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Research paper

In vitro and *in vivo* characterization of a combination chemotherapy formulation consisting of vinorelbine and phosphatidylserine

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Abstract

The purpose of these studies was to design an intravenous drug formulation consisting of two active agents having synergistic *in vitro* activity. Specifically, we describe a novel drug combination consisting of a cytotoxic agent (vinorelbine) with an apoptosis-inducing lipid (phosphatidylserine, PS). *In vitro* cytotoxicity screening of PS and vinorelbine, alone and in combination, against human MDA435/LCC6 breast cancer and H460 lung cancer cells was used to identify the molar ratio of these two agents required for synergistic activity. PS and vinorelbine were co-formulated in a lipid-based system at the synergistic molar ratio and the pharmacokinetic and antitumor characteristics of the combination assessed in mice bearing H460 tumors. The cytotoxicity of the lipid, and the synergy between the lipid and vinorelbine, were specific to PS; these effects were not observed using control lipids. A novel formulation of PS, incorporated as a membrane component in liposomes, and encapsulating vinorelbine using a pH gradient based loading method was developed. The PS to vinorelbine ratio in this formulation was 1/1, a ratio that produced synergistic *in vitro* cytotoxicity over a broad concentration range. The vinorelbine and PS dual-agent treatment significantly delayed the growth of subcutaneous human H460 xenograft tumors in Rag2M mice compared to the same dose of free vinorelbine given alone or given as a cocktail of the free vinorelbine simultaneously with empty PS-containing liposomes. These studies demonstrate the potential to develop clinically relevant drug combinations identified using *in vitro* drug–drug interactions combined with lipid-based delivery systems to co-formulate drugs at their synergistic ratios.

Keywords: Combination therapy; Synergy; Phosphatidylserine; Vinorelbine; Drug delivery

1. Introduction

It is widely recognized that the transformation of normal cells to the neoplastic state involves multiple molecular lesions [1]. Importantly, these molecular alterations may involve parallel pathways such that therapeutic strategies directed against a single target of any given pathway may not be sufficient to achieve control over tumor cell proliferation and survival. In this regard, many investigators have pursued the use of drug combinations, targeting two or more deregulated pathways within a cancer cell population, in anticipation that effective treatment will require the use of therapies that act in an additive or greater-than-additive (i.e., synergistic) manner [2,3]. We are developing drug carrier formulations that incorporate multiple

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therapeutic agents known to exhibit synergistic anticancer activity as determined by *in vitro* cell-based screening assays [4]. Given the clinical success of lipid-based drug carrier systems for improving the therapeutic effects of anticancer drugs such as doxorubicin [5], daunorubicin [6], mitoxantrone [7], vincristine [8] and lurtotecan [9] as well as the capacity of these formulations to incorporate more than one therapeutic agent [10], it is reasonable to pursue this concept by developing liposomal formulations that incorporate two or more therapeutic agents. We believe that there is considerable potential for developing combinations of a cytotoxic agent entrapped in liposomes that incorporate therapeutically active lipids. In this paper, we have characterized a formulation containing the antimitotic drug vinorelbine and the apoptosis-inducing lipid phosphatidylserine (PS).

PS is an anionic phospholipid involved in one of the early triggering events of apoptosis [11]. PS is normally sequestered on the inner leaflet of the plasma membrane, but becomes exposed on the external surface of the cell when the execution phase of apoptosis is initiated, as would be the case when caspase activation has occurred [12]. It has been suggested that PS externalization occurs as a mitochondrial-dependent event and that other apoptotic signaling pathways may not cause translocation of the PS from the inner to outer leaflet of the plasma membrane [13]. It has also been shown that caspase inhibitors fail to prevent PS externalization in lymphocytes following exposure to apoptosis promoting drugs [14]. Thus, the role of PS in the apoptotic process remains controversial.

While it could be viewed that PS externalization is a consequence, but not a cause of apoptosis, several studies have shown that treatment with PS initiates apoptosis and cell death, and this cytotoxicity was not observed for other anionic or neutral phospholipids, [15–17]. In addition, PS has been reported to inhibit the incorporation of thymidine into L1210 leukemia cells, but inhibition was not observed for another anionic lipid, phosphatidic acid (PA) [18]. PS-containing liposomes were significantly more toxic to a panel of human cancer cells than were liposomes composed of phosphatidylcholine (PC) [19]. PS in liposomes was also reported to inhibit the proliferation of the MOLT-4 human leukemia cell line [20]. Therefore, regardless of what induces PS externalization, the presence of this anionic lipid on the outside of tumor cells can result in both direct and indirect killing of cancer cells. The latter may be due to enhanced recognition of tumor cells by the immune system. This recognition process may be mediated by a putative PS-receptor, a protein expressed on the plasma membrane of numerous cells [21], which has been reported to facilitate the phagocytosis of cells with externally exposed PS by activated macrophages [22].

Since PS can be readily incorporated into lipid-based formulations of anticancer drugs, we have explored the potential for using PS as an apoptosis-promoting lipid in combination with the anticancer drug vinorelbine. Vinorelbine is a very potent antimicrotubule agent that results in cell-cycle arrest in M phase and is also associated with the induction of apoptosis [23]. More specifically, it has been suggested that microtubule depolymerizing drugs such as vinorelbine can trigger changes in intracellular signaling pathways known to promote bcl-2 phosphorylation and, subsequently, apoptosis [23]. It has also been reported that the ability of vinorelbine to induce apoptosis is reduced in mitotic spindle checkpoint-proficient cell lines [24] and argued that activation of the spindle assembly checkpoint provides a means to protect cancer cells against vinorelbine-induced cytotoxicity. The relationship between the spindle checkpoint and cell survival signals is becoming better understood and it appears that cancer cells frequently over-express survivin, a microtubulin-associated protein that acts as an apoptosis inhibitor and mitotic regulator [25]. With this in mind, it is reasonable to suggest that the therapeutic activity of drugs like vinorelbine could be enhanced when combined with an apoptosis-promoting agent that acts independently of microtubule function and stability.

In this study, we have extended earlier reports demonstrating PS-mediated cytotoxicity and provided new data demonstrating that PS potentiates or synergizes with the antitumor activity of vinorelbine in H460 human lung cancer cells *in vitro*. These data were used to help design a lipid-based delivery system with PS and vinorelbine. The resulting co-formulated PS/vinorelbine exhibited significant antitumor activity in the H460 xenograft tumor model established in Rag2M mice.

2. Methods and materials

2.1. Materials, cell lines and animals

The NIH 3T3, HL60, H460, P388 cell lines were obtained from the National Cancer Institute. MDA-4335/LCC6 human breast cancer cells were obtained from Dr. Robert Clarke, Georgetown University Medical School, Washington DC. Female Rag2M mice were obtained from Taconic (Hudson, NY). All animal protocols were approved by the University of British Columbia Institutional Animal Care Committee and all studies were performed according to guidelines of the Canadian Council of Animal Care in the Joint Animal Facility of the British Columbia Cancer Research Center.

The PS-receptor antibody (Mab 217) was purchased from Cascade Biosciences (Winchester, MA). PS, PA, PC, phosphatidylglycerol (PG), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(poly(ethylene glycol))-2000] (PEG_{2000}-DSPE), sphingomyelin (SM) and cholesterol (Chol) were obtained from either Northern Lipids (Vancouver, Canada) or Avanti Polar Lipids (Alabaster, AL). Vinorelbine ditartrate (Navelbine) is a product of Glaxo Wellcome (Mississauga, Canada). Tritium-labeled cholesterol hexadecyl ether (^{3}H-CHE) was obtained from New England Nuclear and ^{14}C-dioleoyl-PS (^{14}C-DOPS) was obtained from

Amersham. All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. In vitro cytotoxicity assays

The standard 3-(4,5-diethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric cytotoxicity assay was utilized [26]. MDA-435/LCC6 human breast cancer cells or H460 human lung carcinoma cells (passage number <20) were grown in RPMI medium at 10^4 cells/mL and added into 96-well cell culture plates at 10^3 cells/well. The cells were incubated for 24 h at 37 °C, with 5% CO_2. Serial dilutions of the drugs in RPMI medium were added as follows. Liposomes, prepared as described below, and/or vinorelbine were added directly to the wells; dual-agent combinations were mixed at a specific molar ratio and added in volume increments of 20 μL using a Latin square design or "checkboard" dilution method. The total well volumes were made up to 200 μL with fresh RPMI media.

Cells were treated with the single-agents, or drug combinations, for 72 h and then 50 μL MTT reagent (1 mg/mL in RPMI) was added to each well. MTT was allowed to incubate with the cells for 3–4 h then the well contents were aspirated and 150 μL of dimethylsulfoxide (DMSO) was added to each well. After agitation of the 96-well plates to solubilize the cells, they were read on a microplate spectrophotometer set at 570 nm. The optical density readings were recorded and the corresponding values of the blank wells (containing just media) were subtracted from all the wells containing cells. Cell survival at the end of treatment was calculated as a percentage of the control wells. All assays were performed in triplicate.

2.3. Median-effect analysis for drug combinations

For the drug combination interaction analysis, CalcuSyn (Biosoft, Ferguson, MO, USA), a software program based on the median-effect principle described by Chou and Talalay [4,27], was used. The fixed ratios for the dual-agent combinations were initially derived from the ratios of the IC_{50} values from single-agent cytotoxicity profiles. Subsequently, other fixed ratios were chosen based on considerations for developing a co-formulated drug product as described below. Using the mean cell survival percentages from the MTT assay as a function of drug concentration(s), the CalcuSyn program provides a measure of whether the combined agents interact in an additive, synergistic or antagonistic manner. Specifically, CalcuSyn produces a combination index (CI) value, based on the median-effect principle, which defines the interaction between two or more agents as being synergistic ($CI < 1$), additive ($CI = 1$) or antagonistic ($CI > 1$). The CI value is dependent on the proportion, or fraction, of cells that have been affected by treatment; the "f_a" value. For example, in a cytotoxicity assay the survival of 25% of the cells would indicate death of 75% of the cells, representing an f_a value of 0.75.

2.4. FACS assay for PS-receptor status

The PS-receptor levels on cells were determined by staining with mouse monoclonal IgM kappa-chain antibody against the PS-receptor (Mab 217; Cascade Biosciences, Winchester, MA) and a secondary goat anti-mouse IgM-Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA). Controls included both receptor-positive (NIH 3T3) and receptor-negative cells (HL60). To control for non-specific binding, each cell line was also stained with an isotype control group consisting of myeloma IgM isotype control (Sigma–Aldrich, St. Louis, MO) and staining with secondary antibody as well as a secondary antibody-only control group. Tissue culture cells were obtained from flasks of exponentially growing cells. Suspension cells (HL60 and P388) were collected by centrifugation and washed twice with Hanks' balanced salt solution (HBSS) containing 0.2% FBS, without phenol red, before exposure to the antibody. Subconfluent monolayer cells (NIH 3T3 and H460) were rinsed with HBSS, without Ca^{2+} and Mg^{2+}, and then harvested in HBSS containing 1 mM EDTA at room temperature. Cells were collected by centrifugation, washed and re-suspended in HBSS with Ca^{2+}, Mg^{2+} and 0.2% FBS but without phenol red. P388 cells were recovered from ascitic tumors growing in BDF-1 mice by peritoneal lavage. Before staining, the ascites fluid was depleted of macrophages by culturing overnight in plastic tissue culture plates.

Cells were stained as follows. Approximately 10^6 cells were suspended in 100 μL of HBSS and incubated for 20–30 min with either Mab 217 at 100 μg/mL or isotype control at 30 μg/mL. The concentration for the Mab 217 antibody was based on titration to achieve matched background staining with the isotype control antibody in the PS-receptor negative HL-60 cells and an optimal signal-to-noise ratio in the PS-receptor positive H460 cells. The concentration of the isotype control antibody was recommended by the manufacturer based on their in-house testing. Cells were washed twice by centrifugation and re-suspension in HBSS then incubated with the goat anti-mouse IgM-Cy3 secondary antibody at the recommended dilutions for a further 20–30 min. Cells were washed two more times by centrifugation and re-suspension in HBSS then prepared for FACS by suspension in 300 μL of phosphate-buffered saline containing 2% FBS. FACS analysis was performed using Epics Elite ESP flow cytometer (Beckman Coulter, Miami, FL) equipped with INNOVA Enterprise 621 laser (Coherent, Santa Clara, CA).

2.5. Preparation of empty liposomes for in vitro cytotoxicity assays

Liposomes for *in vitro* cytotoxicity assays were prepared from the lipids dimyristoyl-PC (DMPC), dimyristoyl-PS (DMPS), dilauroyl-PS (DLPS), palmitoyl-oleoyl-PS (POPS), dioleoyl-PS (DOPS), dipalmitoyl-PS (DPPS), distearoyl-PS (DSPS), dilauroyl-PG (DLPG), distearoyl-PG (DSPG), dimyristoyl-PA (DMPA), dilauroyl-PA

(DLPA). Lipids were dissolved in chloroform/methanol (95/5, v/v) and combined at the indicated molar ratios. Trace quantities (0.5 μCi) of the radioactive lipid ^{3}H-CHE were added to serve as a marker to quantify total lipid [28]. The bulk solvent mixture was removed under a stream of N_2 gas then residual solvent was removed under high vacuum overnight. The lipid films were hydrated by the addition of 20 mM Hepes 150 mM saline, pH 7.4, to achieve 50 mg lipid/mL, and then vortexed extensively. The resulting multilamellar vesicles were extruded 10 times through two 100 nm filters using a Thermobarrel Extruder (Northern Lipids, Vancouver) at 42 °C (DMPC and 80% DMPC-containing liposomes) or 65–70 °C (DLPS, DPPS, DSPS, DOPS, POPS, DSPG and DLPG liposomes) [29] to produce large unilamellar liposomes. Cytotoxicity was determined relative to the concentration of PS or PA in the added liposomes.

2.6. Preparation of co-formulated PS/vinorelbine liposomes

Liposomal formulations consisted of mixtures of the lipids distearoyl-PC (DSPC) or SM, Chol, PEG_{2000}-DSPE and either POPS, DOPS, DPPS or DSPS, in the specified molar ratios. Individual lipid stock solutions (chloroform at 100 mg lipid/mL) were combined in the appropriate molar proportions for each formulation as specified. For lipid mixtures containing either DPPS or DSPS, the total lipid was dissolved to 25 mg/mL in chloroform/methanol/water/0.3 M citrate (pH 4.0) [12/6.3/0.6/0.6, by volume]. The radioactive lipid ^{3}H-CHE was added to quantify total lipid [28] and trace quantities of ^{14}C-DOPS were added to quantify PS. The bulk solvent mixture was removed under a stream of N_2 gas then residual solvent was removed under high vacuum overnight. The resulting lipid films were hydrated by addition of 0.3 M citrate buffer (pH 4) to achieve a final lipid concentration of 25 mg/mL. After extensive vortexing, the resulting multilamellar vesicles were extruded 10 times through two 100 nm filters using a Thermobarrel Extruder (Northern Lipids, Vancouver) at 65–70 °C [29] to produce large unilamellar liposomes. Liposome sizes were measured using a NICOMP 270 submicron particle sizer (Particle Sizing Systems, Santa Barbara, CA).

Vinorelbine was loaded into these formulations using the pH gradient loading method [30] as follows. A known amount of lipid was mixed with the amount of vinorelbine necessary to achieve a vinorelbine/lipid ratio of 0.1/1 (w/w). At this drug/lipid ratio, the molar ratio of PS/vinorelbine base in the formulation was 1/1. This mixture was equilibrated at 60 °C for 15 min, then vinorelbine loading was initiated by the addition of 0.2 M Na_2HPO_4 (10 times the volume of the citrate buffer) to establish a transmembrane pH gradient. Vinorelbine loading into the liposomes was allowed to proceed for 10–60 min at 60 °C. Quantitative drug loading into the formulations was confirmed by centrifuging 100 μL of the sample over a 1 mL Sephadex G-50 size exclusion column and then measuring the eluant for lipid (using liquid scintillation counting, LSC) and vinorelbine (absorbance at 270 nm). The detergent *n*-octyl glucopyranoside was used to solubilize the vinorelbine loaded lipid formulations.

2.7. Analytical methods

Vinorelbine concentrations in plasma were determined by high performance liquid chromatography (HPLC) following solid-phase extraction by modification of the method for quantification of vincristine sulphate in human plasma [31]. Each plasma sample contained 20 μg/mL vincristine as an internal standard prepared by mixing 80 μL of plasma sample with 20 μL of a 0.1 mg/mL vincristine stock solution. To each of the experimental samples and plasma standards, 100 μl of cold (−20 °C) HPLC-grade methanol was slowly added during continuous vortexing. Samples were then centrifuged at 10,000 rpm for 10 min.

Solid-phase extraction cartridges (Oasis® HLB, 1 mL, 10 mg, Waters, Milford, MA) were used for preparation of plasma samples prior to HPLC analysis. After conditioning of the cartridges with 1 mL of HPLC-grade methanol followed by 1 mL of HPLC-grade water, the plasma extracts were loaded onto individual cartridges and then washed with three 1.0 mL aliquots of HPLC-grade water and dried under vacuum (approximately 5 in. Hg) for 1 min. The analytes were eluted with 1 mL of methanol into 16 × 100 mm disposable glass culture tubes then evaporated to dryness under a nitrogen stream at approximately 40 °C. All plasma extracts were reconstituted in 100 μL of mobile phase (25 μL of 1.428% (v/v) diethylamine (pH 7.5) plus 75 μL of methanol) by vortexing for 15 s.

The HPLC system consisted of a Waters Model 717 plus autosampler, a Model 600E pump and controller and a Model 996 Photodiode Array Detector (Waters, Milford, MA, USA). Data were acquired and processed with the Millennium32® chromatography manager (Version 3.20, Waters). Separation was carried out using a Symmetry® C8 cartridge column (10 nm pore size, particle size 5 μm; 250 × 4.6 mm I.D., Waters) with a Symmetry® Sentry™ C8 guard column (particle size 5 μm; 20 × 3.9 mm I.D., Waters). The column temperature was held constant at 30 °C. The mobile phase consisted of methanol (1.428%, v/v)/diethylamine (adjusted to pH 7.5 with *o*-phosphoric acid) (75/25, v/v), filtered through 0.45- and 0.22-μm filters, respectively, and degassed. The flow rate was 1.0 mL/min; peak detection was performed at 270 nm (i.e., λ_{max}). For sample analysis, 70 μL of each reconstituted sample and aqueous standard was loaded into 1-mL HPLC sample vials (Waters) with 200-μL inserts (Chromatographic Specialties Inc., Brockville, Ont., Canada), and 50 μL was injected onto the column. The autosampler temperature was set to 5 °C. Complete sets of eight plasma standards and aqueous standards were prepared prior to each analysis on the same day. Calibration curves were obtained by plotting the vinorelbine:internal standard chromatographic peak area ratio as a function of the nominal vinorelbine concentration of the plasma standards. Linear least squares regression was carried out to calculate the best-fit line and

regression coefficient. Vinorelbine concentrations in plasma samples were obtained by applying the resulting linear function to peak area ratios (vinorelbine:internal standard) for each sample.

2.8. Pharmacokinetics

Prior to intravenous administration of the liposomal formulations, the extra-liposomal solution comprising the bulk of the sample (predominantly 0.2 M Na_2HPO_4 and 0.3 M citrate – see Section 2.6 above) was exchanged with 20 mM Hepes (pH 7.4) and 150 mM NaCl using tangential flow filtration. Female Rag2M mice ($n = 12$/group) were injected intravenously via the tail vein with either free vinorelbine at 10 mg/kg or with vinorelbine encapsulated in SM/Chol/DPPS/PEG_{2000}-DSPE liposomes at 100 mg lipid/kg (representing 10 mg/kg of vinorelbine). At various times after injection, mice ($n = 4$/timepoint) were sacrificed by CO_2 asphyxiation and blood was recovered into EDTA-containing Microtainer tubes (BD Biosciences, Oakville, ON, Canada). After centrifugation, plasma aliquots were taken to measure the concentrations of total lipid (^{3}H-CHE using LSC), PS (^{14}C-DOPS using LSC) and/or vinorelbine (HPLC). To support a valid pharmacodynamic comparison (pharmacokinetics vs. efficacy), the doses used in the pharmacokinetic studies (10 mg/kg vinorelbine; 100 mg/kg of lipid) were identical to the highest dose evaluated in the efficacy studies. Representative data from three independent experiments are shown.

The plasma data were modeled using WinNONLIN Version 1.5 pharmacokinetic software (Pharsight Corporation, CA), to calculate pharmacokinetic parameters of free and formulated vinorelbine according to standard equations. Calculated pharmacokinetic parameters included the area under the concentration-time curve ($AUC_{0-24\ h}$) and the half-life of elimination ($T_{1/2}$) using a non-compartmental elimination model.

2.9. Antitumor efficacy and statistical analysis

H460 cells (1×10^6 cells) were injected subcutaneously into the flank of female Rag2M mice ($n = 4$/group). Tumors were allowed to grow until about 50 mg in size then the animals were treated once every 4 days, for a total of three treatments, using 200 μL tail vein injections and four mice per group. Animals were treated at either 10 mg/kg of vincristine, which was defined as the maximum tolerated dose based on preliminary dose range-finding studies, as well as at 5 mg/kg to establish a dose–response relationship. Animal weights and tumor dimensions were measured until the estimated tumor mass exceeded 10% of the animal's original body weight or until the tumors showed any sign of ulceration. Tumor volume was calculated using: $\text{volume} = [(\text{width})^2 \times (\text{length})]/2$. Antitumor activity was quantified by calculation of the treatment/control (T/C) ratio using tumor volumes measured on day 28 (1 week after the last treatment). Although many animals survived beyond day 28 of the study (there were no long-term survivors), day 28 was chosen for the endpoint analysis as it represented the last day on which tumor measurements could be reliably obtained from the control groups. A post hoc comparison of T/C values was performed using ANOVA and differences were considered significant at $p < 0.05$. Representative data from 3 to 4 independent experiments are shown.

3. Results

3.1. In vitro activities of vinorelbine and PS

Although liposomes containing phospholipids are generally regarded as safe and well-tolerated, it is known that some phospholipids can be cytotoxic *in vitro*, with IC_{50}s ranging from micromolar to millimolar concentrations depending on the headgroup and acyl chain composition [15–20]. These observations were confirmed by our cytotoxicity assays. In our initial experiments, phosphatidylserines were compared to control phospholipids, either PC or anionic PA having identical acyl chain compositions, for activity against the MDA-435/LCC6 human breast cancer cell line. The estimated IC_{50} for DMPC was greater than 1 mM, while DMPC/DMPS (8/2 mol/mol) had an IC_{50} approximately 160 μM (Fig. 1A). Cytotoxicity curves completed for DMPA, DMPS, DLPA and DLPS suggested that the cytotoxic effects observed in these MTT assays were PS-specific. For example, the IC_{50} of DMPA was 1.5 mM almost 10-fold greater than that observed for DMPS. Similarly the IC_{50} of DLPS was 200 μM compared to 1.5 mM DLPA (data not shown).

As it was our intention to design a combination product using vinorelbine for the treatment of lung cancer, further *in vitro* studies were performed with the H460 human non-small cell lung cancer line. Vinorelbine was very potent against H460 cells, with an estimated IC_{50} of 2–3 nM (Fig. 1B). In contrast, various molecular species of PS exhibited cytotoxicity only at higher concentrations. Specifically, DLPS and POPS had IC_{50} values from the MTT assay of 74 and 891 μM, respectively, in H460 cells (Fig. 1B). The remaining PS molecular species (DPPS, DSPS and DOPS) clearly had cytotoxic activity against the H460 cells (Fig. 1B), but the IC_{50} values were >1 mM.

We investigated the possibility that the cytotoxicity of PS was related to expression of the PS-receptor on H460 cells. Mab 217 antibodies directed against the PS-receptor recognized a protein on the surface of NIH 3T3 cells (positive control, Fig. 2B) as well as on H460 cells (Fig. 2C). Human HL-60 cells (negative control, Fig. 2A) or mouse ascitic tumor P388 cells (data not shown) were negative for antibody staining, consistent with a previously published report [21] demonstrating that suspension cells are negative for this receptor while adherent cells are positive.

Drug combination studies designed to assess combination effects between PS and vinorelbine against the H460 cell line were completed and analyzed using the median-ef-

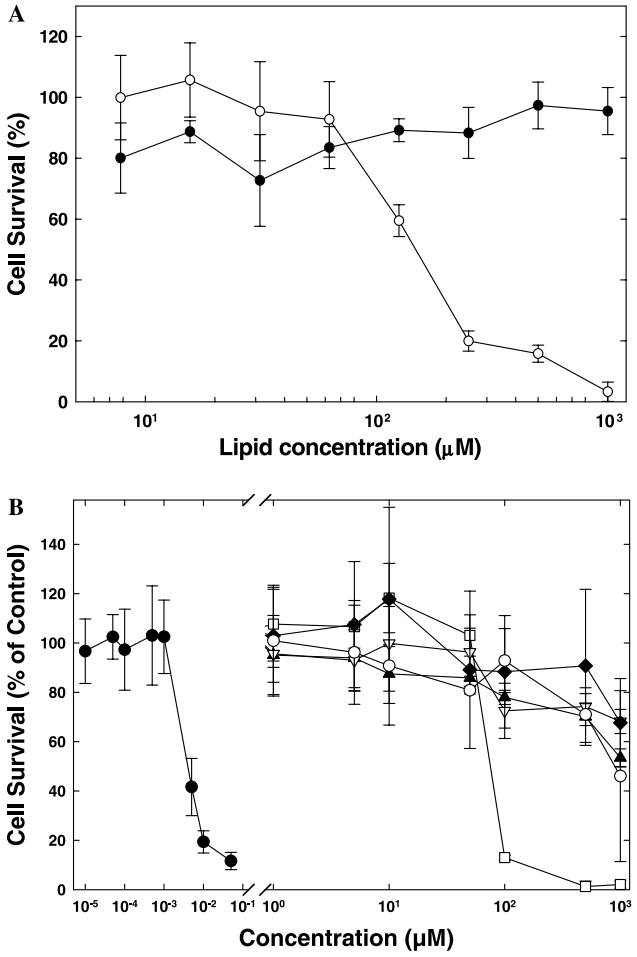

Fig. 1. Cytotoxicity of phospholipids and vinorelbine against the human cancer cells. Panel A: survival of MDA-435/LCC6 human breast cancer cells treated with either DMPC (●) or DMPC/DMPS (8/2, mol/mol; ○) liposomes for 72 h then viability was determined using the MTT assay. Values represent means (± standard deviation) from triplicate samples and are expressed as a percentage of the untreated controls. Panel B: survival of H460 human lung cancer cells treated with vinorelbine (●), DLPS (□), DPPS (▲), DSPS (▽), DOPS (♦) and POPS (○) for 72 h then viability was determined using the MTT assay. Values represent means (± standard deviation) from triplicate samples and are expressed as a percentage of the untreated controls.

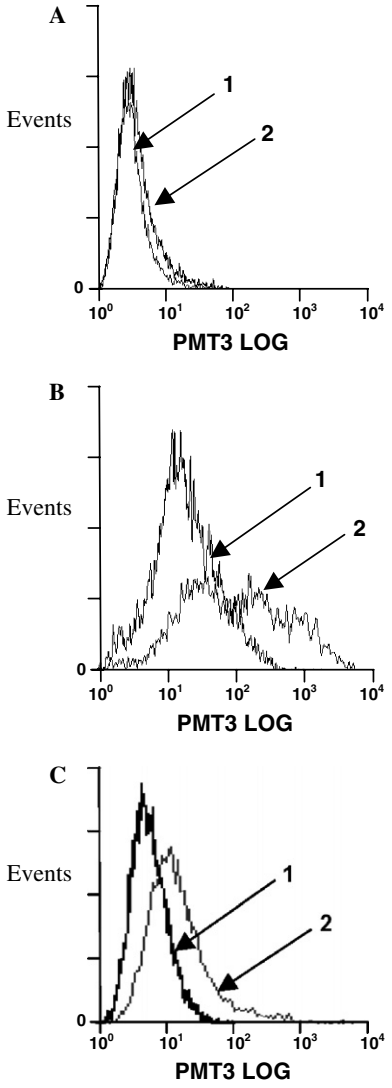

Fig. 2. Flow cytometric analysis of the PS-receptor expression on different cell lines. Each cell line was stained with the either a primary isotype control antibody then stained with a secondary goat anti-mouse IgM-conjugated to Cy3 (indicated as curve 1 in all panels) or stained with a primary mouse IgM monoclonal antibody (Mab 217) directed against the PS-receptor and subsequently stained with a secondary goat anti-mouse IgM-conjugated to Cy3 (indicated as curve 2 in all panels). (A) HL-60 cells (negative control); (B) NIH 3T3 (positive control); and (C) H460 cells.

fect principle at fixed ratios of PS/vinorelbine of 1/1 (Fig. 3A) and 10/1 (Fig. 3B). These ratios were determined based on the expectation that liposomal formulations could be prepared containing PS and vinorelbine at similar molar ratios (see Section 3.2 below). The best-fit curves, shown in Fig. 3, are calculated results obtained following analysis of the sigmoidal cytotoxicity curves for the agents added alone (Fig. 1) and in combinations at fixed molar ratios of 1/1 (Fig. 3A) or 10/1 (Fig. 3B) PS/vinorelbine. The results shown in Fig. 3A and B suggest that the type of interaction with vinorelbine against the H460 cell line was dependent on the molecular species of PS being evaluated. For example, DOPS, DLPS and DSPS had synergistic activity against H460 cells when combined with vinorelbine, as shown by CI values <0.8 at the proportion or fraction of cells affected (f_a) between 0.2 and 0.8 (Fig. 3A). In contrast, POPS and DLPS had significant single-agent activity *in vitro* (Fig. 1B) and, when combined with vinorelbine, showed synergistic interactions at f_a levels greater than 0.5 (Fig. 3A). Changing the PS/vinorelbine ratio from 1/1 (Fig. 3A) to 10/1 (Fig. 3B) had negligible effect on the type of interaction observed between the various PS species and vinorelbine. That is, strong synergism was observed for all PS species (Fig. 3B).

The synergistic interaction of vinorelbine with anionic lipids in the H460 human lung cancer cells was specific to PS. Control studies of the interaction of vinorelbine with the anionic lipids DSPG and DLPG (Fig. 4) as well as

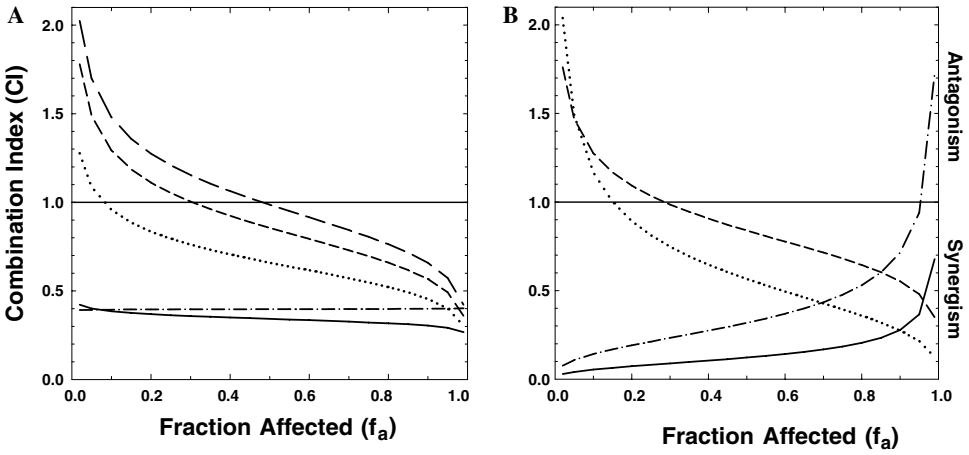

Fig. 3. Plot of the combination index (CI) as a function of the fraction of cells affected (f_a) for H460 human lung cancer cells treated with vinorelbine in combination with POPS (– –), DPPS (- -), DLPS (···), DSPS (·–·–·) or DOPS (——) as described in the Section 2. CI values >1 indicate an antagonistic interaction, values of 1 indicate an additive interaction and values <1 indicate a synergistic or potentiating interaction. Ratio of PS/vinorelbine (mol/mol) was 1/1 (A) or 10/1 (B).

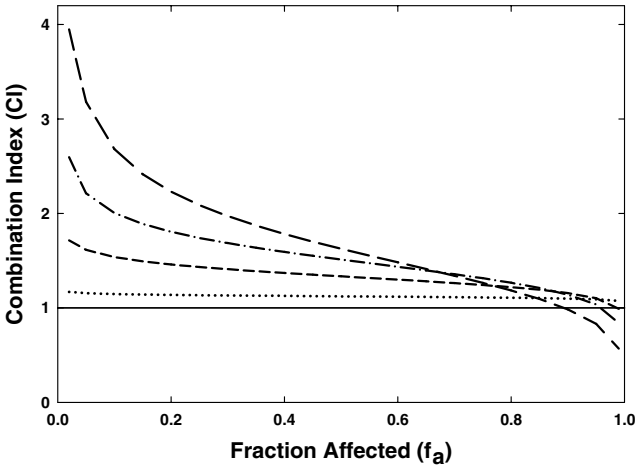

Fig. 4. Plot of the combination index (CI) as a function of the fraction of cells affected (f_a) for H460 human lung cancer cells treated with vinorelbine in combination with DSPG or DLPG. Cells were treated as described in the Section 2 at molar ratios of DSPG/vinorelbine of 1/1 (– –) or 10/1 (– –) or with DLPG/vinorelbine at 1/1 (···) or 10/1 (·–·–·) molar ratios. CI values >1 indicate an antagonistic interaction, values of 1 indicate an additive interaction and values <1 indicate a synergistic or potentiating interaction.

DSPA and DLPA (data not shown) showed that these lipids had additive or antagonistic interactions with vinorelbine (Fig. 4) at the same lipid/vinorelbine ratios and effective concentrations at which PS had synergistic or potentiating interactions (Fig. 3).

3.2. Formulation design

The PS liposomes used for the *in vitro* assays described above were unsuitable for *in vivo* use due to the very rapid clearance from the circulation characteristic of anionic liposomes [32]. Conversely, the liposomes designed for *in vivo* use, as described below, would have had little *in vitro* utility because the PEG-DSPE would have precluded the *in vitro* interaction of the PS with the PS receptor of the H460 cells. Consequently, a novel liposomal formulation for the *in vivo* co-delivery of vinorelbine and PS to tumors was designed based on a combination of empirical results and practical considerations as outlined below.

During preliminary formulation studies, PS incorporated into a liposomal formulation lacking cholesterol was not retained with the formulation after intravenous administration (data not shown). The inclusion of 45 mol% of cholesterol in the liposome was associated with the complete retention of PS after i.v. administration to mice. We also examined different molecular species of PS for their compatibility with the pH-gradient based loading of vinorelbine. The unsaturated molecular species of PS, specifically DOPS and POPS, inhibited the stable loading of vinorelbine into DSPC/Chol/PS/PEG_{2000}-DSPE based carriers (data not shown). In contrast, the higher transition temperature molecular species of PS, specifically DPPS and DSPS, were associated with stable, 100%, encapsulation of vinorelbine into these formulations. While DLPS was the most active molecular species of PS investigated (Fig. 1) and had synergistic cytotoxicity in combination with vinorelbine (Fig. 3A and B), it was not retained with the formulation after intravenous administration, even in formulations containing 45 mol% cholesterol (data not shown). Based on these loading results, the limited availability of DSPS for extensive *in vivo* studies as well as difficulty formulating liposomes containing DSPS, all further studies were performed using DPPS as the molecular species of PS for incorporation to the formulation.

Poly(ethylene glycol) modified lipids were included in the formulation as a means of controlling the circulation lifetime of the formulation. The use of 10 mol% of PEG_{2000}-DSPE in the formulations prepared here was based on previous work [33]. Specifically, the rapid elimination of PS-containing formulations from the circulation

after i.v. administration was effectively ameliorated by the inclusion of 10 mol% of PEG_{2000}-DSPE [33]. Lower molar proportions of PEG_{2000}-DSPE were less effective at prolonging the residence time of the PS-containing formulations in the circulation [33]. Finally, preliminary experiments described above used DSPC as the principal structural component of the liposomal formulation. In subsequent experiments to optimize *in vivo* performance, SM replaced DSPC as the primary bilayer-forming lipid due to the improved chemical stability of SM, compared to DSPC, and the potential for enhanced drug retention in a SM-based liposomal formulation [34].

This design and optimization process resulted in a liposomal formulation comprised of SM/Chol/DPPS/PEG_{2000}-DSPE (35/45/10/10, mol/mol/mol/mol). In response to a transmembrane pH gradient, vinorelbine loading to a final drug/lipid ratio of 0.1/1.0 (w/w), representing >95% encapsulation efficiency, was rapidly achieved within 10 min at 60 °C (data not shown). It should be noted that effective loading of vinorelbine could also be achieved using alternative methods, including uptake in response to an ammonium sulphate gradient [35] or by an ionophore-mediated method [36]. The mean liposomal diameter after formulation and vinorelbine encapsulation was 90–130 nm. Analysis by differential scanning calorimetry provided no evidence for phase transition or lipid immiscibility over the temperature range of 15–70 °C (not shown).

3.3. Pharmacokinetic characterization

The pharmacokinetics of free vinorelbine and of vinorelbine formulated in the SM/Chol/DPPS/PEG_{2000}-DSPE liposomes were characterized in Rag2M mice. Plasma concentrations of vinorelbine after the i.v. administration of the free drug were very low (Fig. 5), indicating rapid elimination from the circulation. In contrast, the liposomal vinorelbine had plasma concentrations that were substantially higher than those of the free drug (Fig. 5). This increase in the concentration and duration of increased plasma vinorelbine levels was most clearly demonstrated by the calculation of the plasma $AUC_{0-24\ h}$ values. The free vinorelbine $AUC_{0-24\ h}$ of 0.112 μg h/mL was increased to 125.3 μg h/mL by formulation in the SM/Chol/DPPS/PEG_{2000}-DSPE liposome (Fig. 5, insert), representing an 1120-fold increase of vinorelbine in the plasma.

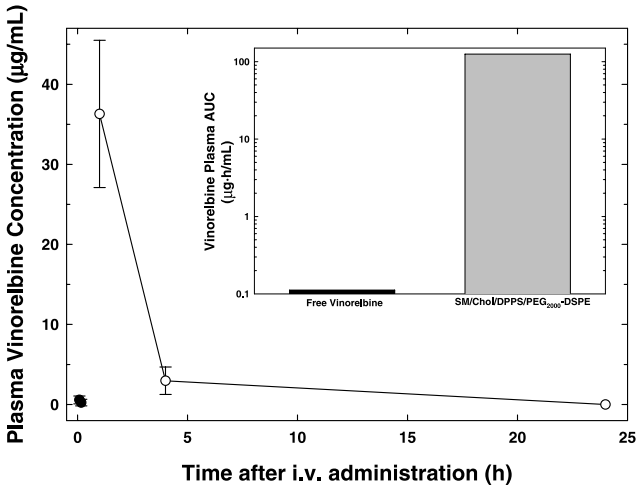

Fig. 5. Comparison of the pharmacokinetics of vinorelbine administered intravenously to Rag2M mice as either free drug or formulated in the SM/Chol/DPPS/PEG_{2000}-DSPE complex. Plasma concentrations of drug is shown as a function of time after i.v. administration of either free (●) or SM/Chol/DPPS/PEG_{2000}-DSPE-formulated (○) vinorelbine. Insert: calculated area-under-the-curve (AUC) values for vinorelbine in the plasma of mice after i.v. administration of either free or SM/Chol/DPPS/PEG_{2000}-DSPE-formulated vinorelbine.

3.4. Antitumor activity

H460 human lung tumor xenografts grown subcutaneously in immuno-compromised Rag2M mice were treated with: (i) free vinorelbine; (ii) empty SM/Chol/DPPS/PEG_{2000}-DSPE liposomes; (iii) free vinorelbine given simultaneously with empty SM/Chol/DPPS/PEG_{2000}-DSPE liposome (i.e., a "cocktail"), or; (iv) vinorelbine encapsulated in SM/Chol/DPPS/PEG_{2000}-DSPE liposome (i.e., the dual-agent formulation). The results from these studies are summarized in Table 1.

The H460 tumor had a strong and statistically significant dose–response to treatment with free vinorelbine, with T/C values of 0.742 and 0.531 at doses of 5 and 10 mg vinorelbine/kg, respectively (Table 1). In contrast, empty DPPS-containing liposomes (i.e., without encapsulated vinorelbine) had no statistically significant effect ($p = 0.246$) on the growth of the H460 xenograft tumor when given at the corresponding lipid dose (Table 1). This result is consistent with the *in vitro* results (Fig. 1) demonstrating that the cytotoxic activity of DPPS given alone is negligible compared to vinorelbine. When the activity associated with free vinorelbine treatment was compared to that achieved with a cocktail of free vinorelbine plus empty PS-containing liposomes, the latter treatment was not significantly more efficacious at the 5 mg vinorelbine/kg dose ($p = 0.771$) and only marginally significant ($p = 0.047$) at the 10 mg vinorelbine/kg dose (Table 1). That is, the free vinorelbine and the cocktail of free vinorelbine plus empty PS-containing liposomes achieve very similar levels of activity against the H460 tumor (Table 1). These data, taken together, suggest that DPPS has limited ability to potentiate the antitumor activity of vinorelbine when the two agents are administered independently (i.e., as a cocktail) and have markedly different elimination profiles from the blood compartment (preceding section). Experiments were also performed to determine the antitumor activity against the H460 tumor when the DPPS and vinorelbine were co-formulated, by liposomal encapsulation. When vinorelbine was co-formulated with DPPS by encapsulation in the liposomes, the H460 tumor growth was significantly delayed ($T/C = 0.473$ and 0.277 at 5 and 10 mg vinorelbine/kg, respectively; Table 1). This antitumor activity was statisti-

Table 1
Summary of the *in vivo* antitumor efficacy studies in the H460 human lung cancer xenograft grown subcutaneously in Rag2M mice

Treatment type	Doses (mg/kg)		*T*/*C*	p^a	p^b	p^c
	Vinorelbine	Lipid				
Free VNB	5	–	0.742	0.009	–	0.771
Empty liposomes	–	50	0.847	0.246	–	–
Free VNB + empty liposome cocktail	5	50	0.764	0.021	0.771	–
Dual-agent PS-encapsulated VNB	5	50	0.473	0.032	0.003	0.008
Free VNB	10	–	0.531	0.001	–	0.047
Free VNB + empty liposome cocktail	10	100	0.411	0.027	0.047	–
Dual-agent PS-encapsulated VNB	10	100	0.277	0.014	0.0004	0.026

Mice with established H460 tumors were treated q4d × 3 with free vinorelbine (VNB), empty SM/Chol/DPPS/PEG_{2000}-DSPE liposomes, free VNB plus SM/Chol/DPPS/PEG_{2000}-DSPE liposomes (i.e., the cocktail) or VNB encapsulated in SM/Chol/DPPS/PEG_{2000}-DSPE liposomes (i.e., the dual-agent formulation). Treatment/control (*T*/*C*) ratios were calculated from the tumor growth curves obtained for four mice per group as described in the Section 2.
[a] *p* value for the ANOVA comparison with the saline-treated control group.
[b] *p* value for the ANOVA comparison with the "Free VNB" treatment group at the same dose.
[c] *p* value for the ANOVA comparison with the "Free VNB + empty liposome cocktail" treatment group at the same dose.

cally significant compared to the efficacy achieved by the same dose of free vinorelbine ($p = 0.003$ and 0.0004 at 5 and 10 mg vinorelbine/kg, respectively; Table 1) and that achieved by the cocktail of the free vinorelbine and empty DPPS-containing liposomes ($p = 0.008$ and 0.026 at 5 and 10 mg vinorelbine/kg, respectively; Table 1).

4. Discussion

The clear superiority of combination chemotherapy, compared to single-agent therapy, in the treatment of cancer, has been well known for several decades. This reality of the clinical setting is consistent with the more recent realization that the transformation and proliferation of normal cells to the cancerous state requires multiple genetic and molecular defects. Taken together, these facts strongly argue that the development of new treatments for cancer should be based on the premise that highly effective therapeutics will require the simultaneous targeting of the multiple lesions of the neoplastic cell. This perspective prompted the development of a multi-component formulation for the co-delivery of two active agents, vinorelbine and PS. We have taken a mechanistic approach to this work in that we have: (i) demonstrated PS cytotoxicity in the MDA-435/LCC6 and H460 human cancer cell lines *in vitro*; (ii) shown the expression of the PS receptor on the H460 human cancer cells; (iii) characterized drug/drug ratios at which *in vitro* synergy occurs between PS and vinorelbine in H460 human cancer cells; (iv) developed a liposomal system that co-formulates PS and vinorelbine at the same drug/drug ratios identified *in vitro*; and (v) demonstrated that this liposomal formulation of the PS and vinorelbine active agents has significant anticancer activity against solid tumors of the PS-receptor expressing H460 human xenograft model.

The observation of PS-induced cytotoxicity in LCC6 human breast cancer cells and in H460 human lung cancer cells (Fig. 1) is consistent with the reports of cytotoxicity and apoptosis inducing many difference cancer cell lines by PS-containing liposomes [15–20]. Our findings concur with those reported previously that the cytotoxicity of PS was not shared by other phospholipids, including phosphatidylinositol [15,17], PC [15,18,19] and PA [15,18] (Fig. 1). Interestingly, the apoptosis induced in CHO-K1 cells by PS occurred by a pathway involving PKC inactivation and was also distinct from the ceramide-mediated apoptotic pathway [15]. It has also been reported that PS-mediated apoptosis is independent of the apoptotic pathways involving mitochondrial membrane depolarization, cytochrome *c* release as well as caspases-1, -3, -8 and -9 [16]. Other investigators have reported that PS-induced apoptosis proceeds via cell detachment, actin disorganization, caspase activation and focal adhesion kinase cleavage [17]. Recent reports suggest that PS liposomes can suppress nitric oxide production via an ERK-mediated increase of TGF-β [37].

This molecular species of PS (dipalmitoyl-PS; DPPS) used in this study has acyl chains with lengths closely matching those of the SM, the predominant lipid in the formulation. Therefore, it was expected this PS would be well anchored in the liposome by hydrophobic forces and would not exchange out of the carrier in the circulation. Indeed, comparable dipalmitoyl phospholipids that are conjugated to large hydrophilic moieties such as poly(ethylene glycol) (Mw = 2000), and which have substantially greater propensity to exchange out of the liposome, are well retained after intravenous circulation [38]. Therefore, it is likely that the *in vivo* cytotoxicity of PS against H460 tumors occurs after the PS-containing liposomes that have extravasated from the circulation to the interstitial spaces of the tumor. These liposomes would be capable of a PS-dependent interaction with the PS receptor on H460 cells (Fig. 2) leading to macropinocytosis and liposome uptake [39] delivering both the PS and the vinorelbine active agents intracellularly to the H460 tumor cells *in vivo* at the synergistic ratios defined by the *in vitro* cytotoxicity assays (Fig. 3). PS-containing liposomes may also trigger a "bystander" uptake of cells that are bound to, but not engulfed by, phagocytes [39]. It should also be noted that these mechanisms by

which PS is directly cytotoxic to cancer cells, based on *in vitro* studies, may be complemented *in vivo* by PS-mediated recruitment and activation of phagocytic cells expressing the PS receptor and enhancement of the macrophage-dependent clearance of apoptotic cells having externally exposed PS [21,22]. There is a theoretical risk that this PS-containing liposomal formulation of vinorelbine could be taken up by macrophages, mediated by PS receptors, and cause increased toxicity towards macrophages. However, this would not necessarily translate into increased tumor growth since the contribution of circulating macrophages to the inhibition of tumor growth is not known. Most importantly, the *in vivo* antitumor efficacy data presented in this manuscript (Table 1) clearly show significant inhibition, not growth, of tumors in treated Rag2M mice, which have normal macrophage production.

PS has been used by a number of previous investigators as a component of liposomal delivery systems for a number of drugs, including doxorubicin [40], lipopeptides [41] and muramyl dipeptide [42]. However, none of these studies considered PS as an active agent and with the further potential to synergize with the encapsulated drug; a novel feature of the work described here. A specific characteristic of the lipid-based formulations described here is their ability to achieve comparable pharmacokinetics of the active agents, PS and vinorelbine. Efficacy studies in which PS and vinorelbine were administered as separate drugs, and were subject to independent elimination from the plasma, showed a negligible improvement in antitumor activity compared to free vinorelbine alone (Table 1). In contrast, when treatment of the H460 tumor with PS and vinorelbine as a dual-agent formulation, the antitumor activity compared to the unformulated vinorelbine and the cocktail was significantly different at two tested doses (Table 1).

We have described an approach to the design of lipid-based formulations allows disparate agents to be administered at drug/drug ratios having synergistic *in vitro* cytotoxicity. In this paper we have specifically described the co-formulation of DPPS and vinorelbine, which were demonstrated to have a synergistic interaction over the ratio range of 1/1 to 10/1 of PS/vinorelbine (mol/mol; Fig. 3). The SM/chol/DPPS/PEG_{2000}-DSPE formulation containing vinorelbine was formulated and administered to mice at a PS/vinorelbine base ratio of 1/1 (mol/mol); a ratio identified as synergistic based on the *in vitro* assays with H460 cells (Fig. 3A). This formulation has been optimized for vinorelbine loading, pharmacokinetics and has enhanced activity against the H460 human lung cancer xenograft in Rag2M mice when compared to the unformulated drug or compared to control groups, including a comparison against the drug cocktail comprised of free vinorelbine plus the PS-containing formulation. These studies also demonstrate an effective approach to the development of clinically relevant drug combinations using *in vitro* and *in vivo* drug–drug interactions.

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