Drug–Drug Interactions Arising from the Use of Liposomal Vincristine in Combination with Other Anticancer Drugs

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INTRODUCTION

Chemotherapeutic agents for cancer treatment typically are given as combination therapies from multiple classes of drugs (1–4). Historically, this approach has yielded far superior results than has single agent therapy (5). There are several liposomal anticancer drugs approved for human use or being tested in clinical trials. These include a liposomal doxorubicin (Myocet®) being marketed in Europe for treatment of metastatic breast cancer; liposomal doxorubicin (Doxil®) used for treatment of ovarian cancer (refractory to paclitaxel and platinum-based therapies [approval 1999]) and AIDS-related Kaposi's sarcoma (approval 1995) (6); liposomal daunorubicin (DaunoXome) for AIDS-related Kaposi's sarcoma (approval 1996) (7,8); and liposomal vincristine, being tested in the treatment of non-Hodgkins lymphoma (9).

Liposomally encapsulated chemotherapeutic agents will increasingly be incorporated into treatment regimens utilizing nonencapsulated drugs, necessitating elucidation of the potential for altered pharmacokinetics of both free and encapsulated drugs when they are given in combination. An example of this was published by Mayer *et al*. (10) in which the potential for a nonencapsulated agent to be taken up into circulating liposomes *in vivo* was demonstrated. In addition, we recently demonstrated encapsulation of both vincristine and mitoxantrone in the same liposomes, a procedure that under certain conditions resulted in release of entrapped vincristine *in vitro* (11). These two studies provided the basis of the studies described herein, which address whether *in vivo* administration of free drugs in combination with liposomal vincristine could effect an increase in the release of vincristine. Liposomal vincristine is more active against lymphomas than free vincristine, an effect that has been attributed to the greater concentrations of vincristine achieved within tumors (9). It stands to reason that increased vincristine drug release rates from liposomes potentially promoted by secondary drugs would result in decreased accumulation at the tumor site, and decreased activity.

The studies summarized in this report assessed whether addition of free anticancer drugs resulted in loss of vincristine from distearoyl phosphatidylcholine (DSPC)/cholesterol (Chol) liposomes, both *in vitro* and *in vivo*. The drugs combined with liposomal vincristine were doxorubicin, daunorubicin, idarubicin, and mitoxantrone. These drugs all load into liposomes in response to a pH gradient (11–13). Also, vincristine, doxorubicin, and other anthracyclines or anthracycline derivatives are often paired in the treatment of a variety of cancers (14–18).

DSPC was chosen for liposome formulation as it has proven increased drug retention properties after loading in response to a pH gradient, as compared to earlier commonly used lipids, such as egg phosphatidylcholine (19). Optimal conditions for loading vincristine and doxorubicin into DSPC/Chol liposomes have been demonstrated previously (19,20). This pH gradient loading method has previously been shown to effect greater than 95% uptake of vincristine, when used at a drug to lipid ratio of 0.05:1.0 (wt/wt). Additionally, we have shown that when vincristine is loaded into DSPC/Chol liposomes in this manner, there is a residual pH gradient of 2.3 units or more. This gradient was sufficient to facilitate >98% encapsulation of mitoxantrone at a drug to lipid ratio of 0.2:1.0 (wt/wt), when coincubated with liposomal vincristine. This uptake of mitoxantrone, however, was associated with a rapid release of approximately 95% of entrapped vincristine (11). For the reasons already stated, we evaluated the effects of nonencapsulated drugs on liposomal vincristine retention, and also the influence of liposomal vincristine on the pharmacokinetics of the coadministered agents.

MATERIALS AND METHODS

Materials

Vincristine sulfate (David Bull Laboratories, Vaudreuil, QC, Canada), mitoxantrone hydrochloride (Lederle Laboratories Division, Cyanamid Canada, Montreal, QC), idarubicin hydrochloride (Pharmacia, Mississauga, ON, Canada), daunorubicin hydrochloride (Rhône-Poulenc Rorer Canada Inc., Montreal, QC), and doxorubicin hydrochloride (Faulding [Canada] Inc., Vaudreuil, QC) were obtained from the British Columbia Cancer Agency (Vancouver, BC, Canada). DSPC was from Avanti Polar Lipids (Alabaster, AL). [^{3}H]-cholesteryl hexadecyl ether (CHE), [^{14}C]-CHE, and [^{3}H]-vincristine sulfate were obtained from Amersham (Oakville, ON, Canada). Reagents not listed were obtained from Sigma Chemical Company (St. Louis, MO). Female CD1 mice were obtained from Charles River Laboratories (St. Constant, QC, Canada). All experiments involving animals adhered to the Principles of Laboratory Animal Care.

Liposome Preparation

DSPC/Chol (55:45, mol:mol) liposomes were prepared by extrusion (21) through polycarbonate filters of defined pore size. Briefly, the appropriate amounts of lipids were

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ABBREVIATIONS: CHE: cholesteryl hexadecyl ether; Chol: cholesterol; DSPC: distearoyl phosphatidylcholine; HBS, HEPES buffered saline; dH_2O: de-ionized, distilled water.

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combined in chloroform. As required, [^{3}H]– or [^{14}C]–CHE was added as a lipid marker (22). The solvent was removed, yielding a homogenous lipid film. Films were hydrated with pH 4.0 300 mM citrate buffer, then subjected to five freeze-thaw cycles (liquid nitrogen, 65°C). The resulting multilamellar vesicles were extruded ten times through two stacked 100 nm pore size filters (Nucleopore, Pleasanton, CA) using an extrusion device maintained at 65°C (Lipex Biomembranes Inc., Vancouver, BC, Canada). Liposomes produced by this method had a mean diameter of 100–120 nm with a Gaussian distribution, as determined by analysis with a Nicomp 270 submicron particle sizer (Pacific Scientific, Santa Barbara, CA).

Transmembrane pH Gradient Loading of Vincristine

Vincristine was loaded into liposomes by means of a transmembrane pH gradient loading procedure (23,24). The external pH of liposomes was increased to 7.3 by exchange of the external citrate buffer to HEPES buffered saline (HBS) by means of gel filtration chromatography, or by addition of sodium phosphate. Vincristine was added to the liposomes at a drug to lipid ratio of 0.04 to 0.05 (wt/wt). The resulting mixture was incubated at 65°C for 20 min to effect vincristine uptake.

Determination of Drug Encapsulation

The amount of encapsulated drug was determined by running aliquots of the liposome-drug mixture over Sephadex G-50 columns, and analyzing for drug and lipid concentrations in the precolumn and collected void volumes. Lipid was measured by [^{3}H]– or [^{14}C]–CHE DPM. Tritiated vincristine counts or spectrophotometric assay at 297 nm were used to determine vincristine concentration. Typical specific activities were 100,000 DPM/mg lipid and 2,000,000 DPM/mg vincristine. Mitoxantrone, idarubicin, daunorubicin, and doxorubicin concentrations were determined by spectrophotometric assay, at 605 nm, 485 nm, 498 nm, or 480 nm, respectively. For these assays, aliquots were brought up to 100 μl with HBS then 900 μl 1% Triton X-100 was added. Samples were heated to cloud-point of the detergent, then cooled to room temperature. Absorbance at the appropriate wavelength was determined (Du®-64 spectrophotometer, Beckman Coulter, Fullerton, CA) and compared with a standard curve. For the vincristine spectrophotometric assay, samples were brought up to 200 μl with deionized, distilled water (dH_2O), then 800 μl EtOH added. Absorbance was determined, and compared with a standard curve. Radioactivity was assessed by mixing samples with Pico-Fluor 40 scintillation cocktail (Packard Bioscience B.V., Groningen, The Netherlands) and counting with a Packard 1900 scintillation counter (Packard Instrument Co., Meriden, CT).

In Vitro Vincristine Release Characteristics in the Presence of Secondary Drug

Liposomal vincristine was incubated in HBS (pH 7.5) over 2 h in the presence of added idarubicin, doxorubicin, daunorubicin, or mitoxantrone at 37°C and 60°C. These drugs were added at a final drug to lipid ratio (wt/wt) of 0.2:1.0. Drugs and lipid were measured at 5, 10, 15, 30, 60, and 120 min.

In Vivo Vincristine Release Characteristics in the Presence of Secondary Drug

Female CD1 mice were given tail vein injections of liposomal vincristine (2 mg/kg). Idarubicin was injected i.v. 30 min later at 3 mg/kg. At 30 min, and 1 and 4 h following idarubicin injection, mice were killed. Blood was collected by cardiac puncture. Plasma was isolated and vincristine and lipid levels determined.

Plasma Elimination Rate of Idarubicin in Presence or Absence of Liposomal Vincristine

Female CD1 mice were given tail vein injections of either liposomal vincristine (2 mg/kg) or saline. Thirty minutes later, mice were injected i.v. with idarubicin, at 3 mg/kg. At 15, 30, and 60 min post-idarubicin injection, blood was collected as above. Plasma lipid content was determined by radiometric assay. Idarubicin was extracted from plasma as follows: samples were adjusted to 800 μl with dH_2O, followed by addition of 100 μl 10% sodium dodecyl sulfate, and 100 μl 10 mM H_2SO_4. This was mixed, then 2 ml of 1:1 (vol:vol) isopropanol/chloroform was added. Mixtures were vortexed then frozen at –20°C overnight to promote protein aggregation. Samples were brought to room temperature, vortexed, then centrifuged at 3,000 rpm for 10 min. The fluorescent intensity of the organic layer, containing the idarubicin, was determined within 45 min, and recorded as idarubicin fluorescent equivalents. For the excitation wavelength, 500 nm was used, with a 2.5 nm bandpass; emission wavelength was 550 nm, with a 10 nm bandpass. Analysis was on the Perkin-Elmer (Canada) Ltd. LS 50 B luminescence spectrometer (Vancouver, BC), and included idarubicin spiked plasma samples for standard curve generation. Statistical analysis was performed with analysis of variance one-way post hoc comparisons.

RESULTS

In Vitro Vincristine Release Characteristics in the Presence of Secondary Drug

Liposomal vincristine was incubated with several chemotherapeutic agents at 60°C. The addition of free doxorubicin or daunorubicin caused at least 85% vincristine release from liposomes within 15 min (Fig. 1A). At 37°C, neither mitoxantrone nor doxorubicin caused any release of vincristine over the 2-h incubation (Fig. 1B). However, daunorubicin caused loss of 26% of encapsulated vincristine within 2 h. The incubation of idarubicin with liposomal vincristine caused a rapid (within 5 min) release of approximately 30% vincristine, followed by a more gradual leakage of vincristine, to about 70% loss after 2 h.

The elimination profile of the free agent must be considered in free drug/liposomal drug combinations. Injected liposomes retain a significant pH gradient, and drugs with protonizable amines could accumulate in these liposomes *in vivo* (10). Liposomal vincristine was incubated with either idarubicin (Fig. 2A) or daunorubicin (Fig. 2B). When liposome encapsulated vincristine was incubated with idarubicin there was rapid release of vincristine, paralleled by liposomal uptake of over 80% of the added idarubicin. During the 2-h incubation, approximately 70% of the vincristine was lost

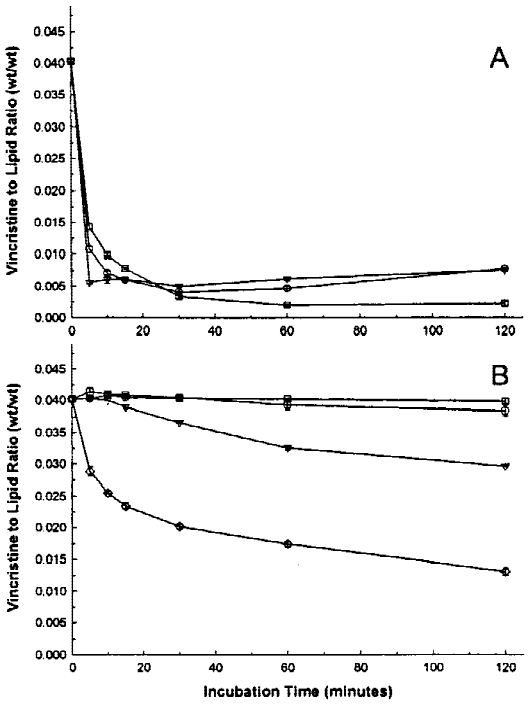

Fig. 1. Release of vincristine from liposomes on *in vitro* incubation with a second chemotherapeutic agent added at a drug to lipid ratio of 0.2:1.0 (wt/wt). (A) Incubation at 60°C with doxorubicin (○), daunorubicin (▽), or mitoxantrone (□). (B) Incubation at 37°C with doxorubicin (○), daunorubicin (▽), mitoxantrone (□), or idarubicin (◇). Error bars represent standard error of the mean from a minimum of three replicate experiments.

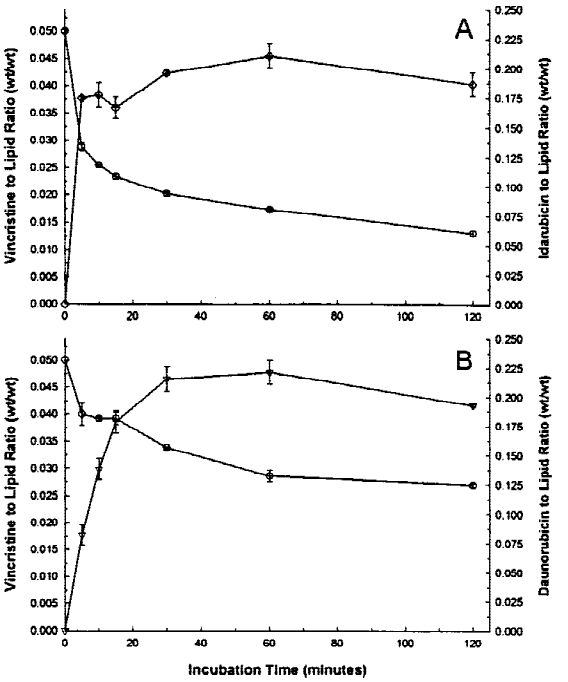

Fig. 2. *In vitro* incubation of liposomal vincristine with a second chemotherapeutic agent at 37°C, showing release of vincristine, accompanied by uptake of second drug. (A) Release of vincristine (○) and uptake of idarubicin (◇). (B) Release of vincristine (○) and uptake of daunorubicin (▽). Error bars represent standard error of the mean of data from a minimum of three replicate experiments.

from liposomes, with a concomitant uptake of greater than 99% of the added idarubicin. Daunorubicin (Fig. 2B) caused a gradual decrease in the level of encapsulated vincristine, with more than 50% vincristine remaining encapsulated following the 2-h incubation. This release was associated with the >99% uptake of daunorubicin into the liposomes, with maximal uptake achieved by 30 min of incubation.

In Vivo Vincristine Release Characteristics from Liposomes Coadministered with Nonencapsulated Anticancer Drugs

Idarubicin was chosen to evaluate the potential for interactions of free and liposomal drugs *in vivo*. Female CD1 mice were injected with liposomal vincristine at 2 mg/kg. This was followed 30 min later with an injection of idarubicin at 3 mg/kg, or saline. Blood was collected and analyzed for drug and lipid content at several time points following the second injection. The results (Fig. 3) indicate that 30 min after injection of idarubicin or saline, the amount of vincristine remaining associated with liposomes in the plasma decreased by 20%. By 24 h, the vincristine drug to lipid ratio dropped to approximately 60% of its original value and the amount of drug loss was not affected by the injection of idarubicin. These results indicate that free idarubicin, at this dose level, did not have an impact on the release of vincristine from liposomes in the plasma compartment.

Plasma Elimination Rate of Idarubicin in Presence or Absence of Liposomal Vincristine

We measured the circulating levels of idarubicin at 15, 30, and 60 min post-idarubicin i.v. injection in those mice which had been given either no pretreatment, or an injection of liposomal vincristine 30 min earlier. For those mice that received only free idarubicin, at 15 min postinjection there was slightly less than 0.5% of the initial injected dose of idarubicin remaining in the plasma (0.3 μg/ml) (Fig. 4). Those mice that received a preinjection of liposomal vincristine had 1.8% of the injected dose of idarubicin in their circulation (1.1 μg/ml), representing a 3.6-fold increase ($p = 0.000001$) in the circulating idarubicin concentrations over those mice that received idarubicin only. At 60 min postinjection, the mice receiving a preinjection of liposomal vincristine still had significantly more idarubicin in their circulations ($p = 0.02$).

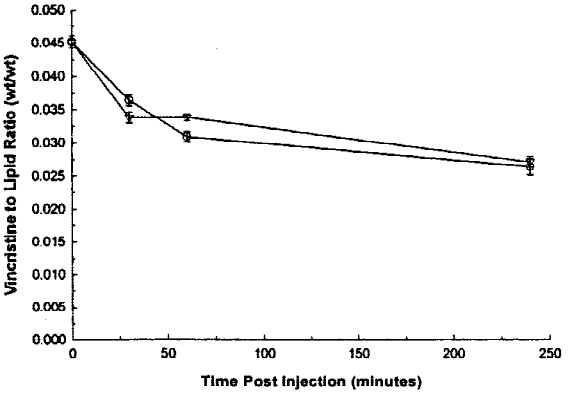

Fig. 3. Plasma vincristine to lipid ratio over time following intravenous injection of liposomal vincristine, and a follow-up i.v. injection 30 min later with saline (○) or idarubicin (▽). Error bars represent standard error of the mean of data from four animals.

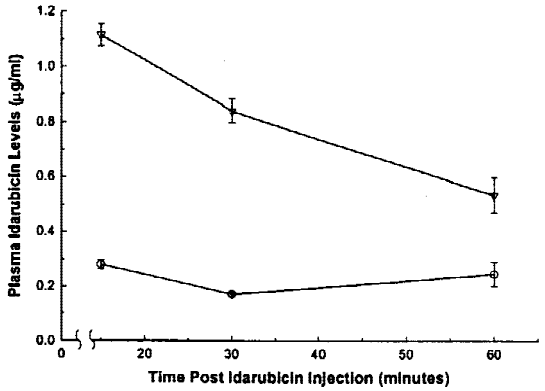

Fig. 4. Plasma idarubicin levels in CD1 mice represented as percentage of initial injected dose, either post i.v.-injection of saline, followed 30 min later by i.v. free idarubicin (○) or i.v. liposomal vincristine at 2 mg/kg followed 30 min later by injection of i.v. free idarubicin (▽). Error bars represent the standard error of the mean of data from four animals.

DISCUSSION

It is possible for independently assessed drugs to have unanticipated adverse interactions when used in combination (25). In this regard novel interactions should be considered. This may be particularly true for drug combinations that include a liposomal formulation. The potential of the liposomal drugs to change the pharmacokinetics of a coadministered free drug, and the potential for a free drug to affect the behavior of the liposomal carrier and encapsulated drug needs to be determined. To date, we are unaware of any studies assessing the interaction between liposomal and nonliposomal drugs in an *in vivo* setting.

When designed appropriately, a liposomal drug carrier will be retained in the plasma compartment for extended time periods (26–28), leading to the potential for the circulating liposomes to interact with other drugs given i.v. Interactions may involve hydrophobic interactions (similar to lipoprotein drug interactions) as well interaction via a membrane system that exhibits a transmembrane ion gradient (pH gradient) facilitating loading of the free drug into liposomes (10). These interactions, and others, have the potential to alter the pharmacokinetic properties of the free drug, the encapsulated drug, as well as the lipid carrier. These alterations may in turn, lead to decreased efficacy or increased toxicities (13).

We were interested in altered pharmacokinetic properties of either an encapsulated chemotherapeutic agent or a free drug, when these drugs were coadministered *in vivo*. There are currently many protocols in the treatment of cancer that couple the use of vincristine with doxorubicin, mitoxantrone, daunorubicin, or idarubicin (14–18). Hence, it may be expected that with the development of liposomal formulations of either vincristine, or one of these other agents, that future protocols will involve the combination of free drugs with encapsulated drugs. The results presented here demonstrate that idarubicin can elicit complete release of vincristine from DSPC/Chol liposomes incubated at 37°C *in vitro.* This is probably due to accumulation of idarubicin, driven by the residual pH gradient which was present in the liposomal vincristine formulation. This vincristine release, however, was not observed in the *in vivo* studies. The difference between the *in vitro* and *in vivo* results could be due to many reasons including: 1) reduced free drug concentration *in vivo* following rapid elimination of idarubicin; 2) idarubicin binding to serum proteins; and/or 3) protein binding mediated changes in the liposomal carrier that prevent idarubicin accumulation (29,30). The latter point was addressed through studies that measured changes in idarubicin elimination in animals that contained liposomal vincristine in the plasma compartment, results of which suggested that circulating liposomes have the potential to alter idarubicin elimination. It is not clear from these studies whether this effect was due to idarubicin binding to the liposome or drug accumulation in response to a pH gradient. The data presented here demonstrate the potential for altered release kinetics of liposomal vincristine *in vitro,* as well as uptake of free drug into liposomes preloaded with vincristine. Differential release rates of vincristine from liposomes *in vitro* following addition of a second drug are likely due to the different uptake rates of the added drugs. Further, the formation of precipitated forms of drugs, such as the anthracyclines, within the liposomes may play a role in promoting vincristine release.

We have also demonstrated *in vivo* the potential for altered pharmacokinetics of free drug when administered to mice having circulating liposomal vincristine. As drugs in combination regimens are often administered sequentially, it would be advisable to administer a liposomal formulation following free drug injection at a time point when the free drug concentrations are low to minimize drug-drug interactions. This article did not rigorously test all the potential combinations of liposomal and free drugs, nor did we address the many different dosages or schedules that may be employed in a clinical setting. These studies should be performed for each different formulation of liposomal drug, as different formulations affect the pK and release characteristics of the encapsulated drugs. However, it is intended that this article raise awareness of these issues, such that sufficient testing of coadministered drugs may be performed prior to the use of specific combination regimens in humans.

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