Optimization and Characterization of a Sphingomyelin/Cholesterol Liposome Formulation of Vinorelbine with Promising Antitumor Activity

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ABSTRACT: Vinorelbine (VRL) is a particularly lipophilic member of the vinca alkaloids which, as a class of drugs, exhibit improved cytotoxicity and therapeutic activity through increased duration of exposure. Here, we describe and optimize a sphingomyelin/cholesterol (SM/Chol) liposome formulation of VRL to maximize *in vivo* drug retention, plasma circulation time, and therapeutic activity. VRL was efficiently encapsulated (>90%) into 100 nm liposomes using an ionophore-mediated loading method. VRL retention in SM/Chol liposomes after intravenous injection in mice was dependent on drug-to-lipid ratio (D/L), with higher D/L ratios exhibiting increased drug retention ($0.3 > 0.2 > 0.1$, wt/wt) and improved pharmacokinetics. Cryo-electron microscopic examination of a high D/L ratio formulation indicated that the intravesicular regions of these liposomes were electron dense compared with empty liposomes. The optimized, high D/L ratio SM/Chol VRL formulation showed promising activity against subcutaneous B16 melanoma tumors compared with VRL or SM/Chol formulations of vincristine or vinblastine. Finally, the stability of the formulation was excellent (<5% drug leakage, >99% intact VRL, no changes in liposome size after 1 year at 2–8°C). The optimized drug retention properties of the SM/Chol formulation of VRL, combined with its promising antitumor activity and pharmaceutical stability, make this formulation an excellent candidate for future clinical development. J Pharm Sci 94:1024–1038, 2005

Keywords: navelbine; vinorelbine; liposomes; drug delivery; controlled release; cancer; cancer chemotherapy; stability

INTRODUCTION

Vinorelbine (Navelbine®) is a semi-synthetic vinca alkaloid that is marketed for use in advanced non-small cell lung and metastatic breast cancers, and has exhibited a broad spectrum of activity in preclinical studies.[1–4] A chemical modification of the catharanthine ring of vinblastine, converting it from a nine-membered ring to an eight membered ring, generates vinorelbine (VRL), which exhibits increased lipophilicity and membrane permeability compared with other compounds in the vinca alkaloid family.[5,6] This alteration in structure resulted in an altered toxicity profile, differential effects on microtubule populations and dynamics, altered pharmacokinetic properties,

Abbreviations: Chol, cholesterol; cryo-TEM, cryo-transmission electron microscopy; D/L, drug-to-lipid ratio; DSPC, 1,2-distearoylphosphatidylcholine; MTD, maximum tolerated dose; SM, sphingomyelin; VRL, vinorelbine.

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and a higher therapeutic index compared with other vinca alkaloids.[2–4] While the increased lipophilicity and cellular uptake of VRL is one of the several mechanistic considerations proposed for its improved activity profile relative to other vinca alkaloids, this membrane permeability property poses a challenge for developing liposomal formulations of VRL with appropriate drug release characteristics.

VRL is a cell cycle phase specific, anti-mitotic agent that interacts with tubulin to inhibit microtubule formation and promote depolymerization of existing microtubules, resulting in cell growth inhibition in late G_2 and M phases.[4,7] As a consequence of its mechanism of action and cell cycle specificity, prolonged exposure of cells to VRL is important for optimal activity. In this regard, several *in vitro* and *in vivo* cytotoxicity studies have demonstrated that extended exposure to anti-mitotic agents,[8–11] and cell cycle specific drugs in general,[12] results in dramatic decreases in IC_{50} concentrations. Recently, continuous infusion studies designed to increase and optimize VRL exposure in solid tumors have shown minimal success in the clinic, with cumulative toxicities limiting dose escalation and providing a narrow therapeutic index.[13] Consequently, a liposomal formulation of VRL that does not appreciably change the toxicity profile and increases plasma levels of VRL, accumulates in tumors and provides sustained release of drug within the tumor microenvironment would be expected to have significant clinical potential.

The pharmacokinetic and pharmacological advantages of liposomal drug delivery systems in cancer chemotherapy are well documented.[14] Nevertheless, significant work continues in the optimization of formulations for increased circulation times, drug release rates, and efficacy. Recently, a sphingomyelin/cholesterol (SM/Chol) liposomal formulation of another vinca alkaloid, vincristine (VCR), has shown clinical promise in Phase II clinical trials against hematological malignancies.[15,16] Since the recommended dose of VRL in humans is relatively high (30 mg/m^2 vs. 1.4 mg/m^2 for VCR), a drug loading method that allows for relatively high drug-to-lipid (D/L) ratios was thought to be important to minimize lipid dosing. While each of the approved liposomal products used in oncology utilize pH-gradient or ammonium sulfate loading methods, an ionophore loading method has recently been described as an effective method for introducing lipophilic amines into liposomes to achieve high drug loading efficiencies.[17] In this study, we have used the A23187 ionophore loading method to impart maximum flexibility into the optimization and characterization of drug loading and *in vivo* retention of VRL, leading to the development of a stable, single-vial formulation of VRL with promising antitumor activity.

MATERIALS AND METHODS

Materials

Vinorelbine tartrate was obtained from Omni-Chem SA (Louvain-la-Neuve, Belgium). Navelbine® was purchased from a pharmacy. [^{3}H]-VRL was synthesized and purified (radiochemical purity >98%) by Moravek Biochemicals, Inc. (Breas, CA). Vincristine sulfate, vinblastine sulfate, and EDTA were obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). [^{14}C]-cholesteryhexadecylether (CHE) was obtained from PerkinElmer (Boston, MA). Egg SM and Chol were obtained from Lipoid GmbH (Ludwigshafen, Germany) and Solvay Pharmaceuticals (Weesp, The Netherlands), respectively. A23187 ionophore was purchased from Fermentek Ltd. (Jerusalem, Israel). Magnesium sulfate ($MgSO_4$), monobasic sodium phosphate, and dibasic sodium phosphate were obtained from JT Baker (Phillipsburg, NJ). Sucrose was obtained from EM Science (Gibbstown, NJ). All reagents were used without further purification.

Preparation of Liposomes

Liposomes were prepared based on an ethanol procedure described by Boman et al.[18] Briefly, lipids (SM/Chol, 55/45, molar ratio) were dissolved in ethanol, mixed in the appropriate ratios, and added into a solution containing aqueous $MgSO_4$ at 60°C. The resulting liposome dispersion was extruded at 65°C through two stacked 80 nm polycarbonate filters (Nucleopore, Pleasanton, CA) using a heated thermobarrel extruder (Northern Lipids, Vancouver, Canada), as described by Hope et al.[19] The final ethanol concentration was 15% (v/v) and was critical for achieving the target liposome size of 100 ± 30 nm. Residual ethanol and external $MgSO_4$ were removed by tangential flow diafiltration at room temperature, exchanging the external solution for 300 mM sucrose (pH 7), using a Midgee™ HOOP™ ultrafiltration cartridge (MW cutoff 100000; Amersham Biosciences). The liposomes were stored at 4°C in

this form until required for drug loading. EDTA and phosphate buffer were added to the liposomes before drug loading. Quasi-elastic light scattering (QELS) was used to assess the size distribution of the extruded liposomes, using a NICOMP model 380 submicron particle sizer (Particle Sizing Systems, Santa Barbara, CA).

Drug Loading and Ionophore Removal

VRL was loaded into liposomes using the A23187-ionophore loading method described by Fenske et al.[17] A brief description of the loading process is given below, with optimized loading conditions shown in parentheses. VRL was dissolved at 10 mg/mL in 300 mM sucrose. EDTA and phosphate buffer were added to the liposomes (15 mg/mL total lipid, pH 6) to achieve 25 and 50 mM final concentrations, respectively. Liposome suspensions were pre-heated to the appropriate incubation temperature (60°C) and the ionophore (1 μg A23187/mg lipid) was added, followed by the addition of VRL and incubation at the appropriate temperature (60°C) for the indicated times (30 min). A23187 ionophore, EDTA, and unencapsulated VRL were removed by tangential flow diafiltration using a Midgee™ HOOP™ ultrafiltration cartridge (MW cutoff 100000; Amersham Biosciences). The external solution was exchanged against 20 sample volumes of phosphate buffered sucrose (300 mM sucrose, 10 mM sodium phosphate, pH 7). Residual A23187 was measured using a fluorescence assay. Sample (30 μL) was diluted in 665 μL methanol, 300 μL ethanol, and 5 μL 250 mM EDTA. Fluorescence was measured against a standard curve using a SLM Aminco Bowman Spectrometer (excitation, 387 nm; emission, 437 nm). The VRL-loaded liposomes were stored at 4°C until characterized further or used for *in vivo* studies. Formulations were physically and chemically stable at this temperature for at least 1 year.

HPLC Detection of VRL

VRL was measured using a Waters Alliance HPLC system consisting of an Alliance 2695 Separations Module (autosampler, HPLC pump and column heater), a Waters 2996 Photodiode Array detector, and Waters Millenium[32] HPLC software Version 4.0 (Waters Corporation, Milford, MA). Samples (10 μL) were injected onto a reverse-phase ACE C8 column with 3 μm packing, 100 × 4.6 mm (Advanced Chromatography Technologies, Aberdeen, UK) and eluted with a mixture of water and methanol containing 0.1% phosphoric acid. The separation consisted of a gradient method, beginning at 30% methanol and increasing to 65% methanol, while maintaining a constant column temperature of 60°C and a flow rate of 1 mL/min. VRL was detected at 267 nm. For VRL detection in plasma samples, 50 μL plasma was added to 300 μL methanol and the mixture was mixed and centrifuged for 10 min at 13000*g*. The supernatant was removed and aliquots (10 μL) were analyzed as described above.

Cryo-Electron Microscopy

Cryogenic-transmission electron microscopy (cryo-TEM) was performed on empty and drug-loaded SM/Chol liposomes using a Zeiss EM 902A Transmission Electron Microscope (LEO Electron Microscopy, Oberkochen, Germany) operated at 80 kV in the zero loss bright-field mode. Digital images were recorded under low dose conditions with a BioVision Pro-SM Slow Scan CCD camera (Proscan GmbH, Scheuring, Germany) and analySIS software (Soft Imaging System, GmbH, Münster, Germany). In order to visualize maximum detail, an underfocus of 1–2 μm was used to enhance the image contrast. Samples were equilibrated at 25°C and approximately 99% relative humidity within a climate chamber. A small drop (~1 μL) of sample was deposited on a copper grid covered with a perforated polymer film coated with carbon on both sides, and excess liquid was then removed by blotting with filter paper, leaving a thin film of the solution on the grid. Immediately after blotting, the sample was vitrified into liquid ethane and maintained just above its freezing point of −182°C. Samples were maintained below −165°C and protected against atmospheric conditions during both transfer to the TEM and examination. Images at 100000× total magnification were captured for each sample.

Differences in the image intensity of the interior and exterior of the liposomes were quantified in the original digital images using ImageJ (Version 1.33) image processing software (NIH, Bethesda, MD). Pixel density was determined in 25 independent regions, both inside and outside the liposomes. Each region analyzed was of an identical area and shape (essentially the internal area of most liposomes) and consisted of approximately 3000 pixels. On a standard grayscale, black is 0 and white is 256.

Mice

Female ICR or C57Bl/6 mice (6–8 weeks old) were obtained from Harlan (Indianapolis, IN) and used for the *in vivo* drug release studies and B16 tumor studies, respectively. All mice were quarantined for at least 2 weeks prior to use. Animals were maintained in a controlled temperature ($22 \pm 1^{\circ}C$) and humidity ($60 \pm 10\%$) environment. Lighting was maintained on automatic 12 h light/dark cycles. Animal studies were conducted in compliance with the guidelines established by the Canadian Council on Animal Care (CCAC).

In Vivo Drug Release and Pharmacokinetic Studies

VRL, containing ~0.5 μCi [^{3}H]-VRL/mg lipid, was loaded into SM/Chol (55/45, molar ratio) liposomes at initial D/L ratios of 0.1, 0.2, and 0.3 wt/wt using the following loading conditions: 1 μg A23187/mg lipid, pH 6, 60°C and 300 mM internal $MgSO_4$. Liposomes contained 0.1–0.2 μCi [^{14}C]-CHE, a non-exchangeable, non-metabolizable lipid marker.[20] The resulting liposome formulations were dialyzed into phosphate buffered sucrose using tangential flow, as described above, and were subsequently injected intravenously into ICR mice through the tail vein at a lipid dose of 50 mg/kg. Blood and plasma samples were analyzed for lipid and drug content by dual label liquid scintillation counting as described previously.[21] In some studies, VRL concentrations were analyzed by HPLC, as described above. Pharmacokinetic parameters were determined by non-compartmental analysis using WinNonlin Professional (Version 4.01; Pharsight Corporation, Mountain View, CA).

B16 Antitumor Efficacy Studies

B16BL-6 melanoma cells were obtained from the Division of Cancer Treatment and Diagnosis (DCTD; Frederick, MD) and cultured in MEM with 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, non-essential amino acids, and vitamins.[22] B16 cells (3×10^5) were injected subcutaneously into C57Bl/6 mice in the dorsal flank. Once tumors were palpable (approximately day 6 post-cell implantation), animals were randomized into groups and injected with a single i.v. bolus injection of free or liposomal formulations of VRL (D/L, 0.32 wt/wt), vinblastine (D/L, 0.30 wt/wt), or VCR (D/L, 0.10 wt/wt). Animal weights and general well-being were monitored daily. Median tumor volumes for each group were recorded and increases in activity were calculated as %T/C according to Plowman et al.[23]

Accelerated Formulation Stability Studies

Aliquots (300 μL) of VRL and liposomal VRL formulations were sealed in 1 mL glass HPLC sample vials, using Teflon-lined caps, and incubated at 5, 25, 40, and 60°C. At various times, liposome samples were analyzed for size (QELS) and drug retention (spin column method) as described previously.[17] Aliquots of all samples were also stored frozen at −70°C for HPLC analysis of VRL decomposition. The chemical stability of liposomal VRL was compared with two commercially available VRL preparations: Navelbine®, a clinically approved formulation of VRL tartrate (Pierre Fabre Medicament; used as supplied), as well as a solution derived from a lyophilized powder (VRL tartrate; OmniChem SA), which was used in all drug loading studies. The VRL from OmniChem was dissolved at 10 mg/mL in acetate buffer (50 mM sodium acetate, 300 mM sucrose pH 4).

RESULTS

It is well documented that VRL is more lipophilic and is able to permeate across membranes more readily than many of the other vinca alkaloids,[6,24,25] a result that we have confirmed in *in vitro* release assays with various liposome compositions (unpublished observations). Webb et al.[26] have previously demonstrated that a SM/Chol liposome formulation of VCR provides increased drug retention and efficacy in murine and human tumor models relative to other, less rigid formulations (e.g., 1,2-distearoylphosphatidylcholine (DSPC)/Chol). Given that VRL is more lipophilic and membrane permeable than VCR or vinblastine, we were interested in developing and optimizing a SM/Chol formulation of VRL that maximizes its loading efficiency and, ultimately, its *in vivo* drug retention and efficacy properties. To accomplish this, we have adapted an A23187 ionophore loading method described by Fenske et al., as it was previously suggested that this method might be more flexible and permit higher D/L ratios than the citrate (pH-gradient) based loading method used to encapsulate VCR in Webb et al.[17,26]

Optimization of VRL Loading Conditions in SM/Chol Liposomes

In an initial series of studies, the effects of temperature, D/L ratio, external pH, ionophore concentration, and internal $MgSO_4$ concentration on the loading of VRL in SM/Chol liposomes were investigated.

Figure 1A shows the rate and extent of VRL accumulation in SM/Chol liposomes at three different temperatures (25, 47, and 60°C) at a D/L ratio of 0.1 wt/wt after incubation with 0.35 μg A23187 ionophore/mg lipid in loading buffer (300 mM sucrose, 50 mM phosphate, 25 mM EDTA, pH 7). As expected, drug uptake was temperature dependent. VRL accumulated rapidly at 60°C reaching a maximum (≥80%) after 60 min., while uptake was slower or negligible at 47 and 25°C, respectively. At 25°C, less than 10% of the drug had accumulated in the liposomes after 1 h and loading increased only slightly to 30% after 24 h. In the absence of A23187, no significant level of drug uptake was seen at any of the incubation temperatures.

Next, the impact of D/L ratio on loading was investigated. Initially, the rationale for these studies was to obtain a relatively high D/L ratio to minimize lipid doses in animal studies. Typically, mice are treated at or near the maximum tolerated dose (MTD) of the drug formulation. In pilot studies, the MTD for liposomal VRL was approximately 20 mg/kg. To maintain the lipid dose within the range of 50–100 mg/kg, a formulation with a D/L ratio of ~0.3 wt/wt is required. Using the previous set of conditions (0.35 μg A23187/mg lipid, 60°C, pH 7), rapid and efficient loading was only observed at a D/L of 0.1 wt/wt (80% at 1 h). Drug uptake occurred much slower and at lower efficiencies at the higher D/L ratios, with encapsulated drug levels (at 1 h) of approximately 40% and 25% for D/L ratios of 0.2 or 0.3 wt/wt, respectively (Figure 1B).

To obtain higher loading at the 0.3 D/L ratio, subsequent studies examined the effects of modifying the external pH. The ionophore A23187 is a

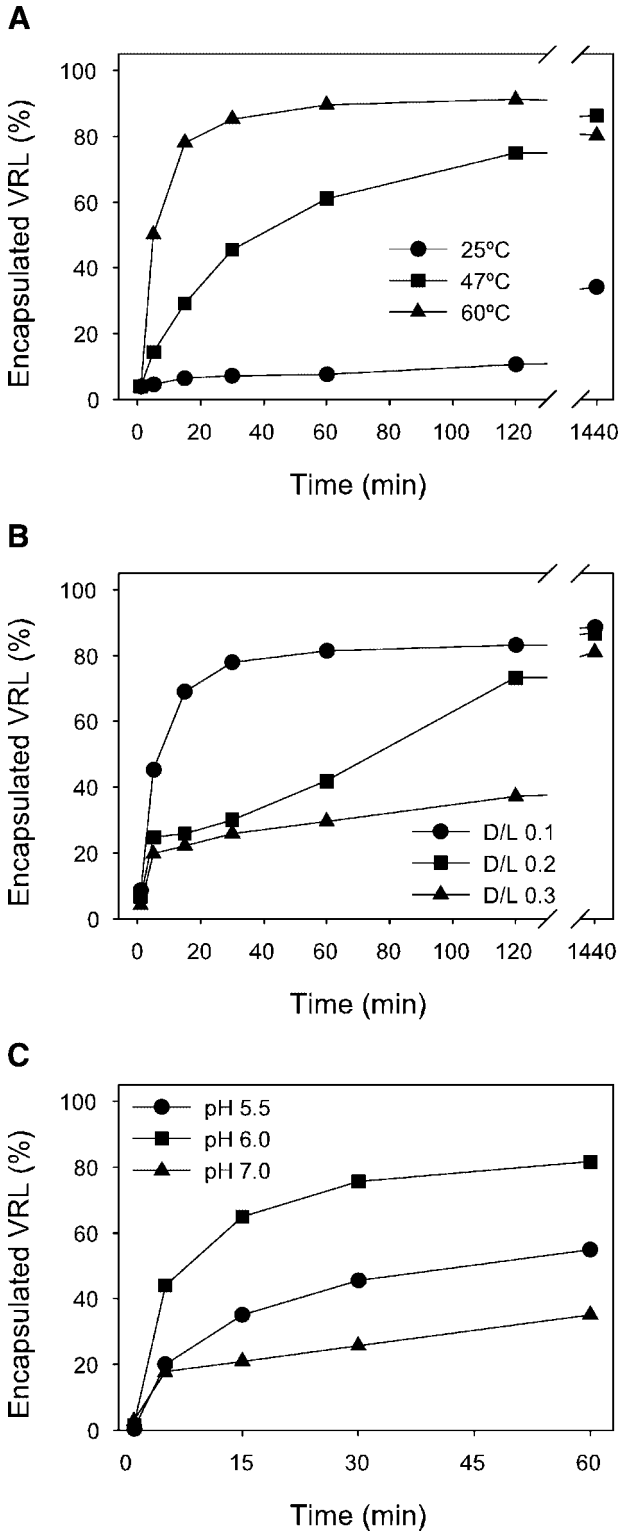

Figure 1. Factors influencing vinorelbine (VRL) uptake into sphingomyelin/cholesterol (SM/Chol) liposomes at low ionophore concentrations. The effects of temperature (A), drug-to-lipid ratio (D/L) (B), and pH (C) on VRL loading into 100 nm SM/Chol liposomes were examined. Except where a particular variable was varied in a given study, standard loading conditions in these studies were 300 mM internal magnesium sulfate ($MgSO_4$), 0.35 μg A23187/mg lipid, pH 7, and 60°C. In panel A, the drug-to-lipid ratio was 0.1 (wt/wt). In panel C, the D/L was 0.3 (wt/wt). The results presented in each panel are representative of at least three independent experiments.

weak acid with a carboxyl group pKa in the range of 5 to 9 depending upon the polarity of the solvent within which it is measured.[27] Additionally, previous studies have shown that the optimal pH for A23187 mediated transport of Mn^{2+} is 6.7–7.0.[28,29] Consequently, the pH of the loading mixture and ionophore concentration might be expected to have a significant impact on drug transport and the rate of drug loading into liposomes. Figure 1C shows the effect of pH on VRL loading at an initial D/L ratio of 0.3 wt/wt (60°C, 0.35 μg A23187/mg lipid). Decreasing the pH from 7 to 6 resulted in a considerable acceleration of VRL loading, with uptake levels of approximately 80% at pH 6 after 1 h as compared to 30% at pH 7. When the pH was lowered further to pH 5.5, loading efficiencies decreased (50% after 1 h). Using a higher concentration of the A23187 ionophore accelerated VRL uptake at a D/L ratio of 0.3 wt/wt and pH 6. Figure 2 shows the effect of increasing the ionophore concentration from 0.2 to 2 μg/mg lipid. At ionophore concentrations ≥1 μg/mg lipid, more than 90% of the drug was loaded within 5 min of incubation at 60°C. All subsequent loading studies were performed using 1 μg A23187/mg lipid.

Using an optimized pH (pH 6) and ionophore concentration (1 μg A23187/mg lipid), rapid and

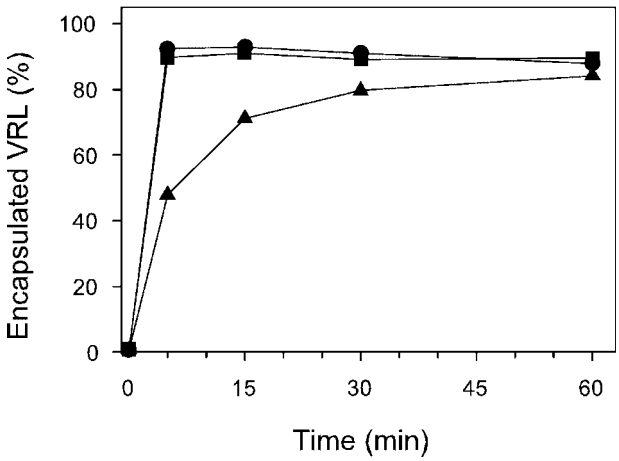

Figure 2. Optimization of ionophore content required for efficient loading of VRL into SM/Chol liposomes at a high initial D/L ratio (0.3, wt/wt). Using SM/Chol liposomes at an initial D/L of 0.3 (wt/wt), VRL was loaded at three different ionophore concentrations: 0.2 μg A23187/mg lipid (▲), 1.0 μg A23187/mg lipid (■), and 2.0 μg A23187/mg lipid (●). The internal $MgSO_4$ concentration was 300 mM, the pH of the external buffer was 6 and the temperature was 60°C. The results presented are representative of three independent experiments.

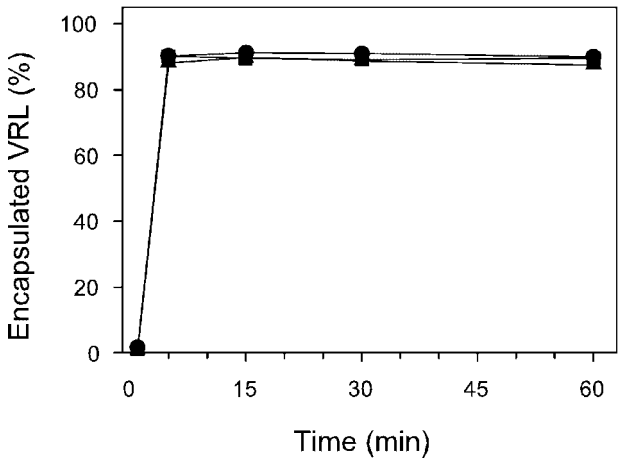

Figure 3. Optimized conditions for encapsulation of VRL at high D/L ratios. VRL was loaded at initial D/L ratios of 0.1 (●), 0.2 (■), and 0.3 (▲) wt/wt using the following loading conditions: 1 μg A23187/mg lipid, pH 6, 60°C and 300 mM internal $MgSO_4$. The results presented are representative of three independent experiments.

efficient loading of VRL (>90%) could be achieved within 5 min of incubation at 60°C at D/L ratios from 0.1 to 0.3 wt/wt (Figure 3). Variability in encapsulation efficiency was sometimes an issue associated with drug loading at higher D/L ratios. In this regard, a series of formulations ($n = 6$) that were prepared at an initial D/L ratio of 0.3 (wt/wt), using the conditions detailed above, showed variable encapsulation efficiencies (80%–100%). Having previously optimized external pH, temperature, and ionophore concentration, the only parameter that remained to be evaluated was the internal $MgSO_4$ concentration. Loading efficiencies were found to be consistently above 90% at 450 and 600 mM internal $MgSO_4$. Consequently, the final optimized set of loading conditions that achieved both reproducible loading efficiencies and superior drug retention properties *in vivo* (see "Discussion") were: SM/Chol liposomes (55/45, molar ratio; 15 mg/mL total lipid, 450 mM internal $MgSO_4$, pH 6), 25 mM EDTA, 50 mM phosphate buffer, VRL in 300 mM sucrose (10 mg/mL), 1 μg A23187/mg lipid, 60°C. This formulation of VRL was named INX-0125 and was used in all subsequent studies.

The divalent cation ionophore A23187 is a low molecular weight, hydrophobic weak acid that readily partitions into lipid bilayers. Since ionophores can be toxic to cells and animals,[30] ensuring appropriate removal of ionophore from the liposome formulation after drug loading is critical.

The tangential flow (TF) process described above typically reduced the level of A23187 to <50 ng/mg total lipid in batches >1 L, as measured either by HPLC/UV or fluorescence methods. We have characterized the removal of A23187 by TF in detail (data not shown). The distribution of ionophore between vesicles and solution was pH-dependent and, at pH 7, approximately 50% of the ionophore was associated with vesicles, either bound to lipid or present as small aggregates. The remainder of the ionophore was present in the bulk solution as monomers and large aggregates. These two main fractions are in equilibrium; consequently, the tangential flow diafiltration process reduces the total ionophore content of the sample. The low levels of residual A23187 do not affect drug retention *in vitro* or *in vivo* and have no detectable effects on toxicity in rodent studies, which is consistent with the fact that, at the maximum tolerated dose of the formulation (20 mg/kg VRL in mice), the maximum administered dose of residual ionophore in these studies is still >1500-fold lower than its LD_{50} in rodents.[30]

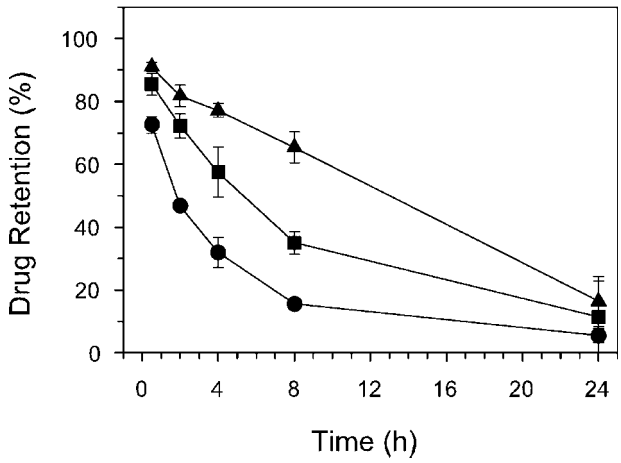

Figure 4. *In vivo* retention of VRL. VRL was loaded at initial D/L ratios of 0.1 (●), 0.2 (■), and 0.3 (▲) wt/wt using the conditions indicated in Figure 3. Liposomal VRL formulations containing [^{3}H]-VRL and a [^{14}C]-CHE lipid marker were injected intravenously into ICR mice at a lipid dose of 50 mg/kg. Blood samples were analyzed for lipid and drug content by dual label liquid scintillation counting. Each data point represents the mean drug retention ± standard deviation ($n = 4$) and the results were consistent between two independent studies.

Drug Retention and Intravesicular Drug

The primary goal of the formulation optimization studies was to achieve drug loading conditions that would allow efficient loading over a range of D/L ratios (e.g., 0.1–0.3, wt/wt), since these were to be used to evaluate and optimize drug retention *in vivo*. VRL retention in the SM/Chol liposomes after intravenous administration in mice was dependent on the D/L ratio with formulations having higher D/L ratios showing better drug retention (Figure 4). The lipid dose in these studies was held constant at 50 mg/kg. Approximately, 65, 35, and 15% of entrapped VRL was retained at 8 h post-injection at D/L ratios of 0.3, 0.2, and 0.1, respectively, and the calculated drug release half-lives for these formulations were 11, 5.1, and 1.8 h, respectively. *In vivo* drug retention was identical for liposomes that initially contained 300 mM or 450 mM $MgSO_4$, while liposomes that contained 600 mM $MgSO_4$ released drug more rapidly and had approximately 25% less entrapped drug than the other formulations at 8 h post injection (data not shown).

An examination of drug-loaded (D/L ratio 0.25, wt/wt) and drug-free $MgSO_4$ (450 mM) liposomes by cryo-TEM revealed the presence of electron dense vesicles in the drug-loaded samples (Figure 5B), which was not observed for the drug-free liposomes (Figure 5A). To quantify this apparent difference in electron density of the liposomes, an analysis of pixel density was performed on 25 independent regions both inside and outside the liposomes. The pixel density in drug loaded liposomes (Figure 5B) was 137 ± 11.9 (mean ± standard deviation), whereas the areas outside the liposomes had a pixel density of 169 ± 9.34. On a grayscale, black is 0 and white is 256. Consequently, a quantitative increase in pixel density/darkness was observed for the drug-loaded liposomes ($p < 0.0001$, t-test). No significant difference in pixel intensity was observed for the empty liposomes (Figure 5A; 185 ± 6.38 inside vs. 181 ± 3.67 outside). The greater electron density of the VRL-loaded samples indicates that these liposomes may have formed an amorphous gel or precipitate within the vesicles.

Apart from the differences in electron density, no other obvious differences were observed between the drug-loaded and drug-free liposomes. The liposomes were spherical and the majority (>75%) of vesicles were unilamellar. The average diameter of the liposomes was 78 ± 24 nm by particle counting and measurement, which was slightly smaller than the results typically observed by QELS (105 ± 30 nm). In our experience and that of others, it is common to observe a small but consistent difference in mean particle sizes using

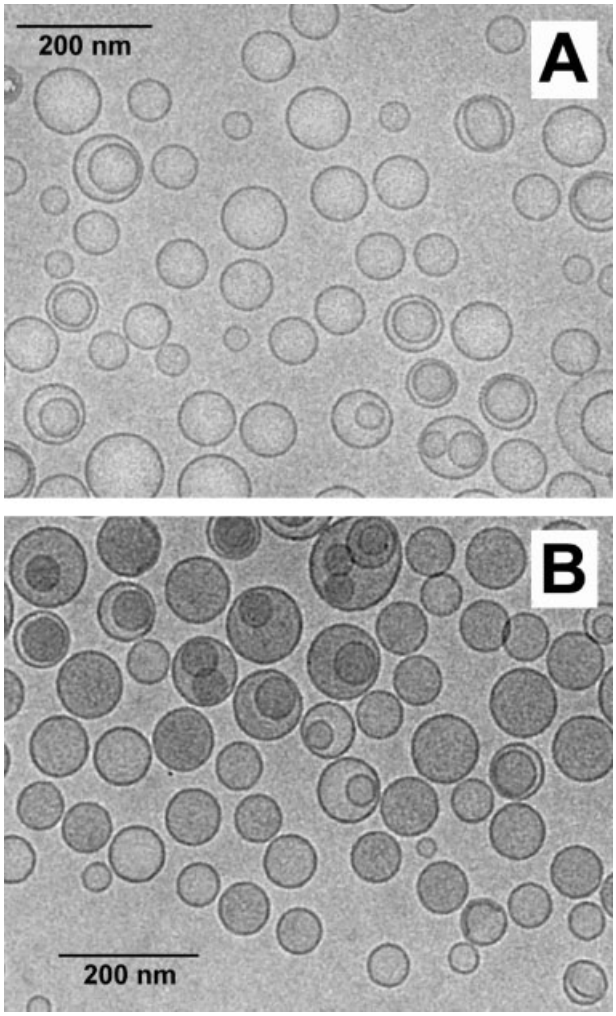

Figure 5. Cryo-electron microscopy of liposomal VRL. Cryogenic-transmission electron microscopy (cryo-TEM) was performed on empty SM/Chol liposomes (panel A; 450 mM internal magnesium sulfate) and a drug-loaded SM/Chol formulation containing VRL (panel B; final D/L ratio, 0.25, wt/wt). The micrographs reveal the presence of electron dense amorphous precipitates (darker intravesicular regions) in panel B. Magnification is 100000×.

the two sizing methods, which are based on different types of diameters.[31,32] For liposomes <400 nm, diameters measured by QELS (Z-average) are often larger (up to 30%) than those obtained using cryo-TEM or freeze-fracture (number average). QELS measures time-dependent fluctuations in scattered light intensity that result from particle diffusion, allowing for calculation of hydrodynamic size of the vesicles, which includes surface-bound water layers. In contrast, electron microscopy measures only the diameter of the lipid membrane and also allows discrimination of larger, non-liposomal particles that can have a significant influence ("over-weighted") in QELS measurements.

Comparative Pharmacokinetics of INX-0125 and VRL

To determine whether the improved drug retention observed in Figure 4 translated into a relative improvement in plasma pharmacokinetics compared with VRL, mice were injected intravenously with either VRL or INX-0125 at 20 mg/kg. A large increase in the circulating plasma concentration of VRL was observed over 48 h when injected as INX-0125 (Figure 6). At 48 h post injection, the plasma drug concentration for INX-0125 was 1.35 μg/mL, while a similar concentration was obtained after only 1–2 h for non-liposomal VRL. Using non-compartmental analysis, the plasma AUC_{0-48h}, clearance and volume of distribution (V_d) for INX-0125 were determined to be 2790 h × mg/L, 0.00713 L/h/kg, and 0.0532 L/kg, respectively. Pharmacokinetic parameters for VRL were not determined as the later time points were below the limit of detection. However, using different methodologies, these parameters have been reported previously in different strains of

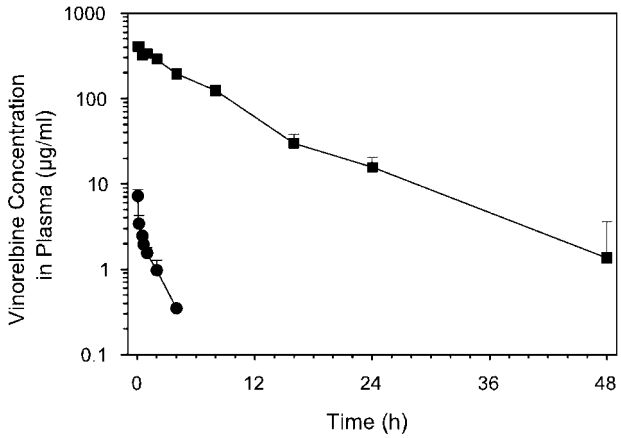

Figure 6. Comparative pharmacokinetics of INX-0125 and VRL in ICR mice. VRL was encapsulated in SM/Chol liposomes (INX-0125; 55/45, mol%) at a D/L ratio of 0.3 (wt/wt) as described in "Materials and Methods." ICR mice were injected intravenously with VRL (●) or INX-0125 (■) at 20 mg/kg. Plasma concentrations of VRL were determined by HPLC as described in "Materials and Methods." Each time point represents the mean plasma VRL concentration ± standard deviation ($n = 5$). Beyond 4 h, the plasma drug concentration for mice treated with VRL alone was below the lower limit of measurability for the assay.

mice as 5.3–8.4 h × mg/L (dose normalized), 2.39–3.78 L/h/kg and 12.7–40.1 L/kg, for plasma AUC, clearance and V_d, respectively.[33,34]

Antitumor Activity of INX-0125 Compared with Other Vinca Alkaloids

The biological activity of the INX-0125 liposomal VRL formulation (D/L ratio, 0.3 wt/wt) and its relative antitumor activity compared with other vinca alkaloids was evaluated in a subcutaneous murine B16 melanoma model after a single bolus injection (Table 1). This model has been used previously for VRL[35,36] and other vinca alkaloid formulations[37,38] and is a non-immunogenic, highly aggressive tumor model that is relatively insensitive to most treatments and has been used extensively in early drug screening studies.[39,40] SM/Chol formulations of VCR, vinblastine, and VRL, each loaded using the ionophore method and having similar drug release rates, were injected intravenously into B16 tumor-bearing mice at various doses. Based on NCI criteria for tumor growth inhibition and activity,[23] none of the free drugs were active (i.e., %T/C were all >42) at or near their reported maximum tolerated doses (MTD). A single injection of SM/Chol liposomal VRL (INX-0125) or liposomal VBL formulations were considered active by NCI criteria, with %T/C values of 18% (22.0 mg/kg) and 18% (9.0 mg/kg), respectively; while liposomal VCR (1.8 mg/kg) was marginally active in this model with a %T/C value of 34% at its MTD.

Formulation Stability

Having optimized the INX-0125 VRL formulation for its *in vivo* drug retention properties and having further demonstrated the antitumor activity of this formulation, we next wanted to evaluate whether the INX-0125 formulation of VRL would meet certain stability criteria for use as a single vial, wet formulation and clinical product. We have previously shown that the lipid components of SM/Chol liposomes are highly stable in the presence of vincristine[26]; however,

Table 1. Antitumor Activity of Liposomal Vinorelbine (INX-0125) and Other Vinca Alkaloids in Murine B16 Melanoma

			Tolerability and Antitumor Activity	
Formulation	Drug Dose (mg/kg)	Lipid Dose (mg/kg)	Maximum Mean % Weight Loss	%T/C
Liposomal VRL	22	69	−3.4	18
(D/L 0.32, wt/wt)	11	34	+[a]	33
	5.5	17	+	51
VRL	22	–	−4.3	76
	11	–	+	66
Liposomal VCR	1.8	18	−3.4	34
(D/L 0.10, wt/wt)	0.9	9.0	+	34
VCR	1.8	–	−6.9	61
	0.9	–	+	57
Liposomal VBL	9.0	30	−6.5	18
(D/L 0.30, wt/wt)	4.5	15	−3.3	29
	2.2	7.3	+	54
VBL	9.0	–	−4.4	74
	4.5	–	+	63

B16 tumor bearing mice were injected with a single intravenous bolus injection of free or SM/Chol liposomal formulations of VRL, vincristine (VCR), or vinblastine (VBL). Tumor growth inhibition (%T/C) was calculated from the median tumor volumes of treated and control groups ($n = 8$) on day 14 after tumor cell implantation.

in that particular formulation, the VCR was not stable for >1 year at 4°C, necessitating the use of a multi-vial, kit format for clinical applications, which enables drug loading immediately prior to administration.

The stability of the optimized liposomal VRL formulation (INX-0125; SM/Chol, 55/45 molar ratio; D/L ratio 0.3, wt/wt; initial internal $MgSO_4$ concentration, 450 mM) was compared with that of free VRL, using a solution of VRL (OmniChem SA) in acetate buffer (pH 4), as well as the commercial formulation, Navelbine® (supplied as a solution). Samples were incubated at 5, 25, 40, and 60°C in sealed vials and the integrity of the drug was analyzed at various time intervals using HPLC. The results of the accelerated degradation studies are summarized in Table 2. As expected, the rate of VRL decomposition increased at higher temperatures. Moreover, liposome encapsulation of VRL did not alter the stability of the drug. The amount of intact VRL after 3 weeks of storage at 60°C was 76% for both INX-0125 and Navelbine®, and 81% for the VRL solution (pH 4.0). At 40°C, no decomposition of VRL was observed in INX-0125 after 6 weeks, while 11% and 8% decomposition was observed for VRL (pH 4.0) and Navelbine®, respectively. At the lower temperatures (5 and 25°C), all VRL formulations showed minimal or no decomposition after 6 weeks. Based on these studies, the shelf-life of liposomal VRL was estimated, assuming pseudo-first order kinetics, as >1.5 years (5°C) and was comparable with the commercial Navelbine® formulation. These stability values are consistent with real time stability assessments conducted at 2–8°C, in which 100% of the HPLC peak area for intact VRL was obtained after 1 year (Table 3) and indicate that a formulation with >98% intact drug may be achievable at 1.5–2.0 years.

While chemical stability is an important feature of the INX-0125 formulation, particle size and drug retention are also critical for a clinical liposomal product. Liposomal VRL did not show any significant changes in liposome size during storage at the various temperatures used for accelerated degradation. All solutions remained clear and free of particle settling during this study. The size of the liposomal VRL formulation has also been analyzed after 100 day (results not shown) and 1 year storage periods at 2–8°C. The results of this study are summarized in Table 3. The average size and polydispersity (107 ± 33 nm) of

Table 2. Stability of Conventional and INX-0125 Vinorelbine Formulations Under Accelerated Degradation Conditions

		Vinorelbine Integrity in Formulation (%)		
Temperature (°C)	Time (Days)	INX-0125	VRL, pH 4[a]	Navelbine®
5	1	101.9 ± 1.6	100.1 ± 0.9	100.3 ± 0.5
	14	100.9 ± 0.4	102.2 ± 0.2	101.2 ± 0.3
	42	99.1 ± 0.1	97.0 ± 0.5	98.9 ± 0.1
25	7	102.8 ± 0.8	97.9 ± 0.4	102.6 ± 0.3
	14	101.5 ± 0.7	100.8 ± 0.6	102.3 ± 0.1
	42	101.6 ± 0.3	96.6 ± 0.4	100.5 ± 0.1
40	3	102.5 ± 1.6	98.8 ± 0.4	98.8 ± 0.5
	7	104.0 ± 0.5	97.2 ± 1.0	98.2 ± 0.6
	14	101.9 ± 1.1	95.8 ± 0.1	98.0 ± 0.3
	28	98.0 ± 1.9	95.1 ± 0.2	95.0 ± 0.2
	42	102.2 ± 0.5	89.1 ± 0.1	91.6 ± 0.2
60	1	99.4 ± 0.3	100.3 ± 0.3	99.9 ± 0.3
	3	102.6 ± 0.2	99.6 ± 0.3	99.0 ± 0.8
	5	102.6 ± 0.2	100.1 ± 0.1	98.1 ± 0.9
	7	100.8 ± 0.5	97.5 ± 0.1	91.5 ± 0.5
	10	98.4 ± 0.3	95.2 ± 0.1	87.9 ± 0.5
	14	87.5 ± 0.1	91.5 ± 0.1	84.4 ± 0.3
	21	76.4 ± 0.2	81.4 ± 0.2	75.9 ± 0.4

VRL formulations were incubated at 5, 25, 40, and 60°C. At the indicated times, samples were analyzed for the presence of intact VRL using an HPLC assay. Data represent the amount of intact VRL (mean ± standard deviation; $n = 4$), expressed as percentage of the initial drug concentration.

[a]Lyophilized powder from OmniChem SA; dissolved in 50 mM sodium acetate buffer containing 300 mM sucrose, pH 4.

Table 3. Stability of INX-0125 (SM/Chol VRL Formulation) After 1 Year at 2–8°C

Sample	HPLC Analysis Peak Area (%)	Drug Retention (% of total)	Vesicle Size (nm)
INX-0125	100.1 ± 0.5	96.9 ± 1.1	107 ± 33
VRL, pH 4[a]	99.9 ± 0.2	N/A	N/A
Navelbine®	100.2 ± 0.4	N/A	N/A

The data indicate the amount of intact VRL ($n = 4$), % drug retention ($n = 4$), and typical particle sizes after 1 year. Values represent mean ± standard deviation.

[a]Lyophilized powder from OmniChem SA; dissolved in 50 mM sodium acetate buffer containing 300 mM sucrose, pH 4.

the liposome sample remained unchanged over 1 year. In addition, VRL showed excellent retention in the optimized INX-0125 VRL formulation during the accelerated degradation studies. Samples stored at 40 and 60°C released drug slowly at a rate of ~0.08 and 0.7% of encapsulated drug per day, respectively. No drug leakage was detected in samples stored at 25 and 5°C during the 6 week study. Moreover, >95% of the drug was retained in liposomes that were stored for 1 year at 2–8°C (Table 3).

DISCUSSION

Drug retention is a critical feature in the design of liposomal systems with optimal *in vivo* properties and therapeutic activity.[41] VRL is a highly lipophilic member of the vinca alkaloids, exhibiting significantly enhanced membrane permeability and transport across plasma membrane vesicles compared with other vinca alkaloids.[5,6,24,25] Moreover, the increased lipophilicity of VRL has been proposed to account for its large relative volume of distribution and increased plasma clearance in mice.[5,34] This permeability property also presents a challenge for developing liposome formulations that release the drug slowly. Recently, a SM/Chol formulation has been described that provided improved drug retention for another vinca alkaloid, VCR.[26] In this study, we have optimized a SM/Chol formulation of VRL that was pharmaceutically stable for up to 1 year at 4°C, could be loaded at high efficiency (>90%) in small (~100 nm) liposomes, and exhibits suitable drug payout rates *in vivo* and promising activity in a B16 murine melanoma model. To accomplish this, we used an ionophore loading method, previously described by Fenske et al.[17] to achieve D/L ratios of up to 0.3 (wt/wt).

The SM/Chol (55/45, molar ratio) lipid composition is well-suited for the permeability characteristics of VRL. Egg SM is composed primarily of saturated acyl chains and contains a single trans double bond in the sphingosine backbone.[42] Moreover, SM is able to form strong intermolecular hydrogen bonds with neighboring cholesterol molecules (present in this formulation at 45 mol%) to provide a very rigid membrane that is relatively impermeable compared with other lipid compositions.[26,43] The bilayer stabilizing properties of cholesterol at greater than 30 mol% greatly reduces the potential for rapid drug release and interactions with plasma proteins *in vivo*,[44] a feature that is important for maintaining consistent drug payout rates. As a result of these features, SM/Chol liposome formulations of drugs such as VCR and ciprofloxacin have been shown to have longer circulation times and better drug retention properties *in vitro* and *in vivo* compared with other liposomes.[17,26]

It was interesting that optimal *in vivo* drug retention for VRL in SM/Chol liposomes required the use of a high D/L ratio. The rationale for this was initially unclear; however, further examination of empty and drug-loaded liposomes revealed the presence of electron dense intravesicular regions within the drug-loaded liposomes, which were not observed in the empty liposomes, suggesting the presence of an amorphous drug precipitate or gel within these liposomes. Several drugs, including anthracyclines (e.g., doxorubicin, daunorubicin),[45,46] anthracenediones (e.g., mitoxantrone),[47] antibiotics,[48,49] and camptothecins,[50] have been shown to form ordered, crystalline precipitates in the presence of di- and trivalent metal ions. In liposomes, these precipitates typically lead to the formation of ovaloid vesicles with defined linear structures that run along the long axis of the vesicles, giving the vesicles an appear-

ance that is often described as a "coffee bean" structure.[51] Sulfate salts are also possible and have been observed for various drugs, including anthracyclines[52] and camptothecin analogues.[53] Recently, Abraham et al.[50] demonstrated a correlation between topotecan release from DSPC/Chol liposomes and the presence of crystalline structures within the liposomes. Interestingly, in that study, variations in drug release were observed at different D/L ratios despite any obvious differences in the precipitated structures. From this, the authors concluded that more than one type of precipitated structure, including both amorphous precipitates and ordered crystals, might be present within the liposomes and contribute to different drug release rates at higher D/L ratios. The presence of electron dense regions and absence of defined crystal patterns within the liposomal VRL formulation supports the hypothesis that these regions represent an amorphous precipitate and adds additional evidence to support a correlation between intravesicular drug precipitation and extended drug release rates. While drug precipitation represents the most likely explanation for altered release rates, other explanations may also exist. In particular, the increased drug retention at higher D/L ratios could result from the drug acting as a buffer, exhibiting increasingly higher buffering capacities with increasing internal drug concentrations (D/L ratios), thereby delaying the collapse of the proton gradient. The structural features of these liposomes as they relate to drug precipitation and release rates are currently being examined in greater detail.

VRL is an important drug in cancer chemotherapy. While VRL is currently approved for clinical use in advanced non-small cell lung and metastatic breast cancers, it has also shown a broad spectrum of activity in preclinical studies and other clinical indications.[1–3,54] The enhanced activity of INX-0125 in subcutaneous B16 melanoma relative to VRL and a similar liposomal formulation of VCR demonstrates the advantages of both the drug and this type of optimized delivery system. VRL possesses several interesting features and properties, relative to other naturally occurring vinca alkaloids, including its improved activity against solid tumors and its altered toxicity profile.[1,3,4,54] These differences are thought to result from its higher affinity for mitotic versus axonal microtubules. Studies that have examined the effects of VCR, vinblastine and VRL on mitotic and axonal microtubules indicate that a 20-fold increase in VRL concentration is required to depolymerize axonal (neurotoxic potential) versus mitotic (antitumor potential) microtubules, suggesting an advantage for VRL over other vinca alkaloids for minimizing neurotoxicity.[55] In contrast, VCR and vinblastine depolymerized both axonal and mitotic microtubules within a relatively narrow concentration range. Recently, investigators have attempted to optimize the clinical activity of VRL in metastatic breast cancer patients by extending drug exposure through 96 h continuous infusion,[13,56] however, cumulative toxicities (neutropenic fever and stomatitis) necessitated dose reductions in many patients. Consequently, an alternate formulation of VRL with long circulation times, altered tissue distribution, and controlled drug release rates, such as the one described here, may offer clinical advantages and an improved therapeutic index.

A comparison of the pharmacokinetic parameters obtained for the SM/Chol liposomal VRL formulation with previously published values for VRL in mice indicates a large increase in plasma AUC (>330-fold), as well as decreases in clearance (>330-fold) and V_d (>240-fold) for this formulation. We have previously demonstrated, using a SM/Chol liposomal formulation of VCR in which the drug was loaded by a pH-gradient method, that increases in plasma AUC of this magnitude result in significant increases (5 to 15-fold) in tumor drug accumulation relative to free drug.[26] Similar increases were also observed for INX-0125 relative to VRL in MX-1 breast (~9.5-fold) and HT-29 colon (~4.6-fold) tumor xenograft models in nude mice (data not shown). Moreover, these results are generally consistent with preliminary results reported for a pegylated (Stealth™) liposome formulation of VRL, although the relative improvement in antitumor activity in a human pancreatic (AsPC-1) xenograft model was limited for the pegylated liposome formulation.[57]

Stability is a critical feature of any formulation destined for clinical development. One of the potential concerns in using drug formulations composed of saturated phospholipids is the possibility of increased flocculation and aggregation over time. Moreover, degradation of lipid components as a result of oxidation and/or hydrolysis results in poor drug retention. In this study, stability of the optimized VRL formulation with respect to intact VRL, consistent particle size and homogeneity and drug retention was demonstrated over 1 year at 2–8°C. Typical commercial product stability requirements for a wet formulation require drug and excipient stability

for at least 1 year, and preferably 2 years or more. Much of the liposome stability can be attributed to the presence of SM, which is predominantly saturated (limits oxidation) and has acyl chains linked through an amide bond (limits hydrolysis). In this regard, superior resistance to hydrolysis and improved drug retention on storage has been previously demonstrated for a SM/Chol (55/45, molar ratio) liposomal formulation of VCR compared with a DSPC/Chol (55/45, molar ratio) formulation.[26] VRL stability in INX-0125 was predicted to be >1.5 years based on accelerated and real-time (5 and 25°C) degradation studies which, in combination with the lipid stability, make this liposomal formulation of VRL viable as a single vial, wet formulation.

The studies reported herein describe the characterization and optimization of a single vial SM/Chol liposomal formulation of VRL with optimized drug retention properties and promising antitumor activity. Of particular importance, these studies elaborate on the growing list of observations[50,52] that are beginning to define the relationships between D/L ratios, drug precipitation in liposomes, and drug retention. Finally, studies are ongoing to define the therapeutic benefits of this optimized liposomal formulation of VRL in a expanded series of preclinical models, as well as the relationship between drug release rates and efficacy in these models.

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