchased from David Bull Laboratories (Canada) Inc. (Vaudreuil, Quebec, Canada), and its purity was affirmed by HPLC (see below). Valspodar was a generous gift from Novartis (Canada) Inc. (Dorval, Quebec, Canada). DOX metabolite standards were generous gifts (Farmitalia Carlo Erba, Milan, Italy). PEG$_{2000}$-DSPE (>99% purity), EPC (>99% purity), and DSPC (>99% purity) were obtained from Northern Lipids, Inc. (Vancouver, British Columbia, Canada), and cholesterol was obtained from Sigma Chemical Co. (St. Louis, MO). Cholesteryl hexadecyl ether ([$^3$H]), a nonexchangeable, nonmetabolizable lipid marker, was purchased from Amersham Canada (Oakville, Ontario, Canada).

**Liposome and Drug Preparation.** Liposomes composed of EPC/Chol (55:45), PEG2000-DSPE/DSPC/Chol (5:50:45), and DSPC/Chol (55:45) were prepared as described previously (25) using a Lipex Extruder (Lipex Biomembranes, Inc., Vancouver, British Columbia, Canada). [3H]Cholesterylhexadecyl ether was used as a nonexchangeable, nonmetabolizable lipid marker (26). The resulting large unilamellar vesicles exhibited a mean diameter ranging between 100 and 130 nm as determined using a Nicomp 270 submicron particle sizer (Particle Sizing Systems, Inc., Santa Barbara, CA), operating at a wavelength of 632.8 nm.

DOX was encapsulated in the liposomes using the transmembrane pH gradient loading procedure (interior acidic) using sodium carbonate as the alkalinizing agent and a drug:lipid weight ratio of 0.2:1.0 (27). Liposomal DOX preparations were diluted with saline as necessary prior to in vivo administration. Valspodar (for animal studies) was dissolved in a 10:1 mixture of ethanol (95%):polyoxyethylene sorbitan monooleate (Tween 80) and administered in a corn oil vehicle by oral gavage of a 200 μl volume (28). Nonencapsulated DOX was administered in sterile saline.

**Animal Experiments.** Cannulated male Sprague Dawley rats (225–275 g) were purchased from Charles River (St. Constant, Quebec, Canada). All animals were used for experimentation within 10 days of surgery and were allowed free access to food and water. These rats had polyurethane cannulas placed in the JV (0.025-inch inside diameter × 0.040-inch OD, 13.5 cm long, dead volume of 40 μl) and common bile duct (0.025-inch inside diameter × 0.040-inch outside diameter, 28 cm long). The bile duct was cannulated and exteriorized at the base of the neck in such a way that bile flow (from animal’s left to right) into the small intestine could be interrupted and restored. The left half of the cannula was used for the sampling, and the right half was plugged with a metal plug. On receipt of the animals, the JV catheter was flushed with saline and locked with 40 μl of a heparinized saline solution. Animals were housed singly in steel metabolic cages throughout the experiment. Samples were collected by gently restraining the animal or briefly anesthetizing the animal with isoflurane-oxygen (controlled via an anesthetic machine; 2% at 2 l/min for 1 min and reduced to 1% at 1 l/min) at the respective time points.

Rats received DOX (5 mg/kg) through the tail vein alone or 4 h after Valspodar (p.o.). Jugular venous blood samples (max-
imum, 0.25 ml) were collected at the indicated times after DOX administration. An equal amount of saline was infused back along with a 0.04-ml heparinized saline lock. Rats were sampled for bile in tared polyethylene tubes at predetermined intervals immediately after bile flow was interrupted. One ml of lactated Ringer solution was infused via the JVC as fluid replacement at every bile collection point. A rectal probe was used to monitor body temperature. Plasma and bile samples were analyzed for DOX, DOXol, and other DOX metabolites by HPLC. To determine whether bile flow interruption alters the pharmacokinetics of DOX, groups of rats with intact JV catheters, where bile duct catheter loop was left uninterrupted, were sampled for serial blood (plasma) and analyzed for DOX.

The HPLC assay of Andersen et al. (29) was used to analyze DOX and its metabolites with minor modification. Briefly, sample extraction with acetonitrile was followed by isocratic elution from a C18 reverse phase column and quantified by endogenous fluorescence at 515 nm. The mobile phase consisted of a 16 mM ammonium formate buffer (pH 3.5):acetone:isopropanol mixture (75:20:5) delivered at a rate of 1.0 ml/min. Recoveries, using acetonitrile as the extraction solvent, from plasma over a concentration range of 0.05–10 μg/ml of DOX and DOXol were in excess of 95%.

Biliary excretion rate (ng/min) is a function of drug concentration in bile (ng/ml) and biliary flow rate (ml/min). Biliary clearance (Clb) was estimated by plotting the average biliary excretion rate versus the plasma drug concentration at the midpoint of collection interval. The slope of the line provided the biliary clearance in ml/min using the following equation:

\[
\text{Biliary clearance (Clb) = } \frac{\text{Bile flow rate (ml/h) } \times \text{concentration of DOX in bile (mg/ml)}}{\text{Average JV plasma concentration (mg/ml)}}
\]

It should be noted that biliary clearance values for animals receiving liposomal DOX reflect clearance of free drug that has been released from liposomes in the plasma or tissues because intact liposomes are not excreted in bile (confirmed by the lack of detectable lipid levels in bile).

Bile samples were analyzed for unencapsulated DOX and conjugated DOX using two aliquots of the same sample. To one aliquot, a solution of sulfatase in pH 7.1 buffer was added to bile samples before extraction with organic solvent and incubated at 37°C for 3 h (30). To another aliquot, a solution of glucuronidase in pH 5.0 formate buffer was added to bile samples and incubated at 37°C for 3 h. The concentration of conjugated DOX was calculated by subtracting the nonconjugated DOX concentration from the samples measured after hydrolysis.

Urine was collected at predetermined intervals, and urinary flow rate was determined. Blood (plasma) and urine samples were analyzed for DOX and its metabolites. Urine samples were analyzed for unencapsulated DOX and conjugated DOX as described for bile. Renal clearance (Cln) was estimated by plotting the average urinary excretion rate versus the plasma drug concentration at the midpoint of collection interval using the following equation:

\[
\text{Renal clearance (Cln) = } \frac{\text{Urine flow rate (ml/h)} \times \text{concentration of DOX in urine (mg/ml)}}{\text{Average JV plasma concentration (mg/ml)}}
\]

The plasma data were modeled using WinNONLIN version 1.5 pharmacokinetic software (Pharsight Corp., Mountain View, CA), to calculate area under the curve (AUC), terminal elimination half-life (T1/2), and plasma clearance (Clp) according to standard equations. To determine appropriate models to fit the plasma data, the criteria used to evaluate the goodness of fit for each model included a visual assessment of distribution of residuals, rank, and Akaike’s Information Criterion. Data are presented as mean ± SD (n = 3 animals/group). Statistical analyses were performed using ANOVA, and statistical significance was set at P < 0.05.

RESULTS

Plasma Pharmacokinetics. Prior to experimentation with various treatment groups, the effect of bile flow interruption on the plasma pharmacokinetics of DOX was investigated. These control rats containing a JV catheter had their bile duct catheter loop left uninterrupted, enabling only blood collection. There were no significant differences in plasma DOX pharmacokinetics between bile flow interrupted and uninterrupted groups (see below), after administration of free and liposomal DOX formulations, indicating a lack of changes in pharmacokinetics of DOX caused by surgical instrumentation. The following sections will therefore focus on results generated in instrumented rats containing JV as well as BD catheters.

Fig. 1 illustrates the plasma DOX concentration-time profile for nonencapsulated DOX (Fig. 1A), EPC/Chol DOX (Fig. 1B), and PEG-DSPE/DSPC/Chol DOX (Fig. 1C) in the presence and absence of Valspodar. The nonencapsulated DOX plasma concentration-time data, both in the presence and absence of the MDR modulator, were fitted with a two-compartment model, characterized by a rapid phase drug concentration decrease, and a slower terminal elimination phase (Fig. 1A). When nonencapsulated DOX was administered i.v. at a dose of 5 mg/kg, a model-derived estimated Cmax of 17.61 ± 1.79 μg/ml was obtained (Table 1). The pharmacokinetics of nonencapsulated DOX was characterized by an AUC of 5.55 ± 0.3 μg·h/ml, a terminal half-life of 1.41 ± 0.2 h, and a plasma clearance (Clp) of 225.7 ± 11.9 ml/h. In the presence of Valspodar, a 3-fold increase in AUC (significant at P < 0.05) was observed, whereas Cmax values were minimally affected (Table 1). This increase in AUC is consistent with earlier observations in mice (23). Further, Valspodar coadministration caused a 2-fold reduction in elimination T1/2 (Table 1).

When EPC/Chol DOX was administered at a dose of 5 mg/kg, the plasma concentration-time data were best fitted with a one-compartment model characterized by a monophasic elimination profile (Fig. 1B). A Cmax of 129.9 ± 11.9 μg/ml and an AUC of 571.4 ± 102.2 μg·h/ml were obtained (Table 1). Compared with the free DOX treatment in the absence of Valspodar, this represented a significant (P < 0.05) 103-fold increase in AUC, confirming slower DOX removal from the plasma compartment after liposome delivery. An elimination T1/2 of 3.05 ± 0.52 h and a Clp of 2.23 ± 0.42 ml/h were estimated. Again,
compared with nonencapsulated DOX, liposomal delivery of DOX in EPC/Chol liposomes resulted in a 2-fold increase in $T_{1/2}$ and a 100-fold reduction in $CL_p$.

When Valspodar was coadministered with EPC/Chol DOX, a significant ($P < 0.05$) 1.8-fold increase in AUC was observed. This increase in AUC by Valspodar for EPC/Chol DOX is consistent with observations in mice (24) and was not as profound as for nonencapsulated DOX. A 1.9-fold increase in elimination $T_{1/2}$ and a 1.8-fold decrease in $CL_p$ were also observed (Table 1; significant at $P < 0.05$).

PEG-DSPE/DSPC/Chol DOX administered at a dose of 5 mg/kg to rats resulted in a plasma elimination kinetics that were also fitted to a one-compartment model (Fig. 1C), with an estimated $C_{max}$ of $232.8 \pm 38.0 \mu g/ml$, an AUC value of $3981 \pm 618.6 \mu g \cdot h/ml$, elimination half life of $12.01 \pm 1.44 h$, and a $CL_p$ of $0.31 \pm 0.01 ml/h$ (Table 1). Valspodar did not significantly alter any of the pharmacokinetic parameters of DOX when administered in PEG-DSPE/DSPC/Chol liposomes, as indicated by comparable values, in the presence and absence of the MDR modulator, for AUC, $T_{1/2}$, $C_{max}$, and $CL_p$ values (Table 1).

The plasma clearance of liposomal DOX was considerably reduced compared with that of nonencapsulated drug under all conditions studied. Specifically, compared with nonencapsulated drug, administration of DOX in EPC/Chol liposomes and PEG-DSPE/DSPC/Chol liposomes decreased the $CL_p$ by 101- and 728-fold, respectively. These observations are substantiated by concomitant increases in AUC of 103- and 724-fold for EPC/Chol and PEG-DSPE/DSPC/Chol liposome formulations, respectively. Furthermore, at the doses used here, no indications of acute toxicity (e.g., stress, lethargy, diarrhea, abnormal appearance of liver, spleen, and intestines upon necropsy) were observed attributable to the increased presence of liposomal drug in the plasma compartment.

DOX metabolites were below detection limits in the plasma for most of the treatment groups. Although free DOX treatment in the absence of Valspodar resulted in detectable but nonquantifiable concentrations of DOXol, the 13-hydroxy metabolite of DOX, this metabolite was below assay detection limits in the presence of Valspodar as well as in either liposomal DOX treatment groups, both in the presence and absence of Valspodar.

**Urinary Excretion.** Urine samples from rats treated with nonencapsulated and liposomal DOX in the presence and absence of Valspodar were analyzed for DOX, the primary metabolite in rats (DOXol), as well as for glucuronide and sulfate conjugates. The volume of urine collected was determined, which provided the urinary flow rate, from which the drug urinary excretion rate was determined. Urine flow rates averaged 0.5–1.0 ml/h throughout the experimental time course for all study groups. Neither DOX nor coadministration with Valspodar significantly altered urinary flow rate at the doses used here.

The DOX urinary excretion profile (in the presence and absence of Valspodar) is presented in Fig. 2. When nonencapsulated DOX is administered at a dose of 5 mg/kg, intact DOX is readily excreted in urine. The cumulative percentage of DOX excreted in urine was 6.8% of the injected dose over 24 h post-injection. This is consistent with previous observations of

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**Fig. 1** Plasma DOX concentration-time profiles of free DOX (A), EPC/Chol DOX (B), and PEG-DSPE/DSPC/Chol DOX (C) at a DOX dose of 5 mg/kg i.v. in the absence (□) and presence (■) of Valspodar (50 mg/kg, p.o., administered 4 h prior to DOX). Data are represented as means ($n = 3/group$); bars, SD. Note the different X- and Y-axis scale for A, B, and C.
DOX excretion in rats (20, 21). DOXol was the only DOX metabolite present and then, only at low levels, where ~0.1% of the injected DOX dose was excreted as DOXol over the 24-h study period (data not shown). The DOX renal clearance (\(CL_r\)) was estimated at 13.47 ± 9.25 ml/h (range, 5.65–23.69 ml/h; Table 2). In the presence of Valspodar, a significant \((P < 0.05)\) 3.5-fold decrease in \(CL_r\) was observed. The cumulative amount of DOX and DOXol excreted over 24 h after administration of nonencapsulated DOX was not dramatically altered by Valspodar. Specifically, total DOX excreted in urine over 48 h in the absence and presence of Valspodar was 6.8 and 6.4%, respectively. For DOXol, 0.12% of the total DOX dose was excreted in urine without Valspodar, whereas this value increased to 0.47% when the MDR modulator was coadministered with nonencapsulated DOX. This apparent lack of a significant Valspodar effect on total renal excretion was related to the fact that the elevated plasma DOX levels caused by Valspodar treatment combined with nonencapsulated DOX offset MDR modulator-induced decreases in DOX renal clearance rates (compare Tables 1 and 2).

DOX encapsulated in EPC/Chol liposomes administered at a dose of 5 mg/kg resulted in decreased DOX excretion in the urine compared with nonencapsulated drug (Fig. 2). Cumulative DOX excreted in urine was estimated at 4.8% of injected dose after 24 h. As seen for nonencapsulated DOX, Valspodar did not alter the cumulative amount of DOX excreted after injection of EPC/Chol DOX (compare 4.8% for EPC/Chol DOX and 4.14% for EPC/Chol DOX plus Valspodar). The \(CL_r\) for EPC/Chol DOX was estimated as 0.085 ± 0.03 ml/h, which was reduced by 1.7-fold to 0.049 ± 0.009 ml/h in the presence of Valspodar. Comparison of the Valspodar-induced decrease in DOX excretion \((CL_{r, no\ Valspodar} - CL_{r, plus\ Valspodar})\) for nonencapsulated and EPC/Chol encapsulated DOX indicated that the reduction in \(CL_r\) of EPC/Chol DOX by Valspodar was significantly \((P < 0.05)\) less than that caused for nonencapsulated DOX. DOXol was below assay detection limits and, therefore, could not be quantitated in rats treated with either EPC/Chol DOX alone or in combination with Valspodar.

When PEG-DSPE/DSPC/Chol DOX was administered at a dose of 5 mg/kg, DOX excretion in urine was the slowest of the three DOX formulations studied here. As shown in Fig. 2, the urinary elimination of DOX when presented in these liposomes was minimal compared with either nonencapsulated or EPC/Chol encapsulated forms. Specifically, urinary clearance of DOX after injection in the PEG-DSPE/DSPC/Chol encapsulated forms. For nonencapsulated DOX, 1.4% of total dose was analyzed for the presence of glucuronide and sulfate conjugates. For EPC/Chol DOX, 0.75% of the injected dose in urine over 48 h. A CL\(_r\) value of 0.004 ± 0.001 ml/h (Table 2) was obtained, which was not statistically different from zero. Valspodar co-administration did not cause significant alterations in urinary excretion, as indicated by a comparable \(CL_r\) value of 0.003 ± 0.001 ml/h, which was also not statistically different from zero. The percentage of DOX excreted in urine, in the absence and presence of Valspodar (1.1% of injected dose at 48 h), were also comparable, indicating a lack of a Valspodar effect on DOX urinary excretion when the drug is encapsulated in PEG-DSPE/DSPC/Chol liposomes.

Because of limited urine samples, aliquots of pooled urine were analyzed for the presence of glucuronide and sulfate conjugates of DOX. For nonencapsulated DOX, 1.4% of total amount of drug in the urine over 0–6 h was recovered as sulfate conjugated DOX, whereas there were no indication of glucuronide conjugates. For EPC/Chol DOX, 12.1% was recovered as

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**Figure 2** Urinary DOX excretion profiles after administration of free, EPC/Chol, and PEG-DSPE/DSPC/Chol DOX at a DOX i.v. dose of 5 mg/kg, in the absence and presence of Valspodar.

**Table 1** Summary of the plasma pharmacokinetic parameters (mean ± SD, \(n = 3/group\)) for free, EPC/Chol, and PEG-DSPE/DSPC/Chol sterically stabilized (SS) DOX at a DOX i.v. dose of 5 mg/kg, in the absence and presence of Valspodar.

<table>
<thead>
<tr>
<th>PK parameter</th>
<th>Free DOX (5 mg/kg)</th>
<th>Free DOX (5 mg/kg) + Valspodar (50 mg/kg)</th>
<th>EPC/Chol DOX (5 mg/kg)</th>
<th>EPC/Chol DOX (5 mg/kg) + Valspodar (50 mg/kg)</th>
<th>PEG-SS DOX (5 mg/kg)</th>
<th>PEG-SS DOX (5 mg/kg) + Valspodar (50 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC(_a) ((\mu)g·h/ml)</td>
<td>5.55 ± 0.3</td>
<td>16.84 ± 0.18(^a)</td>
<td>571.4 ± 102.2</td>
<td>1038.8 ± 169.1(^b)</td>
<td>3981.0 ± 183.6</td>
<td>3115.9 ± 493.6</td>
</tr>
<tr>
<td>(T_{1/2}) (h)</td>
<td>1.41 ± 0.2</td>
<td>2.8 ± 0.7(^b)</td>
<td>3.05 ± 0.52</td>
<td>5.78 ± 0.22(^b)</td>
<td>12.01 ± 1.44</td>
<td>9.54 ± 1.03</td>
</tr>
<tr>
<td>(C_{\text{max}}) ((\mu)g/ml)</td>
<td>17.61 ± 1.79</td>
<td>13.1 ± 0.66</td>
<td>129.9 ± 11.9</td>
<td>124.2 ± 18.3</td>
<td>232.8 ± 38.0</td>
<td>225.5 ± 11.5</td>
</tr>
<tr>
<td>(CL_r) (ml/h)</td>
<td>225.7 ± 11.9</td>
<td>74.2 ± 0.85(^b)</td>
<td>2.24 ± 0.42</td>
<td>1.22 ± 0.19(^b)</td>
<td>0.31 ± 0.01</td>
<td>0.40 ± 0.06</td>
</tr>
</tbody>
</table>

\(^a\) AUC, area under the curve (0-4); \(T_{1/2}\), elimination half-life; \(C_{\text{max}}\), peak concentration achieved as determined using the iterative WinNonlin program; \(CL_r\), plasma clearance as determined using the iterative WinNonlin program.

\(^b\) Statistically significant from the no Valspodar group at \(P < 0.05\) (ANOVA).
the sulfate conjugate and 13.7% as the glucuronide conjugate over 0–6 h. Although there was no indication of glucuronide conjugates with PEG-DSPE/DSPC/Chol DOX, 6.5% was recovered as sulfate conjugated DOX over 0–6 h. Similar recoveries and distribution of DOX conjugates were observed in the presence and absence of Valspodar.

**Biliary Excretion.** Fig. 3 presents the biliary excretion profile of DOX after injection of nonencapsulated and liposomal encapsulated DOX. DOX is rapidly excreted in bile after administration of nonencapsulated drug at a dose of 5 mg/kg. At the end of 24 h, 17% of the administered DOX was excreted unchanged in bile. The biliary flow rate, monitored throughout the study, was not altered by any of the DOX nor Valspodar treatments. The 13-hydroxy metabolite of DOX, DOXol, is also excreted in bile, where ~1.46% of injected DOX dose is recovered as un-conjugated DOXol at the end of 24 h. A C_{Lb} of 55.16 ± 19.2 ml/h was obtained, which constituted 24% of the systemic plasma clearance (Table 2). This is consistent with the value of 22% reported by Speeg and Maldonado (15). In the presence of Valspodar, the cumulative DOX excreted at 24 h was significantly (P < 0.05) reduced 5.6-fold to 3% (Fig. 3). In the absence of Valspodar, a biliary clearance value of 0.007 ± 0.002 ml/h was obtained, which was not statistically different from zero (Table 2). This is substantiated by the fact that only 1% of the injected dose appeared in bile over 48 h. Valspodar coadministration did not significantly alter the C_{Lb} of PEG-DSPE/DSPC/Chol DOX, as indicated by a C_{Lb} of 0.002 ± 0.001 ml/h, which was also not statistically different from zero.

**DISCUSSION**

Recent studies in several laboratories have correlated MDR modulator-induced alterations of plasma anticancer drug elimination with significant reductions in renal and biliary clearance (13–16). Blockade of PGP by MDR modulators in extratumoral sites, particularly in excretory organs such as the kidney and the liver, therefore, presents potential complications in the use of the PGP inhibitor Valspodar with anticancer agents such as DOX. We investigated here the influence of Valspodar on the plasma clearance as well as renal and biliary clearance properties of DOX encapsulated in liposomes. These studies were conducted to characterize the mechanism(s) whereby liposomes can avoid toxicity complications typically associated with Valspodar-nonencapsulated DOX combinations, an observation made previously in murine models (23, 24).

The instrumented rat model was chosen for the studies here, given the need to perform serial sampling of blood and bile, a feature not feasible in the mouse under the constraints of...
the analytical procedure for measuring DOX and its metabolites. The percentage of DOX excreted unchanged in urine (6.75%) and bile (16.8%) as well as the respective clearance parameters observed here for nonencapsulated DOX correlate well with previous observations in several studies (15, 19–21). Also, Valspodar effects on plasma DOX levels in the murine and rat models used in our laboratory were very comparable (23, 24). These results support the comparisons here between nonencapsulated and liposomal DOX formulations and also provide a basis for extrapolating the influence of liposome encapsulation on DOX excretion in the rat to toxicity and efficacy behavior of these systems in the human xenograft tumor models used previously (24).

Significant differences in the renal and biliary handling of DOX arising from administration of nonencapsulated and liposomal DOX formulations were observed in this study. Reductions in DOX plasma, renal, and biliary clearance caused by Valspodar were very modest for the liposome formulations studied here in comparison to nonencapsulated DOX administration. Liposomal DOX formulations that released 50% of encapsulated drug within 1 h after i.v. injection (EPC/Chol) and long-circulating PEG-DSPE/DSPC/Chol liposomes that exhibit negligible DOX release provided varying degrees of Valspodar-mediated inhibition of DOX clearance. The differences in DOX pharmacokinetics with these liposomes were consistent with literature reports (31–33) and enabled the resolution of DOX-Valspodar interactions between unencapsulated and liposome-encapsulated drug pools. In general, Valspodar effects were more marked with EPC/Chol DOX, where statistically significant alterations of plasma drug clearance and trends toward decreased renal and biliary clearance were observed compared with the negligible effects on all clearance parameters with PEG-DSPE/DSPC/Chol DOX. This is consistent with the DOX release properties of the EPC/Chol formulation, where a component of drug distribution properties similar to nonencapsulated DOX are observed when combined with Valspodar treatment. In contrast, the sterically stabilized liposome system is characterized by an ability to retain DOX for extended periods of time as well as decreased accumulation in liver tissue. It should be noted that the reduced renal and biliary clearance of nonencapsulated DOX over the entire time course studied as well as significantly elevated plasma drug concentrations observed in the presence of Valspodar confirm the persistent effects of this MDR modulator on DOX clearance properties.

Although nonencapsulated DOX was rapidly eliminated in bile, DOX administered in EPC/Chol liposomes led to reduced drug excretion in urine and bile, and PEG-DSPE/DSPC/Chol DOX displayed negligible DOX urinary and biliary excretion. Furthermore, whereas Valspodar caused a 3.6-fold decrease in $CL_{\text{R}}$ and 37.5-fold decrease in $CL_{\text{D}}$ of nonencapsulated DOX, it reduced the $CL_{\text{R}}$ and $CL_{\text{D}}$ of DOX after injection of the EPC/Chol formulation by only 1.7- and 2.0-fold, respectively, and had no effects on DOX excretion after administration of PEG-DSPE/DSPC/Chol liposomes. This is consistent with observations where higher levels of conjugated DOX were observed, particularly with the EPC/Chol DOX system, with a general relationship: EPC/Chol DOX (highest) $>$ PEG-DSPE/DSPC/Chol DOX (intermediate) $>$ nonencapsulated DOX (least). Such observations suggest that increased conjugation of DOX occurs when it is entrapped in liposomes that accumulate in the liver, where they are taken up predominantly by Kupffer cells (30, 34). Also, liposomes do not permeate across hepatocyte membranes directly, and consequently, drug has to be released from the liposomes to enter these cells. Whereas Valspodar coadministration with nonencapsulated DOX resulted in significant increases in DOX, DOXol, and DOXone levels, reductions in aglycone levels were observed with PEG-DSPE/DSPC/Chol liposomes in addition to no DOXol formation. Reductions in toxicity observed with liposomal DOX-Valspodar coadministration reported in previous studies may, therefore, be due in part to the enhanced formation of polar, nontoxic glucuronide and sulfate conjugates, which are readily excreted in urine and bile, compared with the more toxic parent DOX or its aglycone metabolites (35, 36).

The renal and biliary clearance of liposomal DOX (alone or with Valspodar) was much lower than that for the nonencapsulated DOX-Valspodar combination. It is likely that coadministration of Valspodar and nonencapsulated DOX leads to saturation of the pathways for DOX metabolism and excretion attributable to increased DOX exposure mediated by the MDR modulator, whereas administration of liposomal DOX results in lower concentrations of DOX exposed to the excretory organs via slower release of the drug from liposomes and altered metabolism. In support of this, alterations in DOX excretion have been shown to occur at high doses of nonencapsulated DOX ($\sim$40 mg/kg), indicating the limited capacity to eliminate DOX when presented at high concentrations (21). Presumably, Valspodar-mediated increases in nonencapsulated DOX exposure, combined with impaired PGP transport in the liver and kidneys, could result in saturation of the processes required to eliminate DOX. Whether extended PGP blockade with Valspodar infusions could lead to altered excretion of liposomal DOX...
is not known. However, given the extremely limited biliary and renal excretion of DOX after administration of the sterically stabilized liposomal formulation, it is very unlikely that substantial alterations of DOX elimination sufficient to cause adverse toxicological consequences would occur, even under these conditions.

Taken together, these results suggest that the slower urinary and biliary elimination of liposomal DOX may explain the lower propensity of Valspodar to exert a profound inhibitory effect on DOX clearance from the circulation. The utility of liposomes to reduce the toxicity of DOX and effects of Valspodar on DOX pharmacokinetics may be related to the delivery of DOX to phagocytic Kupffer cells, where catabolic processes generate metabolites that are neither toxic nor PGP substrates. Liposomal delivery of DOX to the liver appears to result in much lower DOX and DOX metabolite exposure over extended periods of time, such that even under conditions of Valspodar-mediated inhibition of PGP, the renal and biliary excretion capacity is sufficient to handle the levels exposed to these tissues. These results also suggest that sterically stabilized liposomal DOX may be best suited to coadministration with Valspodar, where potential adverse pharmacokinetic interactions with unentrapped drug in the circulation are minimized. Whether these benefits associated with liposomal DOX are also with unentrapped drug in the circulation are minimized.

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Doxorubicin Encapsulated in Sterically Stabilized Liposomes Exhibits Renal and Biliary Clearance Properties That Are Independent of Valspodar (PSC 833) under Conditions That Significantly Inhibit Nonencapsulated Drug Excretion

Rajesh Krishna, Natasha McIntosh, K. Wayne Riggs, et al.


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