In Vitro and In Vivo Characterization of Doxorubicin and Vincristine Coencapsulated within Liposomes through Use of Transition Metal Ion Complexation and pH Gradient Loading

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ABSTRACT

Purpose: There is an opportunity to augment the therapeutic potential of drug combinations through use of drug delivery technology. This report summarizes data obtained using a novel liposomal formulation with coencapsulated doxorubicin and vincristine. The rationale for selecting these drugs is due in part to the fact that liposomal formulations of doxorubicin and vincristine are being separately evaluated as components of drug combinations.

Experimental Design: Doxorubicin and vincristine were coencapsulated into liposomes using two distinct methods of drug loading. A manganese-based drug loading procedure, which relies on drug complexation with a transition metal, was used to encapsulate doxorubicin. Subsequently the ionophore A23187 was added to induce formation of a pH gradient, which promoted vincristine encapsulation.

Results: Plasma elimination studies in mice indicated that the drug:drug ratio before injection [4:1 doxorubicin:vincristine (wt:wt ratio)] changed to 20:1 at the 24-h time point, indicative of more rapid release of vincristine from the liposomes than doxorubicin. Efficacy studies completed in MDA MB-435/LCC6 tumor-bearing mice suggested that at the maximum tolerated dose, the coencapsulated formulation was therapeutically no better than liposomal vincristine. This result was explained in part by in vitro cytotoxicity studies evaluating doxorubicin and vincristine combinations analyzed using the Chou and Talalay median effect principle. These data clearly indicated that simultaneous addition of vincristine and doxorubicin resulted in pronounced antagonism.

Conclusion: These results emphasize that in vitro drug combination screens can be used to predict whether a coformulated drug combination will act in an antagonistic or synergistic manner.

INTRODUCTION

Combination treatment regimes must take into consideration drug resistance mechanisms (1) and tumor heterogeneity (2). In practice, however, drug combinations usually take advantage of nonoverlapping toxicities and unique mechanisms of action. We believe that there is an opportunity to capture the therapeutic potential of drug combinations through the use of drug delivery technology. This hypothesis is supported by three lines of evidence: (a) appropriately designed drug delivery systems can improve drug delivery and efficacy for single agents (Refs. 3–5; b) polymer, protein, and lipid-based drug carriers have the capacity to coformulate two or more therapeutic agents (6–8); and (c) the pharmacokinetic behavior of the coformulated drugs will be dictated by the pharmacokinetic behavior of the drug carrier system used, thus offering the potential to coordinate the plasma elimination and tissue distribution of the combined agents.

There are a multitude of approved chemotherapy combinations that could be used to test this principle, and this report summarizes data obtained using a novel liposomal formulation with coencapsulated doxorubicin and vincristine. The rationale for selecting these drugs is due in part to the fact that liposomal formulations of the individual agents have already proven to be of clinical value in the treatment of patients with HIV-associated Kaposi sarcoma (9), ovarian cancer (10), breast cancer (11), and various hematological malignancies (12, 13), including relapsed non-Hodgkin’s lymphoma. Furthermore, these liposomal formulations of doxorubicin and vincristine are now being evaluated as components of drug combinations used routinely in the management of patients with cancer. Liposomal formulations of doxorubicin, for example, have been evaluated as part of the vincristine, doxorubicin, and dexamethasone regimens used to treat patients with multiple myeloma (14), and in the cyclophosphamide, doxorubicin, vincristine, and prednisone regimen for treatment of aggressive non-Hodgkin’s lymphoma (15). Alternatively, this regimen has been modified by incorporation of a liposomal vincristine formulation for use in the treatment of a similar patient population (16). A liposomal formulation of doxorubicin has been used in combination with cyclophospha...
mide for treatment of patients with metastatic breast cancer (17), and with cyclophosphamide and fluorouracil for first-line treatment of patients with metastatic breast cancer (18). More recently, liposomal formulations of doxorubicin have been tested in combination with novel agents targeting the multidrug resistance protein Pgp as well as with trastuzumab (Herceptin) for treatment of breast cancer patients with tumors overexpressing Her-2/neu (19, 20).

These examples highlight two points. First, liposomal formulations of anticancer drugs are gaining wider acceptance by the clinical oncology community, which is evaluating how these formulations function when used as part of combination regimens. Second, vincristine and doxorubicin (or other anthracyclines) are still in use together as part of many combination regimens. This is likely a consequence of many decades of research providing data highlighting the different modes of action and nonoverlapping toxicities of these agents.

Previous studies from our laboratory have attempted to coformulate these two drugs into a single liposomal formulation by an established transmembrane pH gradient; however, this effort proved unsuccessful because of the instability of the resulting formulation (21). More recently, a novel doxorubicin encapsulation method, which relies on drug binding to an encapsulated transition metal, manganese, has been characterized. It is proposed here that a manganese metal gradient across liposomes could be used to encapsulate doxorubicin and, in a second step, to form a pH gradient across the doxorubicin-loaded liposomes by addition of the electroneutral ionophore, A23187 (divalent cation/proton exchanger). It is demonstrated here that the resulting pH gradient can achieve the rapid accumulation of vincristine without loss of encapsulated doxorubicin. The coencapsulated formulation exhibited plasma elimination and drug release rates comparable with those observed with liposomes containing the individual active agents. Surprisingly, the coformulated combination of doxorubicin and vincristine exhibited antitumor activity that was no better than that observed using liposomal vincristine alone.

MATERIALS AND METHODS

Materials. Doxorubicin hydrochloride for injection and vincristine sulfate for injection (Faulding; Kirkland, Quebec, Canada) were purchased from the BC Cancer Agency, 1,2-Distearyloylsn-glycero-3-phosphocholine (DSPC) was obtained from Avanti Polar Lipids (Alabaster, AL). Cholesterol (Chol), A23187, Sephadex G-50, and all of the other chemicals were obtained from NEN Life Science Products (Boston, MA). [3H]Cholesterol hexadecyl ether and [14C]-cholesterol hexadecyl ether were obtained from Northern Lipids Inc. (Vancouver, British Columbia, Canada). [3H]Vincristine sulfate was obtained from Amersham Pharmacia Biotech. (Oakville, Ontario, Canada). Pico-Fluor15 and 40 scintillation fluid was purchased from Canberra-Packard (Meriden, CT). BALB/c mice and SCID-RAG-2M mice (8–10 weeks of age) were obtained from the BC Cancer Agency Joint Animal Facility breeding colony and were housed in microisolator units according to established operating procedures. The MDA435/LCC6 human breast cancer cell line was a gift from Dr. Robert Clarke (Georgetown University, Washington, DC). Cell culture medium was composed of DMEM (Stem Cell Technologies, Vancouver, British Columbia, Canada) supplemented with 10% fetal bovine serum from Hyclone Laboratories (Logan, UT), 1% l-glutamine, and 1% penicillin and streptomycin solution (Stem Cell Technologies, Vancouver, British Columbia, Canada).

All of the animal studies were done according to procedures approved by the University of British Columbia Animal Care Committee. These studies meet the requirements outlined in the current guidelines for animal use established by the Canadian Council of Animal Care.

Preparation of Liposomes. Lipids (DSPC/Chol; 55/45; mol%) were dissolved in chloroform, and [3H]CHE or [14C]-cholesterol hexadecyl ether was added to achieve ~5 μCi/100 mg lipid. The chloroform was removed under a gentle stream of nitrogen gas, and subsequently the lipid samples were placed under a high vacuum for at least 3 h to remove residual solvent. Dried lipid films were hydrated with 300 mM MnSO4 (adjusted to pH 3.5 with dilute HCl; 0.1 N) to achieve a final lipid concentration of 100 mg/ml. After hydration the multilamellar vesicles were subjected to 5 freeze-thaw cycles (freezing in liquid nitrogen and thawing at 60°C; Ref. 22). Samples were extruded 10 times through stacked polycarbonate filters with 0.1 and 0.08 μm pore size at 60°C using a water-jacketed Extruder (Northern Lipids Inc., Vancouver, British Columbia, Canada). The mean size distribution of the resulting liposome preparations ranged between 100 and 120 nm as determined by a NICOMP Submicron Particle Sizer Model 270 (Pacific Scientific, Santa Barbara, CA) with an argon laser operating at 632.8 nm.

In Vitro Drug Loading. Before the addition of drug to liposomes, a pH gradient (~3 units) and a transition metal gradient was established across liposome bilayers. To generate the gradients, DSPC/Chol liposomes in MnSO4 buffer (pH 3.5) were eluted on Sephadex G-50 columns equilibrated with a 300 mM Sucrose/20 mM HEPES/15 mM EDTA (SHE) buffer at pH 7.5. To stabilize the pH gradient across the liposomes, the divalent cation ionophore A23187 was added to some of the indicated liposome preparations (23, 24). A23187 is an electroneutral ionophore capable of translocating a divalent cation for the exchange of two hydrogen ions (24). A23187 shuttles protons to the vesicle interior in exchange for Mn2+ ions, which are subsequently chelated by the EDTA contained in the SHE buffer. As described elsewhere (24), doxorubicin was added to liposomes (with or without A23187) at 60°C, to achieve a final drug:lipid ratio (wt:wt) of 0.2:1.0. Unless otherwise stated, the final lipid concentration was 5 mg/ml, and the final doxorubicin concentration was 1 mg/ml. For the vincristine-loaded samples, vincristine in solution was added to liposomes (with A23187 ionophore), and all of the components were preincubated at the specified incubation temperature (20°C, 40°C, 50°C, or 60°C) to achieve a final drug:lipid ratio (wt:wt) of 0.05:1.0. The final lipid concentration was 5 mg/ml, and the final vincristine concentration was 0.25 mg/ml. If A23187 was used, then it was added to the liposomes before addition of drug (after the establishment of the pH and metal gradients), at an ionophore:lipid ratio of 0.2:1.0 (μg:μmol).

For experiments requiring coencapsulation of doxorubicin and vincristine, doxorubicin was encapsulated without A23187. Doxorubicin was added to liposomes at 60°C, to achieve a final...
doxorubicin/lipid ratio (wt:wt) of 0.2:1.0. The doxorubicinloaded liposomes were cooled to room temperature, and A23187 was added to achieve an ionophore/lipid ratio of 0.2:1.0 (µg: µmol). This mixture was incubated at 50°C for 5 min before vincristine addition. Vincristine in solution at 50°C was added to achieve a final vincristine/lipid ratio (wt:wt) of 0.05:1.0 and incubated for 60 min. Unless otherwise indicated, the final doxorubicin concentration was 1 mg/ml, the final vincristine concentration was 0.25 mg/ml, and the final lipid concentration was 5 mg/ml for the coencapsulated liposomes.

Accumulation of the drugs into liposomes was determined at the indicated time points by removing 100-µl aliquots and separating unencapsulated drug from encapsulated drug on 1 ml Sephadex G-50 (medium) spin columns equilibrated with SHE buffer. Sample volume was adjusted to 100 µl with SHE, to which 900 µl of 1% Triton X-100 was added, and the liposomes were disrupted with excess 1% Triton X-100. Before assessing absorbance at 480 nm, the samples were placed in a >90°C waterbath until the cloud point of the detergent was observed. The concentration of doxorubicin in the excluded fraction was determined by measuring absorbance (at 480 nm on a Hewlett Packard 8453 Spectrophotometer). [3 H]Vincristine and liposome lipid (as measured using [14C]-cholesterol hexadecyl ether) concentrations were determined by scintillation counting (Packard 1900TR Liquid Scintillation Analyzer) using the single and Full Spectrum Dual label DPM analysis counting protocols.

**Doxorubicin-Manganese Binding.** To assess doxorubicin binding to and dissociation from manganese, a spectrophotometric assay was developed. Changes in the A480 (∆A480; free doxorubicin) and ∆A550 (doxorubicin complexed to Mn2+) were determined after addition of A23187. Doxorubicin-loaded liposomes were prepared as described above (without A23187) at a final lipid concentration of 1 mg/ml. After doxorubicin loading, the sample was diluted to 0.6 mg lipid/ml, A23187 was added, and the A480/∆A500 measured. The Mn2+ complexed doxorubicin appeared purple in color on visual inspection. As the pH gradient formed, resulting in dissociation of the metal-drug complex, the color changed to an orange-red, and the Aλ maximum shifted from 550 to 480 nm.

**In Vivo Drug Release.** For in vivo studies assessing doxorubicin and vincristine release, drug-loaded liposomes were prepared such that doxorubicin was loaded at a 0.2:1.0 drug: lipid ratio (wt:wt) and vincristine was loaded at a 0.05:1.0 drug: lipid ratio (wt:wt). Coencapsulated doxorubicin and vincristine were loaded at these same ratios. For the doxorubicin loaded samples with or without A23187, doxorubicin in solution was added to liposomes and incubated at 60°C for ~15 min. The final lipid concentration was typically 12 mg/ml, and the final doxorubicin concentration was 2.4 mg/ml. For vincristine loading, vincristine in solution was added to liposomes (with A23187 ionophore:lipid ratio of 0.2:1.0 µg:µmol) to achieve a final drug:lipid ratio (wt:wt) of 0.05:1.0 and incubated at 50°C. The final lipid concentration was 12 mg/ml, and the final vincristine concentration was 0.6 mg/ml.

For the in vivo studies requiring coencapsulated doxorubicin and vincristine, liposomes were prepared as described for the in vitro studies, and the final doxorubicin concentration was 2.4 mg/ml, the final vincristine concentration was 0.6 mg/ml, and the final lipid concentration was 12 mg/ml.

All of the drug loaded samples were fractionated on a Sephadex G-50 column equilibrated with 25 mM HEPES/150 mM NaCl at pH 7.5 to remove the SHE buffer and/or the A23187 (23). The drug-loaded liposomes were diluted with 25 mM HEPES/150 mM NaCl to a lipid concentration required to administer a 10 mg/kg doxorubicin dose or a 2.5 mg/kg vincristine dose in an injection volume of 200 µl. The liposomal lipid dose was ~50 mg/kg. Formulations were injected via the lateral tail vein into 20–22-g female BALB/c mice. At 1, 4, and 24 h after injection the animals (4 mice/group) were killed by CO2 asphyxiation, and blood was collected by cardiac puncture and placed into EDTA coated microtainers. Plasma was prepared by centrifuging the blood samples at ~500 x g for 10 min. The liposomal lipid concentrations in plasma were measured on the basis of incorporated [14C]-cholesterol hexadecyl ether, which is a nonexchangeable and nonmetabolizable marker (26). Plasma vincristine concentrations were measured via the [3H]vincristine marker.

Doxorubicin was extracted by mixing the plasma with 10% SDS and 10 mM H2SO4 (1:1:1; vol:vol), diluting with H2O to 1 ml followed by organic extraction with 1:1 (vol:vol) isopropanol-chloroform (2:1; vol:vol with sample). To precipitate plasma proteins, the samples were frozen at ~80°C for 10 min at room temperature. The doxorubicin-containing organic phase was separated by centrifugation at 3000 x g for 10 min at room temperature. Fluorescence in the organic phase was determined using a Perkin-Elmer LS50B luminescence spectrometer using an excitation wavelength of 470 nm (slit width = 2.5) and an emission wavelength of 550 nm (slit width = 10). The fluorescence readings were compared with a standard curve of doxorubicin that was extracted into the organic phase using the procedure described above.

**Human Breast Cancer Xenograft Model.** Tumors were established in SCID-RAG-2M mice by a single s.c. injection of 2 x 106 MDA435/LCC6 cells with 4 mice total/group. Control mice were treated with 0.9% saline. Tumor growth was noted within 12–14 days after cell injection, and within 18 days, measurable tumors (~0.1 g) were observed. Animal weights and tumor weights were measured daily until the tumor mass exceeded 10% of the original body weight of the animals or until the tumors showed any sign of ulceration. Tumor weight was calculated as weight (g) = [(width (mm))2 x [length (mm)])/2. When the tumors reached ~0.1 g, mice were treated with doxorubicin and/or vincristine (free or liposomal form) via the lateral tail vein. Liposomal drugs were prepared as described above and exchanged into 25 mM HEPES/150 mM NaCl using column chromatography before administration at the specified doses. All of the treatments were given in 200-µl injections; therefore, when two drugs were combined the mice received two 200-µl injections separated by approximately 4–6 h with doxorubicin (free or encapsulated) injected first. Coformulated liposomal doxorubicin and vincristine was administered in one injection of 200 µl. The control group received 200 µl of sterile saline injection.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Cytotoxicity Assays and Drug Combination Studies.** Logarithmically growing MDA435/LCC6 human breast cancer cells were counted and plated onto 96-well mi-
crotiter Falcon plates at a density of $2.0 \times 10^3$ cells/well in 0.1 ml of DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin and streptomycin solution (Stern Cell Technologies, Vancouver, British Columbia, Canada). The perimeter wells of the 96-well plates were not used and contained 0.2 ml of sterile water. After 24 h at 37°C in humidified air with 5% CO2, the media were replaced with 0.2 ml of fresh media containing a range of concentrations of doxorubicin or vincristine. Drug combination studies where doxorubicin and vincristine were added simultaneously at fixed ratios of 4:1, 7:1, or 20:1 doxorubicin to vincristine (mol/mol) were also evaluated. Control cells received 0.2 ml of medium. After 72 h cell viability was assessed using a conventional MTT dye reduction assay. Fifty μl of 1.25 mg/ml MTT reagent in complete medium was transferred to each well, and the plates were incubated for 3.5 h at 37°C. The colored formazan product was then dissolved using 200 μl of DMSO. Plates were read ($A_{570}$) using a microtitr plate reader (Dynex Technologies Inc., Chantilly, VA).

The percentage of cell survival after treatment was normalized with untreated controls. All of the assays were performed at least three times in triplicate to determine the IC50. CalcuSyn software (Biosoft, Ferguson, MO) was used to analyze data from the MTT assays. The program provides a measure of whether the combined agents act in an additive, synergistic, or antagonistic manner. The combination index (CI) equation in CalcuSyn is based on the multiple drug-effect equation of Chou and Talalay (27), and defines synergism as a more-than-expected activity effect and antagonism as a less-than-expected additive effect. Chou and Talalay defined a parameter, CI, which can be used to assess synergism (CI < 1), additivity (CI = 1), or antagonism (CI > 1).

**Statistical Analysis.** A post hoc comparison of means (Scheffé test) was performed with the Statistica software package (StatSoft Inc., Tulsa, OK) on plasma elimination and tumor growth analyses involving the administration of both free and liposomal formulations of doxorubicin. Differences were considered significant at $P < 0.05$.

**RESULTS**

Coencapsulation of Doxorubicin and Vincristine into Manganese-Sulfate Containing DSPC/Chol Liposomes. The primary objective of this study was to coencapsulate doxorubicin and vincristine within one liposome population by taking advantage of two distinct loading methods. The first step relies on complexation of doxorubicin to the transition metal manganese (24, 28). The second step uses an ionophore-generated transmembrane pH gradient (23) driving the encapsulation of vincristine into the manganese-doxorubicin liposomes. Liposomes were prepared in 300 mM MnSO4 (pH 3.5) followed by the exchange of outside buffer to 300 mM sucrose/20 mM HEPES/15 mM EDTA (SHE) at pH 7.5, thereby establishing both a pH and a metal ion gradient. In this system the neutral species of doxorubicin crosses the lipid bilayer, and subsequently doxorubicin is protonated within the interior of liposomes. This causes the interior pH of the liposomes to increase because there is no entrapped solute that can buffer the change in proton concentration. When the internal pH is >6.5, the encapsulated doxorubicin can form a complex with the entrapped transition metal. The complexation reaction is readily observed as the solution turns purple when doxorubicin complexes with manganese.

The entrapment properties of this loading reaction are presented in Fig. 1A, where doxorubicin has been added to the liposomes at a drug/lipid ratio of 0.2:1.0 (wt:wt). Upon incubation at 60°C, >98% of the added drug is sequestered within the interior of the liposomes within 10 min. Doxorubicin will also load into liposomes exhibiting a transmembrane pH gradient. Thus, if A23187 is added to the MnSO4 loaded liposomes to generate a pH gradient, the resulting pH gradient is sufficient to load doxorubicin (Fig. 1B). The time course indicated that both metal complexation and pH gradient loading, >98% loading efficiencies are obtained within 10 min at 60°C for doxorubicin.

In contrast with doxorubicin, vincristine does not interact with Mn2+ in a manner that promotes drug accumulation; <5% loading was observed in the absence of the ionophore (Fig. 1C). However, after addition of A23187, the pH gradient generated promotes vincristine loading (Fig. 1D). Upon incubation with the ionophore at 60°C, >98% of the added drug was encapsulated into liposomes within 10 min.

It is known that the drug accumulation process is temperature- and time-dependent for both doxorubicin and vincristine. Efficient loading of doxorubicin into DSPC/Chol (55/45 mol%) liposomes requires an incubation temperature of 60°C (29). The influence of temperature on vincristine loading into liposomes using A23187 to generate a pH gradient is shown in Fig. 2. The results indicate that efficient vincristine loading occurs only when the incubation temperature is held between 50°C and 60°C. When the incubation temperature was 40°C, <20% of the added drug was loaded into the liposomes at the end of the 1-h time course. It is possible that reductions in vincristine loading efficiencies may be a consequence of reduced vincristine permeation across the lipid bilayer at these temperatures (30, 31); however, the temperature effects could also be attributed to reduced activity of the ionophore (32).

Considering the objective of loading both doxorubicin and vincristine into the same liposome population using two loading methods (transition metal complexation and pH gradient) and knowing that the complexation reaction between doxorubicin and manganese is dependent on pH (greatest at pH >6.5; Ref. 33), it was important to define conditions where vincristine loading occurred before dissociation of the doxorubicin-manganese complex. To determine the rate of this dissociation reaction at different temperatures, MnSO4-loaded DSPC/Chol (55/45 mol%) liposomes were prepared and incubated with doxorubicin at a drug/lipid ratio of 0.2:1.0 (wt:wt). On encapsulation of doxorubicin, we have measured the pH gradient across liposome bilayers using methylene as a pH-sensitive probe (24, 34) and have estimated the interior liposomal pH to be ~7.3 (data not shown). After drug loading, A23187 was added and the conversion of the manganese-doxorubicin complex to uncomplexed doxorubicin was followed spectrophotometrically. We have also measured existing pH gradients across the doxorubicin-loaded liposomes with added A23187 and have estimated that the interior liposomal pH is ~3.9.

The results shown in Fig. 3 were obtained by measuring the decrease in $A_{550}$ as a function of time after addition of A23187
to the doxorubicin-loaded liposomes. A23187 exchanges protons in and Mn$^{2+}$ out of the liposomes reducing the interior liposomal pH. A decrease in absorbance at 550 nm indicates the dissociation of the doxorubicin-metal chelate and reprotonation of the chromophore at the reduced pH. At 40°C (filled squares), no decrease in $A_{550}$ was observed over the 40-min time course, indicating that the manganese-doxorubicin complex was stable under these conditions. As noted in Fig. 2, vincristine does not load into these liposomes at this temperature, and it is likely that the ionophore is not functioning optimally at this temperature. When the incubation temperature was increased to 50°C (filled circle) and 60°C (filled triangle), a decrease in $A_{550}$ indicated dissociation of the manganese-doxorubicin complex. Destabilization of this complex was much slower at 50°C, with the maximal changes in $A_{550}$ observed in 30 min after A23187 addition. The visible color change from purple to orange was not seen in the sample incubated at 40°C.

When considered together, the results in Figs. 2 and 3 suggest that, at an incubation temperature of 50°C: (a) vincristine loading would occur within 40 min after A23187 addition to the MnSO$_4$ containing DSPC/Chol (55/45 mol%) liposomes; and (b) over the same time period and under the same conditions the manganese-doxorubicin complex dissociates. It can be suggested that vincristine encapsulation could be achieved in doxorubicin-loaded liposomes before or during dissociation of the manganese-doxorubicin complex. This loading reaction is shown in Fig. 4. Doxorubicin-loaded DSPC/Chol liposomes (0.2:1.0 drug:lipid ratio; wt:wt) were mixed with A23187 at 50°C just before the addition of vincristine (added at a 0.05 drug:lipid weight ratio; wt:wt). Vincristine loading was complete within 40 min with no loss of doxorubicin from the liposomes during this time frame, indicating that the encapsulation efficiency approached 100% for both drugs using the coencapsulation procedure.
capsulated Doxorubicin and Vincristine. The procedure descriptions were evaluated in these studies: (after i.v. injection are shown. Four DSPC/Chol liposomal formulations were prepared as described in “Materials and Methods,” and the outer buffers were exchanged using column chromatography to create a metal gradient. Doxorubicin was added to the liposomes to achieve a 0.2:1.0 (wt:wt) drug:lipid ratio. A23187 was added in each experiment at t = 0 and incubated at the indicated temperatures; a decrease in absorbance at 550 nm is indicative of the conversion of the manganese complex to uncomplexed oxorubicin in a low pH environment. Data points are representative of at least six replicate experiments.

In Vivo Plasma Elimination of Liposomes with Coencapsulated Doxorubicin and Vincristine. The procedure described above results in efficient encapsulation of both doxorubicin and vincristine into DSPC/Chol (55/45 mol%) liposomes. As noted elsewhere, it is possible that the presence of two encapsulated drugs may alter drug release from liposomes in the plasma compartment (21) and perhaps even the plasma elimination rate of the liposomes themselves. The plasma elimination of liposomal lipid and associated drugs was evaluated after i.v. administration in mice. These data have been summarized in Fig. 5, where liposomal lipid and drug levels at 1, 4, and 24 h after i.v. injection are shown. Four DSPC/Chol liposomal formulations were evaluated in these studies: (a) doxorubicin encapsulated without the ionophore; (b) doxorubicin encapsulated with the ionophore; (c) vincristine encapsulated with the ionophore; and (d) doxorubicin and vincristine coencapsulated as described above. BALB/c mice were injected with lipid doses of ~50 mg/kg, and doxorubicin doses of 10 mg/kg and/or vincristine doses of 2.5 mg/kg. As judged by the plasma levels of liposomal lipid (Fig. 5A), all of the liposomes were eliminated from the plasma compartment at comparable rates, although there are significantly higher levels (P < 0.016) of liposomal lipid at the 24-h time point when liposomes contained both vincristine and doxorubicin compared with either alone. It is known that doxorubicin or vincristine, when loaded into a liposomal carrier, can alter the plasma elimination of the carrier (30, 35); therefore, this result could suggest that the combination of drugs actually enhances this effect. Plasma doxorubicin levels (Fig. 5B) are consistent with previous studies where doxorubicin was eliminated more rapidly when the drug-loaded liposomes were prepared in the absence of the ionophore (filled circle). This is most evident at the 24-h time point, where the plasma doxorubicin levels are almost 10-fold lower when compared with those achieved after administration of the doxorubicin-loaded liposomes prepared with A23187 (filled square). Plasma vincristine levels (Fig. 5C) were comparable with those observed for DSPC/Chol liposomal vincristine prepared using citrate based loading methods (4). When both drugs were coencapsulated within liposomes (Fig. 5D) the plasma levels of doxorubicin and vincristine were comparable (P > 0.1181) to that observed for liposomal formulations containing only one of the agents.

Drug release from liposomes in the plasma compartment can be estimated from the data shown in Fig. 5 by calculating the drug:lipid ratios at the indicated time points. Results for the doxorubicin-loaded liposomes (single agent), shown in Fig. 6A, clearly indicate that doxorubicin release from the DSPC/Chol liposomes is faster when loading is achieved through Mn2+-doxorubicin complexation. The formulation prepared using A23187 in the procedure exhibited no measurable change in drug:lipid ratio over the 24 h time course, consistent with previous data for doxorubicin loaded into DSPC/Chol liposomes using the pH gradient procedure [encapsulated 300 mM Citrate (pH 4.0); Ref. 36]. In contrast, vincristine is readily released from DSPC/Chol liposomes over the 24-h time course (Fig. 6B), where the vincristine:lipid ratio at 24 h is 3.3-fold lower than that of the injected formulation. When the injected liposomes contain doxorubicin and vincristine (Fig. 6C), the drug release profiles are superimposable to those measured for liposomes containing the single agents encapsulated using A23187.

![Fig. 3](Image) Changes in A550 (ΔA550) of doxorubicin loaded 1,2-dioleoyl-sn-glycero-3-phosphocholine/cholesterol liposomes after the addition of the ionophore A23187 at 40°C (■), 50°C (●), and 60°C (▲). Liposomes were prepared as described in “Materials and Methods,” and the outer buffers were exchanged using column chromatography to create a metal gradient. Doxorubicin was added to the liposomes to achieve a 0.2:1.0 (wt:wt) drug:lipid ratio. A23187 was added in each experiment at t = 0 and incubated at the indicated temperatures; a decrease in absorbance at 550 nm is indicative of the conversion of the manganese complex to uncomplexed doxorubicin in a low pH environment. Data points are representative of at least six replicate experiments.

![Fig. 4](Image) Doxorubicin and vincristine encapsulation within 1,2-distearoyl-sn-glycero-3-phosphocholine/cholesterol (55/45 mol%) liposomes. Liposomes were prepared as described in “Materials and Methods,” and the outer buffers were exchanged using column chromatography to create a metal gradient. Doxorubicin (○) was added to the liposomes to achieve a 0.2:1.0 (wt:wt) drug:lipid ratio and incubated at 60°C. A23187 was added to the doxorubicin-loaded liposomes and incubated 5 min before the addition of vincristine (■), which was added to achieve a final drug:lipid ratio of 0.05:1.0 (wt:wt). Vincristine loading was completed using an incubation temperature of 50°C. Doxorubicin was quantitated by the A550 (ΔA550) at 550 nm is indicative of the conversion of the manganese complex to uncomplexed doxorubicin in a low pH environment. Data points are representative of at least six replicate experiments.
Efficacy of the Coencapsulated Liposomal Doxorubicin/Vincristine Formulation Against a Human Breast Cancer Xenograft Model. Vaage et al. (37) have evaluated previously the therapeutic activity of vincristine and doxorubicin encapsulated in sterically stabilized liposomes. In these studies the drugs were not coencapsulated, and the drugs were used alone and in combination to treat murine MC2 tumors. The liposomal drugs were more active than free agents; however, when they were used in combination the liposomal vincristine

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**Fig. 5** The lipid and drug plasma elimination profiles of drug-loaded 1,2 distearoyl-sn-glycerol-3-phosphocholine/cholesterol (55/45 mol%) liposomes after i.v. administration into female BALB/c mice. Liposomes were loaded with doxorubicin, vincristine, or both. Loaded liposomes were adjusted to a concentration such that 10 mg/kg doxorubicin or 2.5 mg/kg vincristine could be administered in an injection volume of 200 μl. A, plasma lipid levels in mice administrated either doxorubicin-loaded liposomes using the manganese sulfate loading procedure (●), doxorubicin-loaded liposomes using the manganese sulfate loading procedure with A23187 ionophore (■), vincristine-loaded liposomes using the manganese sulfate loading procedure with A23187 ionophore (▲), or simultaneously loaded doxorubicin and vincristine liposomes (▼). Lipid levels were determined using: [1H]-cholesteryl hexadecyl ether or [14C]-cholesteryl hexadecyl ether as a liposomal lipid marker. B, the level of doxorubicin fluorescent equivalents in plasma from mice administrated doxorubicin-loaded liposomes using the manganese sulfate loading procedure with (●) or without (○) the A23187 ionophore. C, vincristine levels in plasma were determined from mice that received vincristine-loaded liposomes prepared using the manganese sulfate loading procedure with A23187 ionophore (●). D, doxorubicin (▲) and vincristine (▼) levels were also measured in plasma of mice receiving coencapsulated liposomes. Data points represent mean drug:lipid ratios; bars, ± SD (n = 6). A one-way ANOVA analysis was performed to assess differences among the treatment groups. A P < 0.05 was considered significant.

**Fig. 6** In vivo release of doxorubicin and vincristine from 1,2 distearoyl-sn-glycerol-3-phosphocholine/cholesterol (55/45 mol%) liposomes. Panels represent drug:lipid ratios calculated from Fig. 5. Liposomes containing (A): doxorubicin encapsulated using the manganese-sulfate procedure either without A23187 (●); or with A23187 (■); B, vincristine encapsulated using the manganese-sulfate procedure with A23187 (●); C, doxorubicin (▲) and vincristine (▼) coencapsulated using the manganese-sulfate procedure as described in Fig. 4 after i.v. administration to female BALB/c mice. Drug-loaded liposomes were adjusted to a concentration such that a dose of 10 mg/kg doxorubicin and/or 2.5 mg/kg vincristine could be administered in an injection volume of 200 μl. Drug levels in the plasma were assessed via measurements of doxorubicin fluorescent equivalents or the [1H]vincristine sulfate marker. Data points represent mean drug:lipid ratios; bars, ± SD (n = 6).
and liposomal doxorubicin did not provide improved therapy when simultaneously injected (37). Their results suggested that liposomal vincristine inhibited the activity of liposomal doxorubicin. These authors proposed that reduced activity could have been a consequence of the effects of vincristine on the cell cycle, which prevented delayed progression into S phase in which the impact of doxorubicin could be greatest (37). Alternatively, they argued that inhibition of activity could have been a consequence of dosing schedules and liposome-mediated drug delivery, where liposomal vincristine administration may have interfered with the delivery of liposomal doxorubicin to the tumor. This later concern would not arise if the drugs were coencapsulated; therefore, a therapeutic assessment of this liposomal formulation is described here.

Efficacy was determined in SCID mice bearing solid tumors derived from the s.c. injection of MDA435/LCC6 human breast cancer cells. This is an aggressive breast tumor model that has been described elsewhere (38). The results of these studies are summarized in Table 1. Complete dose titrations were completed; however, the activity observed at the maximum tolerated dose of drug (2.5 mg/kg vincristine +10 mg/kg doxorubicin) resulted in only a 32% delay in tumor growth; thus, only the results obtained at the maximum tolerated dose are presented here. Tumor growth delay values calculated as the time in days for the tumors to reach 0.2 g. Results were averaged from at least 4 mice, and a one-way ANOVA analysis was performed to assess differences among the treatment groups. A P of <0.05 was considered significant.

**In Vitro Cytotoxicity Analysis of Doxorubicin and Vin- cristine Alone and in Combination.** Results presented here as well as those of Vaage et al. (37) suggest that coadministration of liposomal doxorubicin and liposomal vincristine results in less than additive activity or antagonism. In an effort to gain a better understanding about the cytotoxic effects of vincristine and doxorubicin when used in combination, in vitro cytotoxicity studies were completed using MDA435/LCC6 cells as the target cell population. Cytotoxicity effects were determined using the multiple drug-equation developed by Chou and Talalay (27). This method is most readily applied to dose titration data collected for individual agents alone, and the combination of drugs added at fixed ratios and over a broad range of effective doses.

In this study the vincristine:doxorubicin ratios were selected to reflect the drug ratios measured in the plasma after i.v. administration. Three fixed vincristine:doxorubicin ratios of 1:4, 1:7, and 1:20 (mol:mol) were evaluated against the MDA435/ LCC6 cell line. The effects of the drugs alone and in combination were evaluated after a 72-h drug exposure. The MTT results (Fig. 7) were completed in triplicate, three separate times. The resulting data points at each concentration were then analyzed using the CaluSyn Software program. This software package takes the nonlinear dose response curves and applies an unweighted linear regression analysis. Our analysis, which assumed mutually exclusive interactions, provided a correlation coefficient of >0.9 for all of the data sets. The regression analysis provided estimations of the drug concentrations required to inhibit cell growth/proliferation at a variety of effect levels. The IC90 of vincristine alone was ~3.7 nM, whereas doxorubicin was 10 μM (Fig. 7). These data demonstrate that the
Coencapsulation of Doxorubicin and Vincristine

These cells were exposed to doxorubicin (Dox) and vincristine (Vinc) separately and combined in three different ratios (1) 4:1 doxorubicin (DOX):vincristine (VINC) ratio (mol:mol), (2) 7:1 DOX:VINC ratio (mol:mol), and (4) 20:1 DOX:VINC ratio (mol:mol).

The rationale for selecting vincristine and doxorubicin for coencapsulation was based on the fact that these two drugs are commonly used in combination chemotherapy. The drugs exhibit different dose-limiting toxicities and different mechanisms of activity. Because liposomal forms of vincristine and doxorubicin are more active than the respective free drugs, it was anticipated that combining the two liposomal drugs would be beneficial. One significant disadvantage of administering individually encapsulated doxorubicin and vincristine is the potential interference each liposome population may exert on the pharmacokinetic profile of the other, thereby altering drug delivery to tumor cells. We attempted to address this problem with coencapsulation, which would make this an unlikely issue. However, it should be noted that a previous study demonstrated that simultaneous administration of separately encapsulated liposomal doxorubicin and liposomal vincristine exhibited activity no better than that achieved for liposomal doxorubicin alone (37). An explanation as to why codeelivery of vincristine and doxorubicin may result in less than additive activity can be developed when considering the mechanisms of action of the free drugs. It is established that free doxorubicin has some activity against cells in various phases of the cell cycle, but its therapeutic activity of the combination would be dominated by the activity of vincristine. The estimated drug combination concentrations required to achieve 90% inhibition of cell growth/proliferation at the 1:4 vincristine:doxorubicin ratio (mol:mol) indicate that the amount of vincristine required was comparable with that needed when vincristine was used alone (~3 nM). As the ratio changed to 1:20 vincristine:doxorubicin (mol:mol), the estimated amount of the drugs required to achieve 90% cell kill increased substantially for both vincristine and doxorubicin. Specifically, the IC$_{50}$ observed was 4.3 μM for vincristine and 86 μM for doxorubicin.

These data reveal the presence of strong antagonism for this drug combination, and this was reflected in the CI values defined by the CalcuSyn Software (39). It should be noted that a CI value of 0.9–1.1 is indicative of additive activity, whereas CI values <0.9 indicate drug synergy and values >1.1 indicate antagonism. When analyzed in this manner, the combination of vincristine and doxorubicin, at all of the ratios studied, exhibited CI values >1.1 over the entire range of effective concentrations. At the 1:20 vincristine:doxorubicin (mol:mol) the CI value was >20, indicative of strong antagonism.

**DISCUSSION**

The results summarized in this report can sustain two avenues of discussion. Of practical significance, a procedure to coencapsulate two anticancer drugs into a single liposomal formulation has been delineated. However, data are provided that clearly demonstrate that the coencapsulated vincristine and doxorubicin formulation exhibits antagonistic effects. This discussion will consider the potential for encapsulating multiple drugs into liposomes using distinct loading methods as well as providing a rationale as to why the coencapsulated vincristine/doxorubicin formulation exhibited antagonistic effects.

Our initial efforts to encapsulate two drugs into a single liposome population using pH gradient-mediated approaches was hindered in part because the stability of these formulations was poor both in vitro and in vivo (21). More recently our group (24) has defined a unique drug loading method that relies on the formation of a manganese-doxorubicin drug complex rather than a transmembrane pH gradient. Applying this new method to the problem of coencapsulating two anticancer drugs, it is demonstrated here that a stable formulation at drug:lipid ratios shown to be therapeutically relevant (4, 29) can be easily prepared. An established manganese metal gradient across liposomes permitted the efficient loading of doxorubicin (Fig. 1A) due to formation of a drug-metal complex. Sufficient levels of uncomplexed Mn$^{2+}$ remained to support the establishment of a transmembrane pH gradient after addition of A23187, an electroneutral divalent metal/proton pump. The pH gradient formed supported the efficient loading of vincristine. The methods described here are generally applicable to many drugs that have chemical groups capable of complexing transition metals like Mn$^{2+}$ [for example anthracyclines (40, 41), camptothecins (42, 43), and anticancer antibiotics such as bleomycin (44, 45)] and second agents that can be encapsulated using pH gradients (for example mitoxanthrone, camptothecins, and vincalkaloids; Refs. 5, 23, 46, 47). Plasma elimination data suggest that the release rates of coencapsulated drugs observed are consistent with what would be expected for the drugs encapsulated into DSPC/Chol liposomes using pH gradient loading methods (see Figs. 5 and 6). The doxorubicin and vincristine drug release profiles from individually loaded DSPC/Chol liposomes (Fig. 6, A and B) are not significantly different from the drug release rates observed in the coencapsulated formulation (Fig. 6C). This suggests that the drugs are not interacting in a manner that affects their release from the liposomes.
maximal effects occur in early S phase. This action causes a delay in the progression through all phases of the cell cycle except M→G1 (48, 49). Vincreistine causes cells to accumulate in mitosis (50–52). On the basis of this information, it is reasonable to suggest that these two drugs could interfere with each other’s action. The Chou and Talalay analysis of vincreistine and doxorubicin combinations at fixed ratios (where the drugs are added simultaneously) clearly indicated that this drug combination is antagonistic (Fig. 7). These findings (Fig. 7; Table 1) indicate that drug combinations selected on the basis of nonoverlapping toxicity and unique mechanisms of action can provide less than optimal therapeutic effects, even when the pharmacokinetics and therapeutic properties of the individual drugs are improved through use of carefully designed drug carriers.

Accepting the concerns identified above for coencapsulated vincreistine and doxorubicin, there is also a tremendous opportunity to define strategies where the drugs encapsulated within the liposomal formulation are selected using arguments that consider the potential for drug combinations to exhibit supra-additive activity or synergy. On the basis of evidence supporting the mechanism of selected agents on tumor cells, it should be possible to better rationalize the design of a formulation with an effective drug combination. The use of multiple agents has to date been developed based on pragmatic principles, addressing issues of overlapping toxicities, drug resistance, and tumor cell heterogeneity. Yet there are now a variety of in vitro assays, like the Chou and Talalay analysis used here, that can establish whether two drugs act synergistically or antagonistically when used in combination (53). Although these in vitro assays can provide information on drug sequencing effects, typically the drugs are added simultaneously to the cells in culture. Given the fact that we have established a method whereby two agents can be efficiently loaded into a liposomal formulation, which, when given i.v., will dictate the plasma elimination and biodistribution of both agents, it is now reasonable to consider combining data obtained from in vitro synergy assays with liposomal drug formulation methods to define carrier systems capable of delivering coencapsulated drugs in a synergistic manner. Importantly, it is also reasonable to consider the potential of formulated drug combinations, where the drugs selected are encapsulated in different liposomes, each optimized for the individual agents. These could then be combined and administered as a single drug product. If this strategy was pursued it would be important to demonstrate that the pharmacokinetic and biodistribution attributes of the different liposomal carriers are comparable.

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In Vitro and in Vivo Characterization of Doxorubicin and Vincristine Coencapsulated within Liposomes through Use of Transition Metal Ion Complexation and pH Gradient Loading

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