Int. J. Cancer: **85,** 131–141 (2000)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

INCREASED INTRACELLULAR DRUG ACCUMULATION AND COMPLETE CHEMOSENSITIZATION ACHIEVED IN MULTIDRUG-RESISTANT SOLID TUMORS BY CO-ADMINISTERING VALSPODAR (PSC 833) WITH STERICALLY STABILIZED LIPOSOMAL DOXORUBICIN

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We have previously demonstrated that liposome encapsulation of doxorubicin (DOX) can alleviate adverse interactions with non-encapsulated DOX and the cyclosporine multidrug-resistant (MDR) modulator Valspodar. We have now investigated the behavior of different liposomal DOX formulations in MDA435LCC6/MDR-1 human breast cancer solid tumor xenograft models to identify liposome characteristics associated with enhanced therapeutic activity and the mechanism whereby increased chemosensitization is achieved. Toxicity studies incorporating conventional phosphatidylcholine (PC)/cholesterol (chol) and sterically stabilized (polyethylene glycol 2000 [PEG]-containing) formulations of DOX indicated that whereas PC/Chol DOX was approximately 3-fold more toxic in the presence of Valspodar, PEG containing distearoylglycerophosphocholine (DSPC)/Chol DOX was minimally affected. In mice bearing MDR tumors, co-administration of Valspodar and egg phosphocholine (EPC)/Chol DOX resulted in modest MDR modulation and efficacy, whereas the sterically stabilized formulation induced reductions in tumor growth equivalent to that achieved for drug-sensitive tumors treated with non-encapsulated DOX. Pharmacokinetic studies revealed a 2.5-fold increase in plasma DOX area under the curve (AUC) upon co-administration of Valspodar with EPC/Chol DOX whereas no such alterations were observed with the sterically stabilized liposomes. Compared to non-encapsulated DOX combined with Valspodar, improvements in efficacy and toxicity correlated with the extent to which liposomal DOX formulations were able to circumvent pharmacokinetic interactions. Confocal microscopy demonstrated that Valspodar increased cell-associated DOX which correlated with the level of anti-tumor efficacy. ***Int. J. Cancer*** **85:131–141, 2000.**
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The development of second generation multidrug-resistant (MDR) reversing agents alleviated many of the problems caused by earlier PGP blockers which were pharmacological agents with their own inherent toxicities. However, co-administration of conventional anticancer drugs with many of these newer MDR modulators has been shown to elicit drug-modulator interactions by virtue of PGP blockade in normal tissues such as liver, kidney, intestine and brain (Keller *et al.,* 1992*a;* Gatmaitan and Arias, 1993). It may not be surprising then that clearance of several anticancer drugs is inhibited by MDR modulators such as cyclosporine (CsA) (Speeg *et al.,* 1992), verapamil (Nooter *et al.,* 1987), Valspodar (Speeg and Maldonado, 1994, previously named PSC 833) and GW918 (Booth *et al.,* 1998).

The effects of MDR modulators on drug transport proteins that cause alterations in anticancer drug excretion often lead to increased anticancer drug exposure of healthy tissues and have necessitated dose reduction in many preclinical (Krishna and Mayer, 1997; Keller *et al.,* 1992*a;* Nooter *et al.,* 1987) and clinical (Boote *et al.,* 1996; Sarris *et al.,* 1996) studies. Although doses can be adjusted to equal levels of toxicity, it is unclear how such pharmacokinetic (PK) changes may impact therapeutic activity. These interactions have been postulated to play a role in limiting the therapeutic outcome in some patients (Wishart *et al.,* 1994; Miller *et al.,* 1994). While changes in anticancer drug dose and/or schedule may be able to address toxicity or efficacy alterations brought about by MDR modulators, this clearly represents a significant complication in applying PGP blockade strategies to cancer chemotherapy. This is due to the fact that most chemotherapy regimens utilize drug combinations, of which more than one are often PGP substrates. Consequently, an ability to avoid such anticancer drug clearance alterations may be considered to be a significant advantage in MDR modulation strategies.

We earlier reported that liposome encapsulation of doxorubicin (DOX) can reduce non-encapsulated drug-Valspodar interactions, resulting in improved growth suppression of MDR murine solid tumors (Krishna and Mayer, 1997). The liposomal formulation used in these studies was composed of 120 nm diameter distearoylglycerophosphocholine (DSPC)/cholesterol (Chol) (55:45 molar ratio) that retains DOX for extended periods of time (Mayer *et al.,* 1989). The enhanced antitumor activity observed in this study appeared to be a consequence of increased protection from Valspodar-mediated PK changes and toxicity exacerbation. However, the mechanisms by which these effects were achieved are not fully understood, particularly since significant amounts of DOX are delivered to the liver by liposomes without notable toxicological consequences. In addition to alleviation of PK alterations, the increased delivery of DOX to MDR solid tumors using liposome delivery systems was associated with increased anti-tumor activity when co-administered with Valspodar compared with non-encapsulated drugs. These studies were unable, however, to distinguish the degree of DOX bioavailability (liposome entrapped *vs.* released drug) in the solid tumor. Therefore, the relative roles of PGP blockade and tumor drug levels remain unresolved.

In order to address these questions, we compared here the toxicity, efficacy, pharmacokinetics, cellular distribution properties of egg phosphocholine (EPC)/Chol DOX (a system where over 50% of the drug is released in the first hour) and a sterically stabilized PEG_{2000} (PEG) distearoylphosphoethanolamine (DSPE)/DSPC/Chol DOX formulation combined with the MDR modulator, Valspodar. The latter system was chosen on the basis of reports that incorporation of 5 mol% PEG-polymerized lipid in 100 nm DSPC/Chol vesicles results in increased circulation longevity, reduced liver uptake and increased tumor delivery (Papahadjopoulos *et al.,* 1991). Therefore, the effects of altering the drug release, liver uptake and tumor accumulation properties of liposomal DOX on toxicological and therapeutic activity were compared to reveal the processes underlying the improvements in toxicity and efficacy achieved with liposomal systems in a human breast carcinoma

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Received 10 May 1999; Revised 12 July 1999

MDR solid tumor xenograft model. Furthermore, these formulations reflect the 2 liposomal DOX products that are either approved (sterically stabilized) or pending approval (EPC/Chol) for widespread clinical use. Consequently, determining their pharmacological properties in the presence of MDR modulators would be of considerable clinical interest.

MATERIAL AND METHODS

Material

DOX hydrochloride for injection (U.S.P.) was purchased from David Bull (Vaudreil, Canada) and its purity affirmed by high-performance liquid chromatography (HPLC; see below). Valspodar was a generous gift from Novartis (Dorval, Canada) and its purity affirmed by LC/MS-MS (Varian LC-MS-MS system; Fisons, Altrincham, UK). DOX metabolite standards were generous gifts from Pharmacia Carlo Erba (Milan, Italy). PEG_{2000}-DSPE (>99% purity), EPC (>99% purity) and DSPC (>99% purity) were obtained from Northern Lipids (Vancouver, Canada) and Chol from Sigma (St. Louis, MO). Cholesteryl hexadecyl ether (^{3}H), a non-exchangeable, non-metabolizable lipid marker was purchased from Amersham (Oakville, Canada). HPLC grade solvents were obtained from BDH (Toronto, Canada) and used without further purification. Female BDF_1 mice were obtained from Charles River (St. Constant, Canada). Female SCID/RAG2 mice were bred in-house at the BC Cancer Agency animal facility. The MDA435/LCC6 and its transfected MDR-1 line were generously provided by Dr. R. Clarke (Georgetown University, Washington, DC). These cells were maintained in tissue culture in Dulbeco's modified Eagle medium (StemCell Technologies, Vancouver, Canada).

Liposome and drug preparation

Liposomes composed of EPC/Chol (55:45), PEG_{2000}-DSPE/DSPC/Chol (5:50:45) and DSPC/Chol (55:45; mol:mol) were prepared by initially dissolving the lipid mixtures in chloroform (100 mg lipid per milliliter) and hydrating the dried lipid film in a 300-mM citric acid, pH 4.00, buffer. The resulting multilamellar vesicles (MLVs) were subjected to 5 freeze-thaw cycles followed by a 10-cycle extrusion through 2 stacked 100-nm polycarbonate filters (Nuclepore, Pleasanton, CA) using a Lipex Extruder (Lipex Biomembranes, Vancouver, Canada). ^{3}H-Cholesterylhexadecyl ether was used as a non-exchangeable, non-metabolizable lipid marker (Derksen *et al.,* 1987). The resulting large unilammelar vesicles exhibited a mean diameter between 110–120 nm as determined using a Nicomp 270 submicron particle sizer (Particle Sizing Systems, Santa Barbara, CA).

DOX was encapsulated in the liposomes using the transmembrane pH gradient loading procedure (interior acidic) employing sodium carbonate as the alkalinizing agent and a drug-to-lipid weight ratio of 0.2:1.0 (Mayer *et al.,* 1989). 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%):Tween 80 and administered in a corn oil vehicle by oral gavage of a 200-µl volume (Keller *et al.,* 1992*b*). Non-encapsulated DOX was administered in sterile saline.

Toxicity evaluation studies

Toxicity of the indicated DOX and Valspodar dose regimens was evaluated in dose range-finding studies using normal (non-tumor-bearing) female BDF_1 mice. Toxic dose range-finding studies in tumor-free female mice were performed using 3 mice per group with appropriate group repetitions as described below. Briefly, mice were administered increasing (each dose in different groups) doses of i.v. DOX (free or liposomal) *via* the tail vein and oral Valspodar at a fixed dose of 100 mg/kg (4 hr before DOX) on days 1, 5 and 9. Liposomal DOX doses were changed by increments of 5 mg/kg on the day 1, 5, 9 injection schedule. DOX dose escalation was stopped when weight loss exceeded 30% or toxicity-related mortality was observed. Survival and the percent change in body weight was monitored over a 21-day period. Animals were monitored for other toxicity signs such as scruffy coat, dehydration, lethargy, ataxia or labored breathing. Animals which demonstrated significant physical manifestations of distress/toxicity or exhibited a body weight loss in excess of 30% were terminated. At the end of the 21-day study period, mice were terminated by carbon dioxide asphyxiation. Necropsies were performed to identify abnormalities in the major organs. The dose at which the body weight loss (group mean value) was ≤15% and all mice survived for the duration of study was established as the maximum tolerated dose (MTD). A deviation in MTD of ±1.5% was allowed in cases where body weight loss between 15–16.5% lasted only 1 day and where 100% recovery of body weight loss was observed, with 100% survival. The results were collated from at least 2 independent experiments. Select groups were repeated as a quality control measure to ensure reproducibility.

In vivo *anti-tumor activity*

MDA435LCC6 cells were cultured in DMEM and passaged at least 3 times in medium. A cell aliquot containing 5×10^6 cells in 0.5 ml HBSS was injected i.p. into 2 female SCID/RAG2 mice. After 20–25 days, ascites (cells) were removed from the mouse *via* the peritoneal wall using a 20g needle and placed in sterile 15-ml conical tubes containing 5 ml of HBSS without Ca and Mg salts. The cell suspension was then centrifuged at 1000 g for 5 min and the supernatant discarded. Using a 27g needle fitted on a 1-ml syringe, 50 µl of the cell suspension was injected into 2 mammary fat pads on each mouse (2×10^6 cells per pad). A period of 18–21 days was needed for the tumors to become palpable and measurable for drug treatments to begin. The MDR MDA435LCC6-MDR-1 cells were maintained and passaged identically to the WT cells. These MDR cells have had the MDR-1 cDNA introduced into the MDA435LCC6 cells (Leonessa *et al.,* 1996). Cell passaging and inoculation were performed identical to the procedures used in the WT sensitive cell line.

Tumor growth suppression experiments were conducted in SCID/RAG2 mice bearing orthotopic human breast carcinoma MDA435LCC6 (multidrug resistant, MDR-1 and sensitive, WT) solid tumors. After approximately 3 weeks, when the tumors (n = 8 per group) were established, treatment was initiated with dosage regimens incorporating i.v. non-encapsulated or liposomal DOX with or without p.o. Valspodar (given 4 hr before DOX) on days 1, 5 and 9. Experiments were repeated to ensure reproducibility of tumor growth inhibition properties. Caliper measurements of the tumors were performed daily, and the tumor weights calculated according to the formula (Krishna and Mayer, 1997):

$$\text{Tumor weight (g)} = \frac{\text{length (cm)} \times [\text{width (cm)}]^2}{2}$$

This conversion formula was verified by comparing the calculation derived tumor weights to excised and weighed tumors. Animal weights and mortality were monitored daily. Animals bearing ulcerated tumors or where tumor weights exceeded 10% of the animals' body weight were terminated. The weights of the bilateral tumors were averaged for each mouse and mean tumor weights for each treatment group ± standard error of the mean were calculated. Statistical tests for these longitudinal data were performed using repeat measures ANOVA employing Statistica for Windows 4.0 (StatSoft, Tulsa, OK) and statistical significance was set at $p < 0.05$.

Pharmacokinetics and tissue distribution

Female MDA435LCC6/MDR-1 solid tumor-bearing SCID/RAG2 mice received i.v. *via* the tail vein a single bolus of liposomal doxorubicin (^{3}H EPC/Chol or PEG_{2000}-DSPE/DSPC/Chol 0.2:1.0). Valspodar (100 mg/kg) was administered p.o. (in 0.2 ml) and doxorubicin administered 4 hr later. After DOX dosing, groups of 3 mice per time point were anesthetized with 100 µl i.p. of ketamine/xylazine at 30 min, 1, 2, 4, 16, 24, 48 and 72 hr. Blood was collected by cardiac puncture and placed into EDTA-coated

microtainer tubes. Terminal blood and selected tissues were collected from all animals and were processed to determine lipid (using radioactivity) and DOX concentrations (using HPLC). After blood collection, the kidneys, livers and tumors were removed from each animal. Tissues were rinsed in PBS, pat dried on absorbent paper and weighed in pre-weighed 16 × 100-mm tubes. Samples were stored in −20°C pending analysis. A 10–30% homogenate in distilled water was prepared using a Polytron homogenizer (Kinematica, Littau, Switzerland). A 0.2-ml aliquot of the homogenate was digested with a tissue solubilizer, decolorized with peroxide and analyzed for the lipid label by scintillation counting. The specimens obtained from the control animals were used as background samples.

DOX and its metabolites in plasma and tissue extracts were determined using HPLC. The HPLC assay of Andersen *et al.* (1993) was used to analyze DOX and its metabolites with minor modification. Briefly, sample extraction with acetonitrile was followed by isocratic elution from a Nova-Pak C18 3.9 × 150 mm (Millipore, Bedford, MA) analytical reverse phase column and quantified by endogenous fluorescence (emission wavelength of 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. The column was maintained at 40°C. A NEC (Boxborough, MA) Powermate SX Plus Computer and a Systems Interface Module (Waters, Milford, MA) were used for data handling.

Using this system, the retention times of DOXol, DOX, DOXone and 7-deoxyDOXone were 3.6, 5.8, 7.5 and 12.6 min, respectively. Recoveries, using acetonitrile as the extraction solvent, from plasma over a concentration range of 0.05–10 μg/ml of DOX, DOXone, 7-deoxyDOXone and DOXol, were between 80–110%. To protect DOX and its metabolites from photodegradation, all procedures were shielded from direct exposure to light. In addition, DOX was found to be stable in the mobile-phase solvent mixture for at least 96 hr (40°C), on the HPLC autosampler tray for at least 96 hr, in reconstituted form at 4°C for at least 10 days and for at least 4 freeze-thaw cycles.

The plasma data were modeled using WinNONLIN Version 1.5 PK software (Pharsight, Mountain View, CA) to calculate area under the curve (AUC), half-life ($T_{1/2}$) and plasma clearance (CL_p) according to standard equations (Gibaldi and Perrier, 1982). The trapezoidal rule was used to calculate the AUCs in tissue concentration-time profiles employing a computer software AUC (program provided by Dr. W. Riggs, Faculty of Pharmaceutical Sciences, University of British Columbia). Tissue DOX levels were corrected for blood volume to account for material residing in the vasculature of the tissues, using previously published values (Bally *et al.,* 1993).

Select samples were assayed for free and liposome-associated DOX using Microcon-30 filters (Amicon, Oakville, Canada) using the equilibrium filtration method of Mayer and St. Onge (1995). Separation of free from liposomal and protein-bound drug was performed using Microcon-30 (0.5-ml capacity, Amicon) ultrafiltration devices with a m.w. cut-off of 30 kDa. Microcon-30 samples were centrifuged at 4°C, 8,000 g for 20 min in a microcentrifuge (IEC Micromax Centrifuge; International Equipment, Needham Heights, MA). The ultrafiltrate was processed for DOX by HPLC (see above).

Confocal microscopy and imaging studies

For confocal imaging studies, SCID/RAG2 mice bearing MDA435LCC6/MDR-1 tumors were treated with non-encapsulated DOX, EPC/Chol DOX or PEG-DSPE/DSPC/Chol DOX (5 mg/kg) in the presence and absence of Valspodar 100 mg/kg (4 hr before DOX). At the indicated times following DOX administration, tissues were aseptically dissected, bathed in PBS and imaged fresh. Before imaging, thin pieces of tumors were placed on concave slides and observed under a 60× oil immersion lens. These were then viewed under the confocal microscope to determine DOX distribution characteristics. As controls, known amounts of non-encapsulated DOX or PEG-DSPE/DSPC/Chol DOX liposomes were infused into freshly isolated muscle tissues and viewed for DOX fluorescence.

Confocal images were collected on an Optiphot 2 research microscope (Nikon, Tokyo, Japan) attached to a confocal laser scanning microscope (MRC-600, BioRad, Hercules, CA) using COMOS software (BioRad). The laser line on the krypton/argon laser was 488 nm. Filterblock BHS was used to detect DOX (488 nm excitation, 515 nm emission). The numerical aperture was 0.75 on the ×20 air objective and 1.2 on the ×60 oil objective. The images were captured such that the xyz dimensions were 0.4 μm cubed (×20) and 0.2 μm pixel (×60). NIH Image version 1.61 was used for image analysis, and all images were based on maximum intensity projection. Projections made in the NIH Image were saved in TIFF format, then imported to Adobe Photoshop version 4.0 where the different fluorophore images were assigned to individual RGB channels and subsequently merged to provide the final image of the single or multiple sections.

RESULTS

Toxicity

In order to identify the MTD for the day 1, 5 and 9 dosage regimen used in therapeutic experiments as well as to investigate the mechanisms of Valspodar-mediated increases in DOX toxicity, 21-day dose range-finding toxicity studies were conducted with EPC/Chol, DSPC/Chol and PEG-DSPE/DSPC/Chol liposomal formulations of DOX as well as non-encapsulated drug in the presence and absence of p.o. Valspodar in non-tumor-bearing healthy mice. The results of this study are shown in Table I, where the body weight loss is presented, along with the MTDs (as defined in Material and Methods). The 3 liposomal DOX formulations yielded variable toxicity characteristics that depended on their respective abilities to retain DOX.

In the absence of Valspodar, EPC/Chol DOX exhibited an MTD of 15 mg/kg, where the body weight loss was 1.7% with 100% survival (Table I). For the 2 saturated lipid formulations, DSPC/Chol and PEG-DSPE/DSPC/Chol DOX, the MTD was identical at 25 mg/kg, representing a 1.7-fold increase in MTD compared with the more leaky EPC/Chol formulation. When EPC/Chol DOX was

TABLE I – TOXICITY AS A FUNCTION OF DOX DOSE FOR NON-ENCAPSULATED AND LIPOSOMAL FORMULATIONS[1]

Group	Dose (mg/kg)	Day 10 weight loss (% survival)	
		−PSC 833	+PSC 833
Non-encapsulated DOX	2.5	0 (100)	10.2 (100)
	5	3.5 (100)	18.5 (0)
	7.5	10.7 (100)	28.2 (0)
	10	29.2 (0)	
MTD		7.5 mg/kg	2.5 mg/kg
EPC/Chol DOX	5		0.9 (100)
	10	2.8 (100)	23.1 (0)
	15	1.7 (100)	
	20	20.4 (0)	24.4 (0)
MTD		15 mg/kg	5 mg/kg
DSPC/Chol DOX	15	10.8 (100)	12.9 (100)
	20	3.1 (100)	15.0 (100)
	25	16.0 (100)	18.5 (67)
MTD		25 mg/kg	20 mg/kg
PEG-DSPE/DSPC/Chol DOX	15	7.3 (100)	9.8 (100)
	20	6.5 (100)	14.8 (100)
	25	9.3 (100)	14.8 (100)
	30	4.4 (67)[2]	25.3 (0)
MTD		25 mg/kg	25 mg/kg

[1]Data are group mean values (n = 3 mice per group treated i.v. on days 1, 5 and 9).–[2]Body weight loss nadir is on day 19 (−10.2%) with 67% survival.

combined with Valspodar, significant toxicity resulted, necessitating a dose reduction by 3-fold, to 5 mg/kg. This is comparable to the 3-fold decrease in MTD caused by Valspodar for non-encapsulated drug administration (Table I). In contrast, Valspodar necessitated a small 1.2-fold decrease in DOX encapsulated in DSPC/Chol liposomes and no dose reduction was required for the PEG-containing sterically stabilized liposomes (Table I).

Efficacy

The anti-tumor activity of the 3 types of liposomal DOX formulations was evaluated *in vivo* in the absence and presence of Valspodar using the MDA435LCC6 and PGP-overexpressing MDA435LCC6/MDR-1 human breast carcinoma xenograft solid tumor models. Figure 1 presents the tumor growth curves for mice treated with non-encapsulated drug (*a*), EPC/Chol DOX (*b*), DSPC/Chol DOX (*c*) and sterically stabilized PEG-DSPE/DSPC/Chol DOX (*d*) in the presence and absence of Valspodar.

When MDA435LCC6 cells (sensitive, WT or resistant, MDR) are inoculated in the mammary fat pads of SCID/RAG2 mice, solid tumors readily establish (tumor take rates >95%). Figure 1 shows that both MDR and WT-untreated controls exhibit comparable tumor growth rates, with the exponential growth phase occurring between days 5 and 14 (slope of linear regression line = 0.067 for

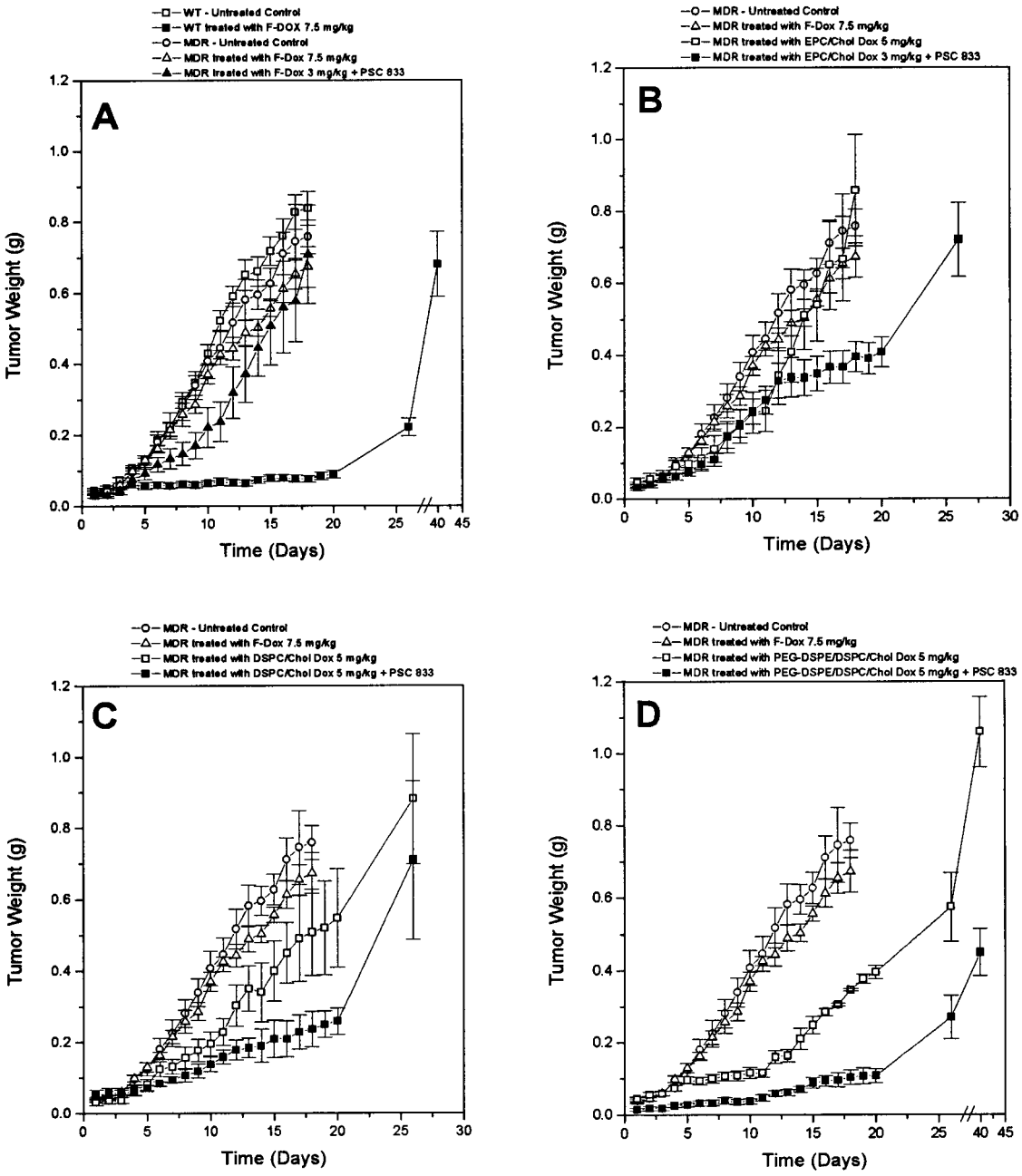

FIGURE 1 – Anti-tumor efficacy of free *(a),* EPC/Chol DOX *(b),* DSPC/Chol *(c)* and PEG-DSPE/DSPC/Chol *(d)* against MDA435LCC6 WT or MDR1 human xenograft solid tumors in the absence and presence of co-administered Valspodar. MDA435LCC6 tumors were grown on mammary fat pads of female SCID/RAG2 mice. Oral Valspodar (100 mg/kg) and i.v. DOX treatments were initiated once tumors were established (20–100 mg) and were given on days 1, 5 and 9 at the indicated doses of free and liposomal DOX. Valspodar was administered 4 hr prior to DOX injection. Data are expressed as mean ± standard error of the mean. For legends, see individual panels.

WT *vs.* 0.056 for MDR tumors). Both groups were terminated on day 18, when tumor weights reached 0.84 ± 0.05 and 0.76 ± 0.05 g for the WT and MDR tumors, respectively. Treatment of WT tumors with non-encapsulated DOX at 7.5 mg/kg on days 1, 5 and 9 (Fig. 1*a*) resulted in significant tumor growth suppression until day 20, when tumors weighed 0.088 ± 0.01 g. These tumors eventually grew to 0.68 ± 0.09 g by day 40. However, administration of non-encapsulated DOX at 7.5 mg/kg on days 1, 5 and 9 in mice bearing MDR tumors did not cause any tumor growth suppression (Fig. 1*a*). When MDR tumor-bearing mice were treated with the MTD of non-encapsulated DOX combined with Valspodar (DOX dose of 3 mg/kg), there was partial tumor growth inhibition until day 11. After this time, tumor growth rates were similar to untreated MDR controls until day 18 when mice were terminated (Fig. 1*a*). Growth of MDR tumors treated with non-encapsulated DOX plus Valspodar was significantly different from both untreated MDR tumors as well as MDR tumors treated with non-encapsulated drug alone between days 7 and 12 post-DOX administration. However, in comparison to WT tumors treated with non-encapsulated DOX 7.5 mg/kg, the MDR tumor growth inhibition caused by non-encapsulated drug and Valspodar was transient (Fig. 1*a*).

When MDR tumors were treated with liposomal DOX formulations in the presence and absence of Valspodar (Fig. 1*b–d*), varying degrees of tumor growth suppression were observed. As seen in Figure 1*b*, EPC/Chol DOX alone (5 mg/kg) was unable to induce substantial inhibition of MDR tumor growth, with tumors weighing 0.86 ± 0.2 g on day 18. In the presence of Valspodar, however, EPC/Chol DOX at 3 mg/kg treatment closely resembled EPC/Chol DOX alone until day 12, after which tumor growth was decreased between days 12 and 20 (Fig. 1*b*). This indicated modest delayed anti-tumor activity. This MDR modulation caused by EPC/Chol DOX and Valspodar was significantly better than that caused by non-encapsulated DOX and Valspodar ($p < 0.05$).

Figure 1*c* illustrates the tumor growth inhibition of DSPC/Chol liposomal DOX in the presence and absence of Valspodar. In the absence of Valspodar, DSPC/Chol liposomal DOX caused a modest reduction in tumor growth. The tumor growth inhibition caused by DSPC/Chol DOX was significantly different from untreated controls until day 12, after which the tumor growth rate increased. Tumor weight for MDR tumors treated with DSPC/Chol DOX alone was 0.51 ± 0.1 g on day 20. In comparison, Valspodar caused a significant increase in DSPC/Chol DOX tumor growth suppression of the MDR solid tumors, where tumor weight was 0.25 ± 0.05 g on day 20. The tumor growth inhibition resulting from DSPC/Chol DOX and Valspodar treatment was significantly ($p < 0.05$) greater than that observed for EPC/Chol DOX and Valspodar.

PEG-DSPE/DSPC/Chol DOX formulations displayed superior anti-tumor activity in the presence and absence of Valspodar when compared with the 2 liposomal DOX formulations described above as well as non-encapsulated drug (Fig. 1*d*). In the absence of Valspodar, PEG-DSPE/DSPC/Chol DOX caused a significant reduction in tumor growth until day 11, when tumor weight was 0.1 ± 0.01 g (compared with a tumor weight of 0.05 ± 0.01 g on day 1), after which tumor growth rates increased. Tumor weight for this group was 0.39 ± 0.02 g on day 20. In the presence of Valspodar, PEG-DSPE/DSPC/Chol DOX significantly inhibited tumor growth and this growth suppression was not significantly different from WT tumors treated with non-encapsulated DOX at its MTD. Specifically, the tumor weights on day 20 for PEG-DSPE/DSPC/Chol DOX in presence of Valspodar were 0.1 ± 0.02 g compared with 0.088 ± 0.01 g for WT tumors treated with non-encapsulated DOX. This suppression of tumor growth for PEG-DSPE/DSPC/Chol DOX and Valspodar was significantly different ($p < 0.05$) from all other treatment groups for mice bearing MDR solid tumors.

Pharmacokinetics and tissue distribution

A comprehensive PK evaluation was performed to correlate toxicity and efficacy data with plasma and tissue (tumor and liver) DOX and DOX metabolite distribution properties determined using HPLC analysis. The comparison of DSPC/Chol DOX and non-encapsulated (free) DOX pharmacokinetics and tissue distribution properties in the presence and absence of Valspodar has been extensively characterized (Krishna and Mayer, 1997). Since the toxicity and efficacy properties of these formulations were very comparable in the current models, the results presented here focused on comparisons between a liposomal system which leaks a significant portion of entrapped drug into the circulation (EPC/Chol DOX), and a sterically stabilized system that exhibits negligible drug release in plasma compartment and increased circulation lifetimes (PEG-DSPE/DSPC/Chol DOX). As a reference comparison, non-encapsulated DOX treatments (with or without Valspodar) were evaluated for DOX concentrations in plasma and the tumor. The goal of these comparisons was to reveal important DOX distribution and metabolism properties that dictate toxicity and efficacy behavior.

Plasma drug kinetics

Figure 2*a* presents the DOX plasma concentrations after i.v. administration of non-encapsulated DOX and the 2 liposomal DOX formulations at a DOX dose of 5 mg/kg. Following administration of non-encapsulated drug, DOX is rapidly eliminated from the circulation. Concentrations of DOX beyond 4 hr were below assay detection limits. The concentration-time profile was characterized by a C_{max} of 1.5 ± 0.1 μg/ml and an AUC of 4.4 μg.hr/ml (Fig. 2*a*). However, when Valspodar was co-administered with non-encapsulated DOX at 5 mg/kg, DOX elimination from plasma was characterized by a prolonged terminal elimination phase (Fig. 2*a*). Valspodar caused significant ($p < 0.05$) increases in C_{max} (3.9 ± 0.5 μg/ml) and AUC (48.1 μg.hr/ml) of non-encapsulated DOX compared to data obtained in the absence of the MDR modulator. This approximately 11-fold increase in DOX AUC caused by Valspodar is consistent with the 10-fold increase in DOX AUC for the non-encapsulated DOX-Valspodar combination observed in the P388/ADR solid tumor model described earlier (Krishna and Mayer, 1997).

As shown in Figure 2*a*, DOX elimination from plasma for both EPC/Chol and PEG-DSPE/DSPC/Chol DOX systems exhibits a monophasic elimination profile characterized by a 1-compartment model with first-order elimination. While EPC/Chol DOX plasma concentration exhibits rapid elimination of the drug within 24 hr, PEG-DSPE/DSPC/Chol DOX displays a prolonged circulation life-time with over 20% remaining at 24 hr. In all cases, parent DOX was the only detectable entity with no indication of any metabolites present. In the presence of Valspodar, the elimination profile remained monophasic, however, DOX concentrations at earlier time points were significantly ($p < 0.05$) increased for EPC/Chol liposomes whereas no such Valspodar effect was observed for PEG containing DSPC/Chol liposomes. This is contrast to observations for non-encapsulated DOX which demonstrated increases in the terminal elimination phase in the presence of Valspodar (Fig. 2*a*). Co-administration of Valspodar and EPC/Chol DOX increased the AUC of DOX by 2.6-fold and C_{max} by 2.3-fold, accounting for the 40% reduction in plasma clearance (Table II; significant at $p < 0.05$). In contrast, Valspodar caused minor changes in the pharmacokinetics of DOX encapsulated in PEG-DSPE/DSPC/Chol liposomes, with small 36% and 25% increases in C_{max} and AUC, respectively (Table II).

Figure 2*b* shows the elimination of liposomal lipid from plasma. These data demonstrate that, similar to DOX pharmacokinetics, liposomal lipid elimination is monophasic, characterized by a 1-compartment model with first-order elimination. The plasma clearance (CL_p) for PEG-DSPE/DSPC/Chol liposomes was 2.4-fold lower than EPC/Chol, and the half-life ($T_{1/2}$) was 2.2-fold higher for PEG-DSPE/DSPC/Chol liposomes compared to EPC/

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