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[2] Preparation, Characterization, and Biological Analysis of Liposomal Formulations of Vincristine

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Abstract

Vincristine is a dimeric Catharanthus alkaloid derived from the Madagascan periwinkle that acts by binding to tubulin and blocking metaphase in actively dividing cells. While vincristine is widely used in the treatment of a number of human carcinomas, its use is associated with dose-limiting neurotoxicity, manifested mainly as peripheral neuropathy. It is known that the therapeutic activity of vincristine can be significantly enhanced after its encapsulation in appropriately designed liposomal systems. Enhanced efficacy is also associated with a slight decrease in drug toxicity. Thus, the therapeutic index of vincristine can be enhanced significantly through the use of a liposomal delivery system. Vincristine may be

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encapsulated into liposomes of varying lipid composition by several techniques, including passive loading, pH gradient loading, and ionophore-assisted loading. However, most research has focused on the encapsulation of vincristine in response to a transbilayer pH gradient, which actively concentrates the drug within the aqueous interior of the liposome. This chapter details the preparation and evaluation of liposomal vincristine. Specifically, we elaborate on the components (choice of lipids, molar proportions, etc.), methods (preparation of liposomes, drug loading methods, etc.), critical design features (size, surface charge, etc.), and key biological endpoints (circulation lifetime, bioavailability, efficacy measurements) important to the development of a formulation of vincristine with enhanced therapeutic properties.

Introduction

Vincristine is a bisindole alkaloid that was initially purified from the periwinkle *Catharanthus roseus* (*Vinca rosea*) in the late 1950s and early 1960s (Svoboda, 1961). The resulting agent (Fig. 1) is a lipophilic amine, a weak base, with pK_as at 5.0–5.5 and 7.4 and a partition coefficient (P) between octanol and water of log $P = 2.82$ (Leo *et al.*, 1971). In its pure form, vincristine is a solid white to off-white powder, with a melting temperature between 218 and 220° and a molecular weight of 824.94 (Budavari *et al.*, 1989).

The activity of vincristine is cell-cycle specific, manifested in metaphase by the inhibition of tubulin polymerization (Rowinsky and Donehower, 1997). Based on this mechanism of action, it is not surprising that vincristine cytotoxicity is observed against a broad spectrum of tumor cell lines including leukemias, lung (small cell and non-small cell), colon, central

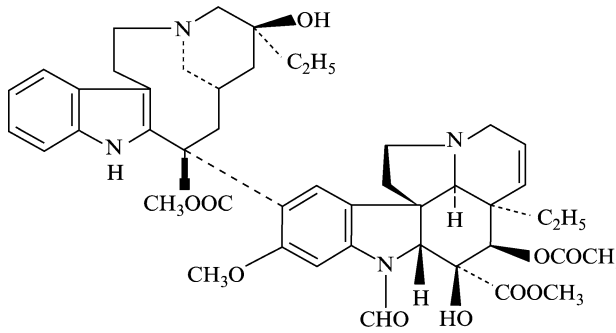

FIG. 1. Chemical structure of vincristine.

nervous system (CNS), melanoma, ovarian, renal, prostate, and breast carcinomas [NCI Cancer Screen Data for NSC 67574 (vincristine)]. Moreover, it might also be anticipated that increased duration of exposure of cells to vincristine would substantially improve the cytotoxicity of the drug. This expectation is supported by the *in vitro* observation that increasing the duration of exposure of L1210 leukemia cells to vincristine from 1 to 72 h was associated with a 10^5-fold increase in cytotoxicity compared with only a 40-fold increase in the cytotoxicity of doxorubicin, whose activity is believed to be less cell-cycle specific (Boman *et al*., 1995; Mayer *et al*., 1995).

Vincristine received its first approval by the U.S. Food and Drug Administration (FDA) in 1963 for the treatment of acute leukemia in children. Since then, vincristine has become one of the most commonly used anticancer drugs. Current approvals for vincristine include the treatment of a variety of adult and pediatric cancers such as acute leukemia, Hodgkin's disease, non-Hodgkin's lymphomas, rhabdomyosarcoma, neuroblastoma, and Wilms' tumor and is also used in the treatment of breast cancer and small cell lung cancer (Rowinsky and Donehower, 1997). Vincristine is rarely used as a single agent; instead, vincristine is almost exclusively used as a component of combination chemotherapy protocols. Currently, approximately 50% of the use of vincristine is in the treatment of lymphomas, for example, as part of the CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) combination for the first-line treatment of non-Hodgkin's lymphomas (Shipp *et al*., 1997). Based on the cell-cycle–dependent activity of vincristine described above, Jackson *et al*. (1981, 1984) attempted to increase its clinical activity by increased duration of exposure to the drug achieved by continuous infusion. Substantial clinical activity was observed in some patients but was also associated with significant to severe toxicities, particularly neurotoxicities (Jackson *et al*., 1984).

An approach to the treatment of cancers that would facilitate a significant increase in the duration of exposure to vincristine would be to encapsulate the drug in a liposomal delivery system. The general principles and benefits of encapsulating drugs in liposomes have been described in detail elsewhere (Bally *et al*., 1998; Tardi *et al*., 1996). Briefly, encapsulation of therapeutic drugs in appropriately designed liposomal carriers can accomplish some or all of the following: (1) significant increases in the plasma concentration of the drug and extension of its circulation lifetime; (2) increased drug accumulation and duration of exposure at disease sites that are characterized by increased vascular permeability, including tumor sites and sites of inflammation and/or infection; (3) significant increases in the efficacy of the drug, coupled with (4) a decrease, or no increase, in the

toxicity of the encapsulated drug compared with the unencapsulated drug. The specific benefits associated with the encapsulation of vincristine have been reviewed previously (Boman *et al*., 1997; Webb *et al*., 1995a) and are all described by these general characteristics. The success of liposomal vincristine in preclinical testing has led to the advanced clinical evaluation of an optimized formulation in advanced refractory non-Hodgkin's lymphomas (Sarris *et al*., 1999, 2000; Webb *et al*., 1998) and in other indications. A pivotal Phase IIb trial on liposomal vincristine (vincristine sulfate liposome injection; VSLI) in treating non-Hodgkin's lymphoma at second or greater relapse has been completed, and an application for registration of this drug by the U.S. FDA has been submitted.

Preparation and Characterization of Liposomal Vincristine

Methods For Encapsulation of Vincristine in Liposomes

There have been numerous published reports of successful entrapment of vincristine into liposomal carriers. This chapter will discuss several of these, with a focus on the most biologically relevant formulations, and those that are most clinically advanced. Initial consideration will be given to the lipid components and liposome formation, followed by specific methodology for vincristine uptake into preformed liposomes.

Choice of Lipids

Inclusion of Cholesterol. Liposomes traditionally contain a significant proportion of cholesterol (Chol) as a stabilizing lipid. The inclusion of cholesterol in liposome formulations has been shown to prevent lipoprotein-induced vesicle destabilization and concomitant release of the encapsulated drug (Kirby *et al*., 1980; Scherphof *et al*., 1978). This leads to increased circulation longevity of drug and can enhance drug accumulation at tumor sites. The cholesterol molecule inserts into a phospholipid bilayer with its hydroxyl group oriented toward the aqueous surface and the planar steroid ring systems parallel to the phospholipid acyl chain orientation. Below the phase transition temperature of the primary phospholipid component of a liposomal formulation, the inclusion of cholesterol has the effect of disrupting acyl chain packing and increasing the fluidity of the gel phase, as well as increasing the membrane permeability. These effects are reversed above the phase transition temperature of the lipids, with reduced chain fluidity and membrane permeability. Cholesterol is typically incorporated at a molar ratio of up to 45% if there is only one other lipid component in the liposome; for example, a distearoylphosphatidylcholine (DSPC)/Chol formulation would have a

molar ratio of 55:45. When other lipids are included in the membrane, it may be either the amount of the primary lipid or the cholesterol that is adjusted accordingly, such as the inclusion of 5 mol% poly(ethylene glycol) (PEG)-conjugated lipid in the above example, giving DSPC/Chol/DSPE-PEG, at a molar ratio of 50:45:5 or 55:40:5.

Choice of Membrane Lipid Components. Liposomes may be prepared with a wide range of lipids, including DSPC, egg phosphatidylcholine (EPC), dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylglycerol (DSPG), sphingomyelin (SM), and others (lipids are obtained from specialty suppliers such as Avanti Polar Lipids Inc., Alabaster, AL or Northern Lipids Inc., Vancouver, BC, Canada). EPC was one of the earliest lipids used in the formation of liposomes but, with the exception of a liposomal doxorubicin formulation approved in Europe and Canada (Myocet), is not used as commonly today as a result of its relatively high membrane permeability coefficients. Consideration must be given to the charge of the lipids used, as well as the degree of saturation and length of the acyl chains. In general, lipids with longer, saturated acyl chains (i.e., DPPC and DSPC) produce liposomes with lower solute permeability and increased stability and blood residence times (Senior and Gregoriadis, 1982). The inclusion of acidic lipids such as PG or phosphatidylserine (PS) lowers the tendency of liposomes to aggregate during formation and enhances *in vitro* uptake by cells, however, it will also lead to a shorter blood circulation half-life following intravenous (iv) injection (Senior *et al*., 1983).

Liposomes often include stabilizing lipids such as the ganglioside G_{M1} (Allen *et al*., 1989; Gabizon and Papahadjopoulos, 1988) or the more commonly used PEG-conjugated lipids (Papahadjopoulos *et al*., 1991). PEG-conjugated lipids serve to provide a steric barrier around the liposome, most likely protecting it from opsonization and clearance by the mononuclear phagocyte system (MPS). Although they can have varying polymer lengths, PEG chains of average molecular weight 2000 are typically employed. Liposomes possessing PEG lipids are usually eliminated less rapidly by the MPS due to the exclusion of macromolecules such as opsonins from the periliposomal space. This increased circulation lifetime may also result in altered biodistribution compared with liposomes without PEG lipids. It has more recently been suggested that the primary effect of liposome steric stabilization is due to elimination of surface–surface interactions that can lead to liposome aggregation or liposome–cell interactions (Johnstone *et al*., 2001).

The decreased interaction with serum proteins observed for liposomes possessing PEG-conjugated lipids or G_{M1} is also seen in liposomes prepared

with the naturally occurring phospholipid sphingomyelin (Webb *et al.*, 1995a). This lipid has the additional advantage of excellent chemical stability. The lability of the ester linkages can limit the "shelf life" of liposomes composed of PC.

Preparation of Liposomes

Preparation of Lipid Films by Solvent Evaporation or Freeze Drying. The amount of material to prepare must depend upon the experimental requirements, and, for simplicity, this chapter will describe formulations with a starting amount of 100 mg total lipid. This may be scaled up or down as required, remembering that if liposomal formulations are to be extruded (see below), a minimum extrusion volume of 1 ml is recommended to minimize sample loss during the procedure. Unless great care is taken, extrusion through a 10-ml extruder can result in a loss of as much as 200 μl (representing 20% of a 1-ml sample).

For a 1-ml preparation containing 100 mg of lipid, the required amounts of lipids, based on the desired molar ratio as described above (e.g., DSPC/Chol 55:45, mol:mol; 71.4:28.6, wt:wt), must be dissolved in organic solvent to ensure homogeneous lipid mixing. This is typically done in chloroform ($CHCl_3$) or $CHCl_3$:methanol mixtures, with a lipid concentration of 10–100 mg/ml, depending on solubility of the individual lipids. We have found that with most lipids, a final solvent volume of 500–1000 μl is sufficient to completely solubilize the lipids. If sphingomyelin is being used, it may be necessary to add several drops of methanol to the $CHCl_3$ to completely dissolve the lipid. The dissolved lipids are combined in one glass test tube, mixing thoroughly to ensure even lipid distribution in the solvent. If liposomes are being used for *in vitro* applications or for pharmacokinetic experiments, a radiolabeled lipid marker can be added to the lipids in chloroform at this stage. We typically use the nonexchangeable, nonmetabolizable marker cholesterylhexadecylether (CHE) labeled with either ^3H or ^{14}C. For specific activity determination, small aliquots are taken from the lipid/$CHCl_3$ solution, with care being taken to prewet the pipette tip to prevent drips during dispensing of small volumes of the organic solvent. It should also be remembered that $CHCl_3$ is a potent quenching agent and must be removed prior to addition of a scintillation cocktail. The dpm values obtained by liquid scintillation counting may then be correlated to the precise amount of lipid known to be in the aliquot taken. If a fluorescent lipid is required as a tracer, we recommend DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Molecular Probes Eugene, OR), a nonexchangeable lipid, the metabolism of which has not yet been established.

Excess solvent is removed from the lipid solution by evaporation under a gentle stream of nitrogen gas. At this point, the lipids should be left in a small volume of $CHCl_3$, in a slurry-like consistency. If too much solvent is removed at this point, it will be difficult to subsequently obtain a completely dried film. The remaining solvent is removed under a high vacuum (approximately 75 cm Hg) for a minimum of 3 h, or until no solvent remains in the tube. If the starting amount of lipid is above approximately 20 mg, the application of a vacuum should result in a lipid "puff" rising to halfway up the test tube. This puff is desirable, as it maximizes the lipid surface during the drying procedure. Alternately, excess solvent may be removed by rotary evaporation, yielding a thin lipid film on the sides of the flask. Lipid surface area may be maximized in rotary evaporation by the use of glass beads in the flask. This step is also followed by removal of residual solvent on a vacuum pump. When mixtures of chloroform and methanol are used initially to solubilize the lipids, care must be taken to ensure that selective precipitation of one lipid component, for example, cholesterol, does not occur during solvent removal when the methanol content in the mixture increases due to preferential evaporation of chloroform.

Following complete removal of solvent, the lipid film is hydrated with 1 ml of aqueous buffer. For employment of the pH gradient loading technique (Mayer *et al.*, 1993), this buffer is typically 300 m*M* citric acid, pH 4.0. Other methods to produce a pH gradient include ammonium sulfate (300 m*M*) gradients or manganese sulfate (300 m*M*) gradients followed by the use of an ionophore such as A23187 (A.G. Scientific, Inc., San Diego, CA), which acts as a divalent cation shuttle or pump and results in an acidified liposomal interior. For other applications, the hydration solution may be saline, distilled water, HEPES-buffered saline (HBS), or sugar solutions, taking care to keep the osmolality of solutions within a physiological range (270–290 mOsm/kg). For optimal lipid hydration, the film and buffer should be preheated to above the phase transition temperature of the lipid with the highest phase transition temperature (T_c) of the formulation (see Table I) and maintained at this temperature during the hydration procedure. Vigorous vortex mixing or agitation aids in optimization of the hydration. This results in the formation of multilamellar vesicles (MLVs), which are large (>1 μm) and have a heterogeneous size distribution. The suspension of MLVs is then transferred to cryovials and subjected to five freeze–thaw cycles, alternating between liquid nitrogen and a water bath set above the phase transition temperature of the lipid employed. This step allows for equilibration of solute across the bilayers of the vesicles, full hydration of the head groups of the lipids, as well as dissociation of large lipid aggregates (Mayer *et al.*, 1985).

TABLE I
AVERAGE TEMPERATURES OF THE MAIN GEL-TO-LIQUID CRYSTALLINE PHASE TRANSITION (T_c) FOR LIPIDS COMMONLY USED IN LIPOSOMAL VINCRISTINE FORMULATIONS[a]

Lipid name	Number of acyl carbons: number of *cis*-unsaturated bonds	T_c (°C)
Dilauroylphosphatidylcholine (DLPC)	12:0	−1.1
Dimyristoylphosphatidylcholine (DMPC)	14:0	23.5
Dipalmitoylphosphatidylcholine (DPPC)	16:0	41.4
Distearoylphosphatidylcholine (DSPC)	18:0	55.1
Dioleoylphosphatidylcholine (DOPC)	18:1	−18.4
Dimyristoylphosphatidylethanolamine (DMPE)	14:0	49.6
Dipalmitoylphosphatidylethanolamine (DPPE)	16:0	64.0
Dioleoylphosphatidylethanolamine (DOPE)	18:1	−16
Sphingomyelin (SM), *N*-palmitoyl-sphingosyl	16:0	40.9
Dipalmitoylphosphatidylserine (DPPS), Na salt	16:0	54
Dipalmitoylphosphatidylglycerol (DPPG), Na salt	16:0	40.9

[a] Data obtained from Marsh, D. (1990). "CRC Handbook of Lipid Bilayers." CRC Press, Boca Raton, FL.

As an alternative to the preceding method, MLVs may also be generated without forming lipid films by direct combination of lipids dissolved in EtOH with aqueous buffer. Briefly, lipids in the appropriate molar amounts (i.e., DSPC/Chol or SM/Chol, 55:45, mol:mol) dissolved in 95% EtOH (1 ml/100 mg lipid) are heated at 60° for 30 min. This ethanol solution of lipids is then added dropwise to preheated 300 m*M* citric acid (3 ml/100 mg lipid)(pH 4.0 or 2.0) with constant vortexing. The solution is heated an additional 30 min, resulting in MLVs that may be taken directly to the size reduction step (without using a freeze–thaw procedure) as discussed below. Following extrusion (below), liposomes must be dialyzed against 300 m*M* citric acid (pH 4.0 or 2.0) to remove ethanol, then further dialyzed against the desired external aqueous buffer, such as HBS, pH 7.5, hence establishing a pH gradient across the liposomal membrane (Boman *et al.*, 1994).

For *in vivo* applications of liposomes, it is important to further reduce the size of these liposomes. It has been experimentally determined that vesicles between 100 and 200 nm in diameter are optimal for intravenous injection (Allen and Everest, 1983). These vesicles are small enough to gain access to tissues in areas of inflammation and at tumor sites due to the fenestrated/leaky vasculature in these areas. Smaller vesicles are also eliminated less rapidly from the circulation, hence increasing the blood

residence time for the encapsulated drug. To obtain smaller vesicle sizes, MLVs may be either sonicated or extruded. We use the Lipex Extruder (Northern Lipids, Inc., Vancouver, BC, Canada) for this purpose, as described below. For formation of approximately 120-nm-diameter vesicles, or large unilamellar vesicles (LUVs), with a homogeneous size distribution, the MLVs are forced through polycarbonate membranes with 100-nm-diameter pores, under high pressure (300–600 psi) and with the thermobarrel of the extruder equilibrated to a temperature 5° above the T_c of the lipids. It is recommended that the filters be prewet with the buffer in which the liposomes were prepared to minimize volume loss during the extrusion procedure. A minimum of 10 passes through the extruder will typically result in homogeneous size distributions of liposomes (Hope *et al.*, 1985) (see discussion of size analysis below).

The extrusion procedure, if the hydration buffer is 300 m*M* citrate, pH 4.0, results in a suspension of liposomes in which both the interior aqueous volume and the external buffer are at pH 4.0. To establish a pH gradient for drug loading, the external buffer must either be raised to a pH of 7.0–7.5 by the addition of, for example, 0.5 *M* Na_2HPO_4, or by passing the liposomes over a gel filtration column such as G-50 Sephadex that has been preequilibrated in the desired buffer, such as 150 m*M* NaCl, 20 m*M* HEPES, pH 7.5 (HBS).

Loading Preformed LUVs with Vincristine. To the formed LUVs with an established pH gradient, sufficient vincristine sulfate solution (e.g., Oncovin, Eli Lilly & Co., Indianapolis, IN) is added for a final drug to lipid ratio (wt/wt) of between 0.05 and 0.2. For a starting solution of 100 mg total lipid, this will be between 5 and 20 ml of a 1 mg/ml vincristine sulfate solution. The drug and liposome mixture is incubated above about 45–65° (the actual temperature required depends upon lipid composition) for a minimum of 10 min to effect drug uptake into the liposomes in response to the pH gradient. Vincristine is highly permeable to lipid membranes in its neutral form. Upon entering the liposome interior, it becomes protonated and is no longer as readily able to cross the lipid bilayer, effectively trapping it within the aqueous core of the liposome. It is important to note that the protonation of vincristine in the liposome interior reduces the hydrogen ion pool; therefore, it is important that the starting pH gradient is sufficient to ensure complete uptake of vincristine (typically pH 4.0 or 2.0 within the liposomes, pH 7.2 to 7.5 in the external buffer). Redistribution of vincristine between the intravesicular medium and the external solution is in accordance with the Henderson–Hasselbach equation. In a simplified form, this predicts that the drug concentration gradient across the liposomal membrane will be equal to the proton concentration gradient. Also, it should be noted that the lower the internal pH

the lower the efflux rate of vincristine from the liposome (Boman *et al.*, 1993). Interior pH values less than 4 are, of course, difficult to employ experimentally due to the potential for lipid and drug degradation in the loaded liposome.

Ionophore (A23187 or Nigericin)-Mediated Loading of Vincristine into Liposomes. It is possible to use DSPC/Chol or SM/Chol liposomes with either the channel ionophore A23187 or nigericin to encapsulate several drugs, including vincristine. DSPC/Chol or SM/Chol (55:45, mol:mol) liposomes are prepared as outlined above, with the lipid film being hydrated in either 300 m*M* $MnSO_4$ or $MgSO_4$ (for A23187-mediated loading) or 300 m*M* K_2SO_4 (for nigericin-mediated loading). Once extruded, liposomes are dialyzed against two changes of 100 volumes of 300 m*M* sucrose to exchange the external buffer, thus creating a salt gradient across the liposomal membrane.

For A23187-mediated uptake of vincristine, the ionophore is added to the liposomes (0.1 μg A23187/μmol lipid) and incubated at 65° for 5 min before the addition of vincristine (0.05:1.0–0.2:1.0 drug-to-lipid ratio, wt/wt) and 200 m*M* ethylenediaminetetraacetic acid (EDTA), pH 7.0 (final concentration of 30 m*M*). The presence of EDTA in this system is important to chelate manganese or magnesium ions effluxing from the liposomes to drive drug uptake to completion. Following a further incubation of 10–60 min at 65°, the vincristine is encapsulated within the liposomes, with entrapment approaching 100% by virtue of the pH gradient created with the coupling of external transport of one manganese/magnesium ion to the internal transport of two protons. Removal of ionophore may be easily accomplished by either dialysis or column chromatography. Vincristine encapsulation using nigericin (1 ng/μmol lipid) is performed in a similar manner, except that a potassium ion gradient is employed and EDTA is not required, with nigericin catalyzing a one-for-one exchange of K^+ for H^+ (Fenske *et al.*, 1998).

Formation of Vincristine Precipitates Using Suramin, Heparin Sulfate, or Dextran Sulfate. It has been determined for some formulations that leakage of vincristine from the liposomal carrier occurs during the plasma distribution phase, thereby diminishing the amount of liposomal drug that will accumulate within the tumor site, as evidenced by faster elimination of vincristine than the lipid carrier. It is possible to extend the plasma distribution phase of vincristine to more closely match that of the liposomal carrier, by causing a polyanion/vincristine precipitate to form within the liposomes. We will not provide extensive detail in this area, since experiments were unable to demonstrate any enhanced efficacy, although precipitates form within the liposomes and the leakage rate is reduced (Zhu *et al.*, 1996). These researchers formed pegylated liposomes using

an ethanol injection method, where lipids dissolved in ethanol were injected into 125 m*M* ammonium sulfate containing heparin sulfate, dextran sulfate, or suramin. Polyanion to vincristine ratios were estimated to be 8.7:1, 1.8:1, and 2.8:1 for vincristine-loaded liposomes containing dextran sulfate, heparin, and suramin, respectively.

Characterization of Liposomal Vincristine

Determination of Trapping Efficiency. To determine the trapping efficiency of vincristine in liposomes, a comparison of the drug-to-lipid ratio is made before and after a column chromatography step to remove unencapsulated drug. Specifically, aliquots are taken both before and after running a portion of the sample down a size exclusion chromatographic column such as G-50 Sephadex, equilibrated in the appropriate buffer, and measured for both lipid and vincristine as outlined above. The drug-to-lipid ratio (wt/wt) in the column eluate is compared with the precolumn drug-to-lipid ratio for determination of percent encapsulation of the drug within the liposomes. Methods for quantifying the drug and lipid are described below.

Determination of both lipid and vincristine concentrations may be either by addition of a radiolabeled marker in the initial formulation or by spectrophotometric assay. We use [^{3}H]- or [^{14}C]CHE as a lipid label as discussed above, bearing in mind that the concentration of CHE must not be so high as to prevent adequate incorporation and [^{3}H]vincristine (Amersham Canada Inc.) for determination of vincristine concentrations. The [^{3}H]vincristine should be pipetted into a clean, dry test tube and the solvent evaporated off prior to thorough mixing with the stock vincristine sulfate solution. Aliquots for determination of specific activity by liquid scintillation counting are taken prior to addition of desired amount to liposomes. This activity is then correlated to the concentration of vincristine in the aliquot and a specific activity (dpm/μmol or dpm/μg) determined. Caution should be exercised when working with [^{3}H]vincristine, as over time the marker becomes less accurate, requiring calibration with a spectrophotometric determination of vincristine concentration. If spectrophotometric assays are preferred for vincristine, the following procedures may be employed. Sample aliquots and standards (0–100 μg/ml Oncovin in H_2O) are brought to a volume of 200 μl with distilled, deionized water. Liposomes are solubilized by addition of 800 μl EtOH. Absorbance is measured at 297 nm and compared with the standard curve for determination of concentration.

Phosphate assays follow the method of Fiske and Subbarow (1925). Standards are prepared from 2 m*M* Na_2HPO_4, from 0–200 nmol total

phosphate. Standards and samples are pipetted into thick-walled test tubes, to which 750 μl perchloric acid is added and heated at 180° for a minimum of 2 h or until solutions become colorless. To prevent sample loss, tubes are topped with glass marbles, allowing venting of excess steam during the heating steps. After cooling to room temperature, 750 μl Fiske solution (150 g $NaHSO_3$ and 5 g Na_2SO_3 dissolved in 1 liter distilled H_2O, plus 2.5 g 1-amino-2-naphthol-4-sulfonic acid; covered and stirred at 40° to dissolve crystals, stored overnight in the dark, filtered, and stored in the dark) and 7 ml ammonium molybdate solution (800 ml distilled H_2O plus 2.2 g ammonium molybdate and 20 ml H_2SO_4, made up to 1 liter with distilled H_2O) are added to the tubes, and the contents are gently mixed. Tubes are further heated at 100° for 20 min for color development, cooled to room temperature, and the absorbance measured at 815 nm.

Determination of Liposome Size. The elimination rate of liposomes from the circulation is very sensitive to liposome size, with larger liposomes eliminated more quickly than small ones. It is therefore critical to measure this parameter prior to administration of a liposomal drug. Several techniques are available, but we have found that quasielastic light scattering (QELS) is the most reliable and efficient when dealing with relatively homogeneous samples. This method provides analysis of the mean size, as well as giving information with respect to the distribution of diameters within the sample. We use the NICOMP model Nicomp 270 submicron particle sizer (Pacific Scientific, Santa Barbara, CA), with an argon laser operating at 632.8 nm according to the manufacturer's instructions. Liposomes prepared by extrusion typically exhibit size distributions in good agreement with a Gaussian fit. Mean diameters can then be expressed on a number, volume, or light-scattering intensity basis. By convention, mean diameter on a volume basis is generally used.

pH Gradient Determination. When utilizing the pH gradient method for loading vincristine into liposomes, there is an anticipated drug loading of close to 100%. Should the experimenter achieve less than this, a possible explanation is the lack of a sufficient transmembrane pH gradient. This may be easily verified by the use of [^{14}C]methylamine (Harrigan *et al.*, 1992). The uncharged form of methylamine ($MeNH_3$) is highly lipid permeable and will rapidly equilibrate across a membrane (Rottenberg *et al.*, 1972) according to the following equation:

$$\frac{[\mathrm{MeNH}]^{+}_{\mathrm{in}}}{[\mathrm{MeNH}^{+}_{3}]_{\mathrm{out}}} = \frac{[\mathrm{H}^{+}]_{\mathrm{in}}}{[\mathrm{H}^{+}]_{\mathrm{out}}} \tag{1}$$

Upon entry to the acidified aqueous interior of the liposome, the methylamine becomes protonated ($MeNH_3^+$) and is hence unable to readily cross

the membrane. The subsequent measurement of trapped versus untrapped probe allows the proton gradient (Δ pH) to be determined.

To measure ΔpH, aliquots of liposomes are diluted into the same buffer used to initially create the gradient (i.e., HBS, pH 7.5), with the addition of 1 μCi [^{14}C]methylamine, to a final lipid concentration of approximately 2 m*M*. Following a 10-min incubation at a temperature just over the phase transition temperature of the lipid, an aliquot is passed over a G-50 Sephadex minispin column (1 ml bed volume, column spun at 2000 *g* for 2 min) then ^{14}C and phosphorus determined in the prespin and eluate samples. The transmembrane ΔpH is then simply determined using the following formula:

$$\Delta\text{pH} = \log \frac{[\text{MeNH}_3]_{\text{in}}}{[\text{MeNH}_3]_{\text{out}}} \tag{2}$$

This method of determining a transmembrane proton gradient has been found to be accurate to a difference of up to 3 pH units given a sufficient interior buffering capacity (i.e., citrate concentration of 20 m*M* or higher) and absence of significant transmembrane osmotic gradient and requires measurement of the interior (trapped) volume of the liposomes, as described previously (Harrigan *et al.*, 1992).

Biological Analysis of Liposomal Vincristine

It has been our experience that the most informative and rapid means of evaluating and characterizing liposomal formulations of cytotoxic agents, including vincristine, is by *in vivo* experiments. For example, it is possible to compare vincristine release rates from different liposomal formulations using *in vitro* methods (Webb *et al.*, 1995a,b). While drug release rates determined *in vitro* may show a good qualitative correlation, for different liposomes, with the *in vivo* drug release rates, the absolute drug release kinetics are significantly faster *in vivo* (Webb *et al.*, 1995a). Similarly, *in vitro* evaluation of the efficacy of liposomal cytotoxic drugs such as vincristine is complicated by the slow or negligible release of drug from liposomes under *in vitro* conditions. For this and other reasons, we focus on the use of *in vivo* assessments for the characterization and optimization of liposomal formulations of agents such as vincristine.

Pharmacokinetics

Pharmacokinetic characterization is the most useful tool for the rapid screening and optimization of formulations of liposomal vincristine, as well as formulations of other liposomal agents. For liposomal vincristine, intravenous administration is the route of choice and is typically achieved

via the tail vein with volumes not exceeding 200 μl per 20-g mouse. Care needs to be taken administering formulations of drug loaded using the citrate-pH gradient method, particularly at high doses and/or low drug-to-lipid ratios, that adverse toxicities are not caused by citrate-mediated chelation of cations in the blood. Formulations in this category may require the exchange of external citrate for saline or dextrose solutions using dialysis, column chromatography, or tangential flow methods prior to administration of the formulation. The choice of mouse strain for appropriate pharmacokinetic studies should reflect the ultimate use of the formulation for planned efficacy studies. While pharmacokinetic parameters are not markedly different in different strains, the dose for the pharmacokinetic study needs to be at or near the maximum tolerated dose (MTD). On single administration of liposomal vincristine, MTDs can vary from about 4–5 mg/kg for outbred mice (i.e., CD1) to 2 mg/kg for immunocompromised mice (i.e., SCID). As a general comment, all *in vivo* studies should be done in accordance with local animal care guidelines, such as those defined in Canada by the Canadian Council of Animal Care (http://www.ccac.ca). As lethal dose assessments are not permitted by these guidelines, we determine MTD values using small dose range–finding studies (involving less than 10 mice in total) in which the MTD is defined as the dose that achieves a nadir weight loss of 15%.

Acceptable pharmacokinetic criteria for liposomal formulations of vincristine are based on all major components of the preparation. Specifically, (1) liposome elimination half-life of at least 8 h, based on the elimination of a nonexchangeable lipid marker such as CHE; (2) half-life for the release of vincristine from the liposomal carrier, based on the change of the vincristine/lipid ratio in the plasma of greater than 15 h; and (3) vincristine elimination half-life of at least 4 h. The best available formulations of vincristine have lipid elimination half-lives greater than 50 h, half-lives for the vincristine release from the liposome in excess of 25 h, and vincristine elimination half-lives of at least 12 h (Boman *et al.*, 1997). To date, no formulations of liposomal vincristine have been described in which the rate of drug release is reduced sufficiently to result in decreased antitumor efficacy.

Since free vincristine is rapidly eliminated from the circulation, it is reasonable to assume that all vincristine observed in the plasma after intravenous administration of liposomal drug represents only that drug that is encapsulated in the liposomes. However, in some instances it may be necessary to quantify the contributions of both unencapsulated and liposomally encapsulated vincristine to the total plasma concentration. To separate unencapsulated vincristine from encapsulated vincristine in plasma after intravenous administration, aliquots of plasma are placed into

Microcon-30 ultracentrifugation devices (Millipore, Bedford, MA) and centrifuged at 10,000 rpm at 4° for 15 min (Mayer and St.-Onge, 1995; Krishna, *et al*., 2001). The resultant ultrafiltrate contains free vincristine only, but does not quantitatively account for protein-bound vincristine. However, as protein-bound vincristine has been experimentally determined to represent 40% of the total nonliposomal vincristine plasma content (Mayer and St.-Onge, 1995), the values of vincristine obtained from the ultrafiltrate represent 60% of the total nonliposomal vincristine concentration in the plasma.

Antitumor Efficacy Endpoints

Therapeutic activity of vincristine encapsulated in liposomal delivery vehicles may be measured *in vivo* in a range of tumor model types and assessed by several different means. Methods of determining the therapeutic activity in animal tumor models are described below.

Solid (Subcutaneous) Tumor Model Evaluation

1. Tumor volume (mass): Mean tumor volumes are determined from vernier caliper measurements of perpendicular length and width measurements (height measurements can often be obtained as well). Tumor volume (mass; units of ml^3 or mg) is calculated from

$$\text{Volume} = (\text{length} \times \text{width}^2)/2 \tag{3}$$

or

$$\text{Volume} = \pi/6 \times (\text{length} \times \text{width} \times \text{height}) \tag{4}$$

Data are plotted with respect to time.
2. Tumor weight inhibition (TWI%): At a defined time point the mean tumor weight of a treated group divided by the mean tumor weight of the control group, minus 1. This value is then multiplied by 100 to define a percent change.
3. Tumor growth delay (T–C): Median time in days for the treated (T) groups to reach an arbitrarily determined tumor size (i.e., 300 mg) minus median time in days for the control group (C) to reach the same tumor size.
4. Tumor regression: Treatment results in reductions in tumor size (mass) often with disappearance of the tumor.

Intravenous and/or Intraperitoneal Model Evaluation

1. Increase in life span (ILS%): Percentage increase in life span (days) of treated groups versus control or untreated groups.

2. Tumor growth delay (T–C): Median time in days for treated (T) group survival minus median time in days for control (C) group survival.
3. Long-term survivors (Cures): Treatment results in long-term survival where treatment groups survive up to and beyond three times the survival times of untreated or control groups.

Conclusions

The preparation of a liposomal drug formulation having enhanced therapeutic value is a challenging process that requires attention to a variety of important physicochemical characteristics (liposome size, trapped volume, transmembrane pH gradient, etc.), biochemical properties of both lipid and drug components (charge, pK_as, solubilities in polar and nonpolar environments, drug stability at acidic pH, etc.), biological performance (drug retention, liposome circulation longevity, efficacy, etc.), as well as methodological issues. In general terms, the processes described in this chapter for liposomal encapsulation of vincristine are similar to those that would be encountered in the development of many liposomal cytotoxic drugs (see Chapter 4). More specifically, the dramatic increases in therapeutic value occurring as a consequence of liposomal encapsulation of vincristine would also be expected to occur for other cytotoxic drugs whose activity is primarily cell-cycle dependent. Agents with this mechanism of action include the additional vinca alkaloids, as well as other agents, such as the taxanes, with activity against microtubules.

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