Improved Pharmacokinetics and Efficacy of a Highly Stable Nanoliposomal Vinorelbine

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ABSTRACT

Effective liposomal formulations of vinorelbine (5′ nor-anhydrovinblastine; VRL) have been elusive due to vinorelbine's hydrophobic structure and resulting difficulty in stabilizing the drug inside the nanocarrier. Triethylammonium salts of several polyanionic trapping agents were used initially to prepare minimally pegylated nanoliposomal vinorelbine formulations with a wide range of drug release rates. Sulfate, poly(phosphate), and sucrose octasulfate were used to stabilize vinorelbine intraliposomally while in circulation, with varying degrees of effectiveness. The release rate of vinorelbine from the liposomal carrier was affected by both the chemical nature of the trapping agent and the resulting drug-to-lipid ratio, with liposomes prepared using sucrose octasulfate displaying the longest half-life in circulation (9.4 h) and in vivo retention in the nanoparticle ($t_{1/2} = 27.2$ h). Efficacy was considerably improved in both a human colon carcinoma (HT-29) and a murine (C-26) colon carcinoma model

when vinorelbine was stably encapsulated in liposomes using triethylammonium sucrose octasulfate. Early difficulties in preparing highly pegylated formulations were later overcome by substituting a neutral distearoylglycerol anchor for the more commonly used anionic distearoylphosphatidylethanolamine anchor. The new pegylated nanoliposomal vinorelbine displayed high encapsulation efficiency and in vivo drug retention, and it was highly active against human breast and lung tumor xenografts. Acute toxicity of the drug in immunocompetent mice slightly decreased upon encapsulation in liposomes, with a maximum tolerated dose of 17.5 mg VRL/kg for free vinorelbine and 23.8 mg VRL/kg for nanoliposomal vinorelbine. Our results demonstrate that a highly active, stable, and long-circulating liposomal vinorelbine can be prepared and warrants further study in the treatment of cancer.

Nanoparticles such as small unilamellar liposomes have been shown to improve the pharmacokinetics and tumor localization of encapsulated drugs, modify the toxicities associated with a particular drug, and ultimately enhance antitumor efficacy compared with the nonencapsulated drug (Drummond et al., 2008). For the success of liposomal drug

delivery, the stable encapsulation of an amphipathic drug in the lumen (Mayer et al., 1985; Haran et al., 1993; Webb et al., 1995; Drummond et al., 2008) of liposomes with long-circulating properties (Allen et al., 2006; Drummond et al., 2008) is preferred, resulting in the ability of such liposomes to localize preferentially in solid tumors through the enhanced permeability and retention effect (Matsumura and Maeda, 1986; Drummond et al., 1999). A liposomal drug is in effect a prodrug, inactive until released from the confines of its carrier, rendering it bioavailable and capable of subsequently acting on its molecular target. Therefore, the ability of the carrier to deliver the active chemical agent to the site of disease, and to subsequently release the drug so as to achieve the desired therapeutic outcome, are equally important. Thus, the rates of drug release from the liposomal carrier in

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ABBREVIATIONS: nLs-VRL, nanoliposomal vinorelbine; DSPC, 1,2-distearoyl-3-sn-phosphatidylcholine; PEG-DSG, methoxypolyethylene glycol-1,2-distearoylglycerol ether; PEG-DSPE, N-(methoxypoly(ethyleneglycol)oxycarbonyl-1,2-distearoylphosphatidylethanolamine; PL, phospholipid; VRL, vinorelbine; CHE, cholesterylhexadecyl ether; HPLC, high-performance liquid chromatography; %ID, percentage of injected dose; MRT, mean residence time in the circulation; AUC_∞, area under the concentration versus time curve in plasma based on the sum of exponential terms; MTD, maximal tolerated dose; CPT-11, 7-ethyl-10-hydroxycamptothecin; TEA, triethylammonium; SOS, sucrose octasulfate; TEA₈SOS, triethylammonium sucrose octasulfate; Pn, poly(phosphate); pLs-VRL, pegylated liposomal vinorelbine; TEA-Pn, triethylammonium poly(phosphate).

vivo are critical for the optimal construction of a liposomal therapeutic.

Vinca alkaloids are potent anticancer agents that act by binding to tubulin and preventing tubulin assembly into microtubules, ultimately leading to mitotic inhibition and induction of apoptosis (Jordan et al., 1991). Vinorelbine (5'-nor-anhydro-vinblastine) is a clinically approved drug that is frequently used in the treatment of various cancers, including metastatic breast cancer (Weber et al., 1995) and non-small-cell lung cancer (Gridelli and De Vivo, 2002). Vinorelbine is better tolerated than many of the other vinca alkaloids, including reduced neurotoxicity (Mathé and Reizenstein, 1985) due to its reduced affinity for axonal microtubules (Binet et al., 1990). Liposome-based delivery may further improve the therapeutic index for vinorelbine through an improved pharmacokinetic profile and specific delivery to solid tumors, similar to that observed for liposomal doxorubicin. Although several liposomal formulations of vincristine have been described in the literature (Allen et al., 1995; Webb et al., 1995; Zhu et al., 1996; Embree et al., 1998), vinorelbine has been considerably more difficult to stabilize in liposomes (Semple et al., 2005; Zhigaltsev et al., 2005), resulting in a majority of the drug being released from the particle before reaching the tumor.

We have recently described a novel and highly effective intraliposomal stabilization strategy for improving the in vivo retention of difficult to formulate anticancer drugs (Drummond et al., 2005, 2006, 2008). We use the term "nanoliposomal drug" to describe a nanocarrier that includes a lipid scaffold encapsulating a nanoscale drug complex that improves drug retention in vivo. Here, we use this technology to prepare multiple nanoliposomal vinorelbine (nLs-VRL) formulations with a wide range of in vivo drug release rates. The development of a successful liposomal drug construct requires tailoring of the liposome formulation to the physicochemical properties of the drug to be encapsulated. Even different drugs within the same drug class often require distinctive formulations for optimal activity. Mitoxantrone, for example, is encapsulated too stably in liposomes typically used to entrap doxorubicin, and it requires a modification to the composition for optimal activity (Lim et al., 1997). Likewise, vincristine is stabilized much more easily in liposomes than vinorelbine (Semple et al., 2005; Zhigaltsev et al., 2005), despite both being vinca alkaloids. This work aimed to optimize the liposome formulation parameters for vinorelbine to produce a long-circulating, stable, and active liposomal vinorelbine.

Formulations of vincristine that included distearoylphosphatidylcholine (DSPC) were previously shown to be unstable, rapidly leaking their drug in the blood upon intravenous administration (Webb et al., 1995). Unexpectedly, the use of triethylammonium sucrose octasulfate gradients to prepare liposomal vinorelbine allowed for remarkable in vivo stability, even using a DSPC-based lipid membrane composition. The in vivo antitumor efficacy of liposomal vinorelbine was shown to be greatly improved upon liposome encapsulation using sucrose octasulfate as an intraliposomal trapping agent. The acute toxicity of this liposomal vinorelbine was also slightly improved compared with free vinorelbine.

Materials and Methods

Materials. DSPC and poly(ethylene)glycol PEG₂₀₀₀-derivatized distearoylphosphatidylethanolamine (PEG-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol was obtained from Calbiochem (La Jolla, CA). Vinorelbine tartrate (10 mg/ml; GlaxoSmithKline, Uxbridge, Middlesex, UK) was obtained from the pharmacy. Sucrose octasulfate (sodium salt) was purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada). mPEG₂₀₀₀-distearoylglycerol (PEG-DSG) was purchased from NOF Corporation (White Plains, NY). Sepharose CL-4B and Sephadex G-75 size exclusion resins, Dowex 50W-8X-200 cation exchange resin, and triethylamine were all obtained from Sigma-Aldrich (St. Louis, MO). [³H]cholesterylhexadecyl ether (48.3 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA).

Preparation of Liposomes. Liposomes with entrapped polyvalent anionic salts were prepared as described previously (Drummond et al., 2006). In brief, triethylammonium salts of sucrose octasulfate and poly(phosphate) ($n = 13-18$) were prepared by ion exchange chromatography from the corresponding commercially available sodium salts. Triethylammonium sulfate was prepared by titration of 250 mM sulfuric acid with concentrated triethylamine and diluted to a final concentration of 200 mM. Ammonium sulfate solution (0.25 M) was prepared by dissolving the commercial salt in water. The prototype lipid composition used in these studies was 3 mol parts of DSPC, 2 mol parts of cholesterol, and 0.015 mol parts of PEG-DSPE. For studies characterizing the effect of liposome PEGylation on the drug loading, PEG-DSPE was included in the lipid matrix at concentrations ranging from 0.5 to 10 mol% of the phospholipid (PL), or PEG-DSG was incorporated simply at 10 mol% of the PL. For in vivo stability studies, [³H]cholesterylhexadecyl ether was included at 0.5 μ Ci/ μ mol PL. Liposomes with the average size range of 8 to 95 nm or 100 to 115 nm were prepared by extrusion of lipid suspensions, hydrated in the polyvalent anionic salt solutions, through polycarbonate membranes (Nucleopore; Corning Life Sciences, Lowell, MA) with the pore size of 0.08 or 0.1 μ m, respectively. Particle size was determined by photon correlation spectroscopy using an N4 Plus particle size analyzer (Beckman Coulter, Fullerton, CA). The size is reported as volume weighted diameter $\pm$ S.D. Unencapsulated multivalent anion salts were removed by gel filtration of on a Sepharose CL-4B column eluted with 5 mM HEPES-Na and 5% dextrose, pH 6.5.

Loading of Vinorelbine. Vinorelbine (VRL) in the form of stock solution of vinorelbine bitartrate (10 mg/ml USP) was added to the liposomes at a drug-to-phospholipid ratio of 350 g/mol, the pH was adjusted to 6.5 using 1 N NaOH, and the mixture was incubated at $60 \pm 2^\circ\text{C}$ for 30 min. For characterizing the effect of the drug-to-PL ratio on drug loading, the amount of drug added was varied from 150 to 550 g VRL/mol PL. In all subsequent studies a VRL-to-PL ratio of 350 g VRL/mol PL was used. The mixture was then chilled on ice for 15 min, and unencapsulated drug was removed by Sephadex G-75 gel filtration chromatography, eluting with HBS-6.5 buffer (20 mM HEPES and 135 mM NaCl, pH 6.5). Aliquots of purified liposomes were then solubilized in a solution composed of methanol/0.1 M phosphoric acid [80:20 (vol:vol)] and analyzed for vinorelbine spectrophotometrically at 270 nm. Liposome phospholipid was quantified using the phosphate assay of Bartlett (1959) directly or after methanol-chloroform extraction for samples containing poly(phosphate) as the intraliposomal trapping agent.

Pharmacokinetics of Liposomal VRL Formulations with Tunable Release Rates. All studies involving animals were in accordance with institutionally approved animal research protocols. Liposomal VRL and lipid pharmacokinetics were studied in female Sprague-Dawley rats (190–210 g), with indwelling central venous catheters. Two rats per formulation were injected with a 0.2- to 0.3-ml bolus of [³H]CHE-labeled vinorelbine liposomes (5 mg of VRL per kg of the body weight). Blood samples (0.2–0.3 ml) were drawn at various times after injection and diluted with 0.3 ml of ice-cold

phosphate-buffered saline containing 0.04% EDTA, weighed, and the blood cells were separated by centrifugation. The supernatant fluids were collected and assayed for VRL by HPLC analysis as follows. The samples were spiked with vinblastine (internal standard), extracted with diethyl ether, and evaporated. The resulting residues were dissolved in the mobile phase consisting of aqueous 50 mM triethylammonium acetate, pH 5.5, and acetonitrile (58:42 by volume). The samples were loaded on a C-18 reverse phase silica column (250 × 4 mm i.d.; particle size, 5 µm; Supelco, Bellefonte, PA) preceded by a C-18 guard column. The column was eluted isocratically with the above-mentioned mobile phase at a flow rate of 1.0 ml/min. VRL was detected by absorbance at 270 nm. Typical retention times for VRL and vinblastine (internal standard) were 9.1 and 7.8 min, respectively, with a resolution of 2.1. Standards (0.25–25 µg/ml) were formed by addition of VRL (10 mg/ml USP stock solution) to blank rat plasma emulating the matrix of the samples. Recovery of VRL from plasma spikes ranged from 101 to 103% as determined by comparison with VRL extraction from phosphate-buffered saline. No interference was observed for either vinca alkaloid during the analysis of blank plasma. The average standard linearity (R^2) was 0.9993 with a detection limit of 0.25 µg/ml. The batch-to-batch precision over the range of standards (indicated as relative S.D.) for VRL retention time and peak area was 3.5 and 4.2%, respectively.

The liposome lipid label was quantitated by scintillation radioactivity counting using conventional methods. The liposome preparations with known drug and [^3H]CHE-lipid concentration were used as standards. Radioactivity standards contain equal amount of diluted rat plasma to account for quenching. The amount of VRL and the liposome lipid in the blood was calculated assuming the blood volume in milliliters as 6.5% of the body weight in grams and the hematocrit of 40%. The total amount of the lipid and the drug in the blood was expressed as percentage of injected dose (%ID) and plotted against post-injection time. The release of drug from the liposomes was determined by monitoring changes in the drug-to-lipid ratio as a function of time. Because free vinorelbine is cleared rapidly from the blood once it escapes the carrier, the drug-to-lipid ratio in the blood is a direct indication of the amount of drug retained inside liposomes. In brief, the VRL and lipid concentrations were determined by HPLC and scintillation counting of [^3H]CHE, as described above. Because both DSPC (Silvius and Leventis, 1993) and [^3H]CHE (Pool et al., 1982) are nonexchangeable lipids, the concentration of liposomal phospholipid in the blood can be determined indirectly by measuring [^3H]CHE using standard scintillation counting and then comparing it directly to a standard curve of the [^3H]CHE where the liposomal phospholipid (DSPC) concentration has been determined by phosphate analysis (Bartlett, 1959). The VRL-to-PL ratio was then determined and expressed as grams of VRL per mole of PL at prescribed time points and then used to calculate the percentage of the preinjection VRL-to-PL ratio (percentage of original drug-to-PL ratio) by dividing the VRL-to-PL ratio determined at a specified time point by the VRL-to-PL ratio of the initially injected sample (time 0).

Because the plots generally showed good agreement with monoexponential kinetics (linearity in semilogarithmic scale), blood half-lives of the drug, the lipid, and of the drug release from the liposomes were calculated from the best fit of the data to monoexponential decay equation using the TREND option of the Excel computer program (Microsoft, Redmond, WA). Pharmacokinetic parameters, including the volume of distribution, clearance, the mean residence time in the circulation (MRT), and the area under the concentration versus time curve (AUC_∞), were all determined by noncompartmental pharmacokinetics data analysis using PK Solutions 2.0 software (Summit Research Services, Montrose, CO).

Acute Toxicity of Liposomal VRL. The acute toxicities of free VRL, nLs-VRL, and nondrug-loaded triethylammonium sucrose octasulfate-encapsulated liposomes were compared by determining the maximal tolerated dose (MTD) following single intravenous injection in regular (immunocompetent) mice (female Swiss-Webster mice). MTD determinations generally followed the protocol adopted by the

United States National Cancer Institute Developmental Therapeutics Program as described in our previous work (Drummond et al., 2006). A brief description of the steps follows. Step 1: A range-seeking step with the dose escalation factor of 1.8 is used until acute mortality or terminal morbidity (within >1 day after injection) is observed in any of the animals. The dose one step below the mortality/terminal morbidity dose is recorded. Step 2: A range-seeking step is used with the dose escalation factor of 1.15 until acute mortality or terminal morbidity (within >1 day after injection) is observed in any of the animals. The dose one step below the mortality/terminal morbidity dose is recorded at tentative MTD. Step 3: Validation step. A group of five animals is injected intravenously (tail vein) with free or liposomal VRL at tentative MTD determined at step 2. The animals are followed for 7 days; the animal body weight is recorded twice weekly and compared with the preinjection weight. General health of the animals is observed (alertness, grooming, feeding, excreta, skin, fur, and mucous membrane conditions, ambulation, breathing, posture). If during the observation period there is no mortality, progressive morbidity, or weight loss in excess of 15% of the preinjection body weight, the dose is considered to be validated as acute single injection MTD. If any of these effects occur, the experiment is repeated at the next lower dose by a factor 1.15. To obtain additional statistics for validation step, the body weight dynamics of surviving animals was followed for up to 11 days after injection. Liposomes containing triethylammonium sucrose octasulfate (TEA_8SOS), prepared and purified as described above, but without the drug loading step, were used as a placebo (liposome-only) control. Free VRL (vinorelbine bitartrate USP, Navelbine; GlaxoSmithKline) was used directly from the vial and diluted with sterile 5% dextrose before injection.

Antitumor Efficacy of Liposomal VRL. The antitumor efficacy of nLs-VRL was studied in a syngeneic C-26 murine colon carcinoma tumor model, as well as in HT-29 (human colon carcinoma), BT-474 (human breast adenocarcinoma), and Calu-3 (human lung adenocarcinoma) xenograft models. All cells were acquired from American Type Culture Collection (Manassas, VA) and cultured according to American Type Culture Collection protocols. Cultured C-26 cells (2×10^5) were inoculated subcutaneously into the flank area of immunocompetent 6- to 8-week-old male BALB/c mice. At day 12 after inoculation, mice were randomly divided into six treatment groups of five animals per group. The tumor-bearing mice were injected through the tail-vein with free vinorelbine at 6, 8, or 12 mg/kg, and with nanoliposomal vinorelbine at 4 or 6 mg/kg every 3 days for a total of three injections. Cultured human carcinoma cells were similarly inoculated into homozygous nude mice (HT-29: BALB/c, female, 6 to 8 weeks old, 1×10^6 cells; Calu-3: NCR *nu/nu*, male, 5 to 6 weeks old, 1.5×10^7 cells; BT-474: NCR *nu/nu*, 5 to 6 weeks old, female, 2×10^7 cells), except that in the BT-474 study, the animals were preimplanted with a 60-day sustained release 0.72-mg 17β -estradiol pellets (Innovative Research of America, Inc., Sarasota, FL). After the development of tumors, the animals were randomly assigned to treatment groups. Treatment by intravenous (tail vein) injection of free or liposomally formulated drug started at day 16 (HT-29: six animals/group, tumors 5–8 mm in diameter), day 25 (BT-474: eight animals/group, tumor volumes 144–309 mm^3), or day 30 (Calu-3: nine animals/group, tumor volumes 70–150 mm^3) according to the schedules and doses indicated under *Results*. Control groups received equal volumes of physiological saline. Tumor volumes (V) were determined twice weekly by caliper measurements of the long (L) and short (W) axis of the tumor and calculated using formula $V = \text{LW}^2/2$ (Geran et al., 1972). Animal body weights were also determined twice weekly, and the general health of the animals was assessed daily to assess the extent of treatment-related toxicities. The average tumor sizes in control and treatment groups were plotted versus time and compared for statistical significant of the difference pairwise at various post-treatment days using Student's *t* test.

Results

Formulation of Vinorelbine in Minimally Pegylated Liposomes. A novel intraliposomal stabilization process for stable and high-capacity liposomal entrapment of difficult to formulate basic amphipathic drugs such as vinorelbine has been developed. The method uses triethylammonium salts of polyanionic compounds such as polyphosphate or sucrose octasulfate as intraliposomal trapping agents to improve liposomal drug encapsulation stability in vivo. The polyanionic compounds are thought to form complexes with varying degrees of stability with vinorelbine, and possibly precipitate or gelate the drug inside the liposomes, thus enhancing its stability even further. Initial attempts to load vinorelbine using any gradient-based loading strategy, into liposomes stabilized with high concentrations of PEG-DSPE (10 mol% total phospholipid), resulted in exceptionally poor encapsulation efficiencies (<25%); thus, all early development efforts were carried out with liposomes containing only minimal concentrations of PEG-DSPE (0.5 mol%). Incubation of vinorelbine with the liposomes containing polyvalent anions and transmembrane gradient of triethylammonium ion resulted in the efficient loading of the drug. The amount of drug loaded per unit of lipid matrix, as well as the percentage of encapsulated drug, depended on the nature of polyvalent ion. At the drug/lipid ratio of 150 g/mol PL, the loading was quantitative (>95%) with all three anions [SO_4 , poly(phosphate) (Pn), and SOS]. At 350 g/mol PL, the loading efficiency of $(\text{TEA})_2\text{SO}_4$ liposomes decreased to 75%, whereas both polymeric (Pn) and nonpolymeric (SOS) multivalent anions provided nearly quantitative loading at 99.3 and 100.7%, respectively. Remarkably, loading in the liposomes with SOS remained practically quantitative for drug/lipid ratios of up to 450 g/mol PL (101.4%) and only slightly decreased (88%) at 550 g/mol PL. The drug/lipid ratio of 350 g/mol PL, accepted for further studies, corresponds to a molar ratio of approximately 1 drug per 3.08 phospholipids, which translates into approximately 30,000 VRL molecules per nanoliposomal particle. This degree of loading compares favorably (is approximately 2-fold higher) with other reported liposomal vinorelbine formulations (Semple et al., 2005; Zhigaltsev et al., 2005) and is more than four times higher than that used for liposomal vincristine (Webb et al., 1995; Embree et al., 1998).

Pharmacokinetics of Liposomal Vinorelbine Formulated with Various Anions. In rats, liposomal encapsulation dramatically reduced the clearance of VRL from the circulation and increased the MRT and AUC_∞ in the blood (Fig. 1; Table 1). The extent of the improvement in the circulation longevity of the drug depended on the anion used to stabilize the drug inside the liposomes. Encapsulation in liposomes containing ammonium sulfate reduced the clearance of VRL from the circulation 220-fold. Encapsulation in the most stable liposome formulations using triethylammonium sucrose octasulfate resulted in an approximately 1800-fold reduction in clearance.

The in vivo stability of liposomal vinorelbine formulations was characterized by monitoring changes in the drug-to-lipid ratio over time as described under *Materials and Methods*. Liposomal vinorelbine formulated using ammonium sulfate was relatively unstable, with a $t_{1/2}$ value for in vivo drug release of only 1.8 h. Although the pharmacokinetics of vinorelbine were improved upon encapsulation in ammonium

Fig. 1. Blood pharmacokinetics of the liposomal VRL after intravenous bolus administration in rats. The liposomes are loaded using pre-entrapped ammonium sulfate (■), TEA-Pn (○, △), or TEA₂SOS (□). The liposomes were loaded at drug-to-phospholipid ratios of 220 μg VRL/ μmol PL for the $(\text{NH}_4)_2\text{SO}_4$ formulation, 200 (△) or 350 (○) μg VRL/ μmol PL for the poly(phosphate) formulation, and 350 μg VRL/ μmol PL for the TEA₂SOS formulation. Free VRL (●) was dissolved in 5% dextrose and injected. The pharmacokinetics of liposomal lipid (A) and VRL (B) were followed as a function of time using scintillation counting of [^3H]cholesterylhexadecyl ether for determination of the lipid label and HPLC analysis for VRL quantitation in the plasma as described under *Materials and Methods*. Concentrations of both drug and lipid are given as %ID. C, dynamics of the drug-to-liposomal lipid ratio in the blood of a rat in vivo after intravenous bolus administration for each of the various liposomal vinorelbine formulations. Two rats were used for determination of the pharmacokinetics, and the average and cumulative errors of those measurements are plotted.

sulfate liposomes ($t_{1/2}$ of 1.54 h versus 0.2 h), the rate of VRL release from the liposomes was considerably more rapid than the time required for maximal tumor localization of long-circulating liposomes (24–48 h). Liposomes loaded with VRL using TEA-Pn gradients were more stable than the $(\text{TEA})_2\text{SO}_4$ formulations and showed increased stability at

TABLE 1

Pharmacokinetic parameters for free and various liposomal vinorelbine formulations in rats

The data used to calculate the pharmacokinetic parameters for VRL when formulated either in the free form or liposomal form refer to the actual drug concentrations measured in the blood that were then used to calculate the %ID values found in the corresponding curves for Figs. 1B and 5A.

Formulation	$t_{1/2}$	AUC _∞	CL	V _d	MRT	$t_{1/2}$ (VRL Release) ^a
	<i>h</i>	$\mu\text{g} \cdot \text{h} / \text{ml}$	<i>ml/h</i>	<i>ml</i>		<i>h</i>
Free VRL	0.20	0.5	10,300	3007	0.3	
Ls-VRL [(NH ₄) ₂ SO ₄]	1.54	107.0	46.7	103.5	2.2	1.8
nLs-VRL [TEA-Pn-220]	3.25	253.2	19.7	92.6	4.7	4.2
nLs-VRL [TEA-Pn-350]	6.62	746.7	6.70	64.0	9.6	15.2
nLs-VRL [TEA-SOS]	9.43	879.3	5.69	77.4	13.6	27.2
pLs-VRL [TEA-SOS]	9.42	956.8	5.23	71.1	13.6	26.6

CL, clearance calculated from exponential terms; V_d, volume of distribution.^a $t_{1/2}$ value for VRL release refers to the half-life of VRL release from the liposomal carrier in the circulation upon intravenous administration.

higher drug-to-phospholipid ratios (Fig. 1C; Table 1). The liposomes loaded with VRL using TEA₈SOS demonstrated the greatest stability, with a $t_{1/2}$ value for drug leakage rate in circulation of 27.2 h.

Acute Toxicity of Liposomal Vinorelbine. The maximal tolerated dose of free VRL, nLs-VRL, and “empty” liposomes following a single intravenous dose was determined in normal Swiss-Webster mice. The MTD was 17.5 mg/kg for free VRL and 23.8 mg/kg for nanoliposomal VRL prepared with either 0.5% PEG-DSPE or 10% PEG-DSG. The relatively low toxicity of empty liposomes has been confirmed in numerous instances and is an attractive attribute for liposomes as drug delivery particles. However, in this study we perceived a need for confirming this low toxicity due to the nature of the trapping agent being used, i.e., sucrose octasulfate. Polyvalent anionic molecules, in particular, polymeric anions (heparin, suramin, dermatan sulfate, heparan sulfate, and dextran sulfate) are known to activate coagulation cascade enzymes and at high concentrations or suboptimal dosing, can even demonstrate considerable toxicity (Astrup et al., 1955; Flexner et al., 1991; Bitton et al., 1995). The MTD of empty liposomes formulated containing just TEA-SOS solution, was not achieved even at the highest administered dose of 583.2 μmol PL/kg. The dose of more than 600 μmol PL/kg was difficult to administer because of concentration and injection volume limitations. By comparison, the phospholipid dose at the MTD for nLs-VRL is 68 μmol PL/kg, and the dose typically used for in vivo efficacy studies (i.e., 5 mg VRL/kg) is 14.3 μmol PL/kg.

Antitumor Efficacy of Liposomal VRL in Murine Models of Cancer. Sparsely (0.5 mol% PEG-lipid) PEGylated liposomal vinorelbine (nLs-VRL) prepared using intraliposomal polyanionic stabilization was more effective than free drug against a variety of syngeneic or xenograft (human) tumors raised subcutaneously in mice. In a syngeneic murine colon carcinoma model (C-26), nLs-VRL prepared by the TEA₈SOS method at 0.5 mol% PEG-DSPE and 350 g VRL/mol PL, and given in four intravenous injections spaced at 3 days in the dose of 4 mg VRL/kg/injection, was considerably more efficacious ($P = 0.009$) in reducing the tumor growth than free drug given in the same schedule at 12 mg VRL/kg (Fig. 2A), indicating a minimal 3-fold improvement in the activity of nLs-VRL compared with free VRL. Due to the rapid growth rate of these tumors, it is possible that this improvement may prove to be even more substantial when nLs-VRL is tested in slower growing tumors, in which the rate of drug release relative to the rate of tumor growth is more substantial. The animal body weights in the course of

treatment showed little change (<10% decrease) consistent with the prior finding that the toxicity of liposomal vinorelbine was not in excess of that of free drug. Similar results were obtained with the same formulation in a human colon carcinoma xenograft model (HT-29) raised subcutaneously in homozygous nude mice. In the treatment regimen of four 5 mg VRL/kg i.v. doses of free or nLs-VRL, spaced at 3-day intervals, and initiated upon reaching an initial tumor diameter of 5 to 8 mm, nLs-VRL was considerably more efficacious in suppressing the growth of HT-29 tumors than free vinorelbine ($P < 0.001$), causing tumors to regress, whereas in the free drug group the tumors always continued to grow (Fig. 2B). There was little change in the animals' body weight during the course of treatment, indicating again the treatment was well tolerated and that liposomalization did not increase drug toxicity.

Effect of PEGylated Lipids on Loading of Vinorelbine in Liposomes. Despite the early success developing a highly stable and long-circulating liposomal vinorelbine formulation that was only minimally pegylated, additional strategies were pursued to overcome the barriers in loading vinorelbine into highly pegylated liposomes similar in lipid compositions used in the commercial pegylated liposomal doxorubicin (Doxil; Alza/Johnson & Johnson, Palo Alto, CA). Previously, we had observed that liposomes loaded with vinorelbine using ammonium sulfate gradients displayed a reduced efficiency of loading when PEG-DSPE was incorporated at concentrations greater than 3 mol% of the phospholipid component (data not shown). Here, we investigated the effect of PEG-DSPE on loading of vinorelbine loaded in DSPC/cholesterol liposomes using the TEA₈SOS gradient method described above. Loading was inhibited when the PEG-DSPE content was increased above 3 mol% (Fig. 3A). Loading in liposomes containing 10 mol% PEG-DSPE (a concentration similar to that used in the clinical preparation Doxil) was only 17.5% at this ratio. Substitution of the nonionic PEG-DSG for PEG-DSPE (Fig. 3B) resulted in quantitative loading at 10 mol% of the PEGylated lipid, suggesting the nonionic nature of PEG-DSG played a role in allowing for efficient VRL encapsulation using our drug loading and stabilization protocol. The pharmacokinetics of PEG-DSG-stabilized liposomes were similar to those of liposomal VRL containing only 0.5 mol% PEG-DSPE (Fig. 4A). Importantly, the inclusion of PEG-DSG in the formulation did not increase the rate of drug leakage from the liposomes (Fig. 4B; Table 1).

Antitumor Efficacy of Pegylated Liposomal VRL in Human Breast and Lung Carcinoma Xenografts. The antitumor efficacy of PEGylated liposomal vinorelbine (10

Fig. 2. Antitumor efficacy of free vinorelbine or liposomal vinorelbine in a syngeneic C-26 murine colon carcinoma model (A) and in HT-29 human colon cancer xenografts (B). A, male BALB/c mice inoculated subcutaneously with C-26 murine colon carcinoma cells were treated by intravenous administration with free VRL at 6 mg/kg (○), 8 mg/kg (□), or 12 mg/kg (■), or with nLs-VRL at 4 mg/kg (△) or 6 mg/kg (▲) every 3 days for a total of three injections (see arrows). Saline (●) designates the mice treated with drug- and liposome-free vehicle only. B, female BALB/c homozygous nude mice inoculated subcutaneously with HT-29 human colon carcinoma cells were treated with free VRL (■) or nLs-VRL (▲) at a dose of 5 mg VRL/kg through the tail vein every 3 days for a total of four injections (see arrows). Saline (●) designates the mice treated with drug- and liposome-free vehicle only. nLs-VRL was formulated using the TEA₈SOS gradient method for the first two studies. *P* value of 0.009 refers to comparison of free VRL with nLs-VRL (4 mg/kg) at 32 days.

mol% PEG-DSG) was studied in a human breast carcinoma (BT-474) model, an estrogen-dependent ductal adenocarcinoma that overexpresses the C-ErbB2 (HER2) receptor (Fig. 5A). pLs-VRL loaded using sucrose octasulfate was noticeably more efficacious than free VRL in retarding tumor growth ($P < 0.001$), resulting in tumor regressions and even one complete cure (one in seven). A final study looked at the efficacy of pLs-VRL in a human lung carcinoma xenograft (Calu-3) model (Fig. 5B). pLs-VRL demonstrated a statistically significant improvement over both free VRL ($P = 0.027$) and saline controls ($P = 0.006$). Similar to the previous two efficacy studies, the animal body weights in the course of treatment showed little change (<10% decrease) upon treatment with pLs-VRL in either of these two subsequent studies.

Discussion

A significant effort has been made toward the development of liposomes with suitable sustained drug release rates, an important consideration for optimal drug delivery (Allen et al., 2006; Drummond et al., 2008). The requirements may vary considerably depending on the location of the therapeutic target and the mechanism of action of the drug to be delivered. For the treatment of solid tumors, highly stable liposome formulations are more desirable due to the need for stable encapsulation until the drug carriers can accumulate in the tumor due to the enhanced permeability and retention effect. Maximal accumulation in solid tumors usually occurs on the time scale of 24 to 48 h (Gabizon et al., 1997; Drummond et al., 1999). Conversely, the treatment of hematological cancers that reside in vascularly accessible locations may

benefit to a lesser extent on stability compared with the treatment of solid tumors. Allen and coworkers have recently observed efficacy with a liposomal vincristine that is superior to that of a significantly more stable liposomal doxorubicin formulation (Sapra et al., 2004). The mechanism of action may also play a role in determining the optimal drug release rate. Drugs that are schedule-dependent, such as vincristine or vinorelbine, will undoubtedly benefit from a different drug-release profile than schedule-independent drugs, including doxorubicin.

A variety of liposomal properties or formulation methods play important roles in determining the degree of stability and hence the rate of drug release from the liposomal carrier. A careful choice of liposomal lipids, including the inclusion of highly saturated phospholipids (Bally et al., 1990; Gabizon et al., 1993), the presence of cholesterol (Papahadjopoulos et al., 1972; Drummond et al., 1999), and appropriate mixtures of sphingomyelin and cholesterol (Kirby and Gregoriadis, 1983; Webb et al., 1995), all regulate the permeability of the liposomal membrane to encapsulated drugs. Drug retention is equally dependent on the physicochemical properties of the drug to be encapsulated and the use of transmembrane gradients to both load and stabilize liposomal formulations of weakly basic amphipathic drugs.

Transmembrane gradient-loading methods have been used to actively encapsulate amphipathic weak bases into liposomes at relatively high efficiencies. These include simple pH gradients (Mayer et al., 1985; Webb et al., 1995), ammonium gradients (Haran et al., 1993), and MnSO_4 gradients (Cheung et al., 1998). Although stable formulations of lipo-

Fig. 3. Effect of inclusion of PEGylated lipids on VRL encapsulation in liposomes loaded using the TEA₈SOS gradient-loading method. Liposomes containing TEA₈SOS were incubated with VRL at pH 6.5 and 60°C for 30 min and then purified by gel filtration chromatography. The amount of drug loaded was determined relative to the theoretical drug entrapment to determine the loading efficiency (percentage). The liposomes tested had increasing concentrations of PEG-DSPE (0.5–10 mol%) or 10 mol% PEG-DSG present in the formulation (A). The chemical structures of PEG-DSPE and PEG-DSG are shown in B.

somal doxorubicin have been prepared using these methods, other drugs have proven more difficult to entrap with a similar degree of stability. A liposomal vincristine formulation has recently shown efficacy in a phase II clinical trials in lymphocytic leukemia (Thomas et al., 2006). This formulation resulted from the combination of a pH gradient remote-loading strategy for vincristine and the use of a formulation-stabilizing sphingomyelin and cholesterol lipid composition (Webb et al., 1995; Embree et al., 1998). Although the relatively stable encapsulation of vincristine in this case represents a significant advance in liposome technology, it is notable that the *in vivo* stability of the formulation remains significantly less than that observed for liposomal formulations of doxorubicin (Sapra et al., 2004). Vinorelbine and vinblastine present even greater challenges to stable encapsulation due to their more hydrophobic chemical structure (Semple et al., 2005; Zhigaltsev et al., 2005).

Ammonium gradients of citrate or dextran sulfate as counterions were successfully used to load vincristine into PEGylated phosphatidylcholine-based liposomes (Allen et al., 1995; Zhu et al., 1996). Liposomal vincristine formed using the polyanion suramin was nearly as stable as liposomal doxorubicin formulations prepared using ammonium sulfate gradients (Zhu et al., 1996). However, the increased stability did not readily translate into increased efficacy, with the more rapidly releasing ammonium citrate formulation demonstrating the greatest efficacy. This result illustrates the need to develop methods for regulating the drug-release rates of liposomal drug formulations to allow for stability in the circulation, but release of the active agent upon reaching the tumor.

We have recently described a method for stably encapsulating drugs with weakly basic amines that uses substituted ammonium salts of various polyanions (Drummond et al.,

Fig. 4. Pharmacokinetics of vinorelbine and lipid matrix of minimally PEGylated (PEG-DSPE, 0.5 mol%; ○) and highly PEGylated (PEG-DSG, 10 mol%; ●) liposomes. The liposomes were loaded at a drug-to-phospholipid ratio of 350 μ g VRL/ μ mol PL using the TEA₈SOS formulation method. The pharmacokinetics of VRL (A) were followed as a function of time using HPLC analysis for VRL quantitation in the plasma and the dynamics of the drug-to-liposomal lipid ratio in the blood of a rat (B) in vivo following intravenous bolus administration for each of the various liposomal vinorelbine formulations was also determined. Two rats were used for determination of the pharmacokinetics, and the average and cumulative errors of those measurements are plotted. Liposomal lipid concentrations in the blood were determined by scintillation counting of the nonexchangeable lipid marker [³H]cholesterylhexadecyl ether.

2005, 2006). For nanoliposomal vinorelbine formulations prepared using this technology, we show that the degree of in vivo stability can then be controlled by modifying the chemical nature of the polyanionic trapping agent used (i.e., SOS > poly-Pn > SO₄) or by modifying the encapsulated drug-to-phospholipid ratio (350 μ g VRL/ μ mol PL > 220 μ g VRL/ μ mol PL). Using this strategy, we were able to effectively improve the in vivo drug-release rates over a wide range (Fig. 1C; Table 1). The most stable of these formulations used a polysulfated nonpolymeric sugar, sucrose octasulfate. Using liposomally entrapped triethylammonium salt of sucrose octasulfate allowed to load vinorelbine into liposomes at quite high drug-to-phospholipid ratios (>450 g VRL/mol PL). The high degree of in vivo stability of these formulations suggests that the drug is retained long enough to remain within the liposomes following their accumulation in tumors. However, as demonstrated in multiple antitumor efficacy studies (Figs. 2 and 5), the rate of release is not so slow as to preclude its antitumor activity, and it is similar to that observed for remotely loaded doxorubicin liposomes (Sapra et al., 2004).

We hypothesize that increased stability of nLs-VRL prepared by the TEA-SOS method may be at least partially attributed to strong ionic interaction between sucrose octasulfate anion and the drug cation, resulting in stable complexation, potentially with gelation and/or precipitation of the complex within liposomes. In our experiments, mixing vinorelbine bitartrate at 1 to 5 mg/ml with the TEA₈SOS solution at pH 5 to 7 resulted in gelation and development of turbidity, suggesting such complexation. Interestingly, re-

duction of intraliposomal solubility of vinorelbine by forming a salt with entrapped aromatic sulfonate, in particular, *p*-hydroxybenzene sulfonate, resulting in prolonged in vivo drug retention within sphingomyelin liposomes was reported recently (Zhigaltsev et al., 2006).

We hypothesized that stable encapsulation of vinorelbine in liposomes, and selective delivery to solid tumors, could increase its anticancer efficacy and potentially reduce its toxicity. We chose to use our most stable liposomal vinorelbine, loaded using a TEA₈SOS gradient, due to the perceived requirement for stable encapsulation in treating solid tumors. Our results here demonstrated a slightly improved MTD (23.8 versus 17.5 mg VRL/kg) when vinorelbine was administered as a liposomal formulation compared with the free form of the drug. The drug-free liposomes were nontoxic at the highest achievable lipid dose. Although liposome encapsulation of schedule-independent drugs, such as doxorubicin, often result in a substantial decrease in the MTD when studied in animal models, schedule-dependent drugs have often displayed an increase in the MTD due to the sustained release of the drug from its carrier (Tardi et al., 2000; Drummond et al., 2005, 2008). However, the substantial increase in efficacy is enough to compensate and allow for an improvement in the therapeutic index for the liposomal drug. Thus, the minimal improvement in the MTD upon stable encapsulation of the schedule-dependent vinorelbine is not surprising.

Liposomal vinorelbine was also consistently and significantly more active than free vinorelbine in the treatment of colon and breast carcinoma models. A multidose study in a C-26 colon carcinoma model indicated liposomal vinorelbine

Fig. 5. Antitumor efficacy of PEGylated liposomal vinorelbine in (A) human breast (BT-474) tumor and (B) human lung (Calu-3) xenografts. A, female NCR *nu/nu* mice were subcutaneously implanted with 60-day sustained-release estrogen pellets and in 2 days were inoculated subcutaneously with a 0.1-ml suspension containing 2×10^7 BT-474 cells. At day 25 after tumor cell inoculation, when the tumors reached approximately 200 mm³ in size, the mice were randomized into three groups of eight animals per group and treated i.v. with saline (●), 5 mg/kg free VRL (■), or pLs-VRL with SOS as a counter-ion (▲), at a dose of 5 mg VRL/kg weekly for a total of three injections. The control group received an equal volume of normal saline. B, male NCR *nu/nu* mice were subcutaneously inoculated with 1.5×10^7 Calu-3 cells and subsequently treated weekly (see arrows) with saline (●), 6 mg/kg free VRL (■), or 4 (△) or 6 (▲) mg/kg VRL of pLs-VRL with SOS as a counter-ion when the tumors reached an average size of 105 mm³.

was a minimum of 3-fold more active than free vinorelbine in the treatment of this model (Fig. 2A). Together, the observed improved toxicity and antitumor efficacy suggest a significant widening of the therapeutic window by stable encapsulation of vinorelbine in liposomes.

Finally, a highly PEGylated liposome formulation of vinorelbine without the loss of loading efficiency or in vivo drug encapsulation stability was developed. Attempts to encapsulate VRL using an ammonium sulfate gradient indicated a significant inhibition of loading when PEG-DSPE was incorporated at greater than 3 mol% of the total phospholipid. We observed a similar inhibition when using a TEA₈SOS gradient to load VRL (Fig. 3A). However, substitution of the nonionic PEG-distearoylglycerol (Fig. 3B) for PEG-DSPE resulted in quantitative loading, even at 10 mol% PEG-DSG. Interestingly, at the studied dose of 10 mg/kg, the pharmacokinetics of 10% PEG-DSG nLs-VRL in rats was nearly identical to that of minimally PEGylated (0.5 mol% PEG-DSPE) nLs-VRL. In addition, the rate of drug release from the liposome was not affected by inclusion of the PEGylated lipid. In previous studies, the use of the nonionic PEG-ceramide resulted in a significant improvement over PEG-DSPE; nevertheless, increased drug leakage was observed compared with non-PEGylated liposomes for several vincristine liposome formulations (Webb et al., 1998). Because the

inclusion of PEGylated lipids was shown to decrease dose dependence of the pharmacokinetic parameters of liposomally encapsulated drugs, thus improving or limiting the heterogeneity of the pharmacokinetics of liposomal drug formulations, the possibility of highly PEGylated VRL liposomes without the loss of formulation quality is valuable. This PEGylated nLs-VRL formulation prepared using the TEA₈SOS gradient method was considerably more active than free VRL in a BT474 human breast tumor xenograft model, resulting not only in notable tumor growth suppression but also in a complete tumor regression in one of seven animals (Fig. 5).

We believe the development of this new highly stable and active nanoliposomal formulation of vinorelbine provides an opportunity to improve the therapeutic index of this drug, and improve the quality of life for patients being treated with it. Based on these considerations and the data presented here, the nLs-VRL formulation described in this work has entered clinical development.

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