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Advances in Brief

Liposomal Vincristine Which Exhibits Increased Drug Retention and Increased Circulation Longevity Cures Mice Bearing P388 Tumors

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Abstract

Prolonged exposure to vincristine correlates with improved therapeutic activity. In this work, two methods are used to increase the circulation longevity of liposomal formulations of vincristine. The first involves incorporation of the ganglioside GM1, which acts to increase the circulation lifetime of encapsulated vincristine. The second involves decreasing the pH and incorporates into the liposomes the monosialoganglioside GM1'. Both approaches act synergistically to significantly enhance the circulation lifetime of encapsulated vincristine. The therapeutic activity of the resulting liposomal vincristine preparation is dramatically improved and results in cures (70-day survival) in over 50% of mice inoculated with P388 lymphocytic leukemia.

Materials and Methods

Oncovin (vincristine sulfate) was obtained from the B.C. Cancer Agency (Vancouver, British Columbia). DSPC was purchased from Avanti Polar Lipids and was greater than 99% pure. Monosialoganglioside GM1, cholesterol, and all salts were obtained from Sigma Chemical Co. (St. Louis, MO). Cholesteryl hexadecyl ether (CH(2)CH(2)OH), a lipid marker that is not exchanged or metabolized in vivo (20), was synthesized by Amersham (Oakville, Ontario). Female BDF1 mice (6–8 weeks old) were purchased from Charles River Laboratories (Ontario).

DSC/Chol (55:45 mol:mol) or DSC/Chol/GM1 (45:5:10 mol:mol) solutions were prepared by dissolving the lipid mixtures in 95% ethanol (0.1 μm diameter pores (21)). The extrusion device, obtained from Lipex Biomembranes (Vancouver, British Columbia), was also maintained at 60°C. Ethanol was removed from the liposome preparation by dialyzing (Spectra/Por 2 dialysis tubing, 12,000–14,000 MWCO) against two changes of 100 volumes of 250 mM citric acid (pH 4.0 or 2.0) over a 24-h period. More than 99.9% of the ethanol was removed using this procedure. Thin-layer chromatography analysis of all lipids, including GM1', showed no degradation at either pH 4.0 or 2.0 (results not shown).

Vincristine was entrapped in the liposomes using the transmembrane pH gradient (pH) loading procedure described elsewhere (9). Briefly, vesicles (25 mg/ml) were added to a vincristine solution (Oncovin; 1 mg vincristine/ml) to achieve a drug/lipid ratio of 0.1:1. The exterior pH of the liposome/vincristine mixture was raised to pH 7.0–7.2 with 0.5 M NaHPO4 and immediately heated to 60°C for 10 min. High-performance liquid chromatography analysis (isocratic DEA:methanol mobile phase on a C8 column with spectrophometric detection at 297 nm) of vincristine at both pH 2.0 and 4.0 showed no degradation over 24 h (results not shown).

Plasma clearance studies were performed by injecting 20 mg lipid/kg body weight of drug-loaded or empty liposomes via a lateral tail vein to female BDF1 mice (18–22 g). The vincristine dose was, therefore, typically 2 mg/kg. Previous studies have shown that this dose of vincristine, when entrapped in liposomes, exhibits measurable levels of antitumor activity in L1210 and P388 ascites tumor models (9). Four mice were used per time point. The mice were anesthetized at the indicated time points (i.p. mixture of 160 mg/kg ketamine and 20 mg/kg xylazine). Blood was collected via cardiac puncture and placed into EDTA-coated microtainer tubes (Becton Dickinson). The samples were then centrifuged (500 x g for 10 min) to pellet the blood cells and obtain...

Received 2/3/94; accepted 4/21/94.

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1 This research was supported by the Natural Cancer Institute of Canada. N. L. B. is a recipient of an MRC Fellowship. M. B. B. is a B.C. Health Research Foundation Scholar.

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Accession Numbers

The abbreviations used are: PEG, polyethylene glycol; pH, interior pH.

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plasma samples. Liposomal lipid and/or vincristine were then assayed using scintillation counting.

Biodistribution studies were performed on the same mice used for plasma clearance studies. Following heart puncture, animals were killed by cervical dislocation, and selected tissues were removed from each animal and weighed. Saline was added to each organ to achieve a 10% (w/v) ratio and homogenized using a Polytron homogenizer (Brinkmann Instruments, Rexdale, Ontario). Tissue homogenates (500 μl) were digested with 300 μl of "Solvable" (Du Pont, Inc.) for 3 h at 50°C. Subsequently, the samples were cooled to room temperature before decolorizing with 200 μl of 30% hydrogen peroxide. Samples were then counted using Picofluor (Packard) scintillation cocktail.

The statistical significance of both the plasma clearance and biodistribution results were determined using the Student t test.

Results

Plasma Clearance and in Vivo Drug Release Studies. The influence of GM1 incorporation and entrapped vincristine on the circulation lifetime of 100-nm diameter DSPC/Chol liposomes is shown in Fig. 1A. Two important conclusions can be derived from this data: (a) the circulation lifetime of liposomes containing vincristine is greater than control, drug-free liposomes. This effect cannot be achieved by pretreatment with free drug (data not shown) and is consistent with previous results demonstrating that the presence of encapsulated doxorubicin significantly increases the circulation longevity of associated liposomal carriers (22, 23); (b) incorporation of 10 mol% GM1 in 100 nm DSPC/Chol liposomal vincristine results in a further increase in carrier circulation lifetime. Plasma liposomal lipid levels are increased approximately 2.5-fold 24 h after i.v. administration when GM1 is incorporated into DSPC/Chol liposomal vincristine as compared to DSPC/Chol. Liposomal lipid accumulation in liver and spleen (Fig. 1, B and C) is reduced significantly when DSPC/Chol/GM1 liposomes are used to encapsulate vincristine. This data is consistent with results which indicate that incorporation of GM1 decreases uptake of liposomes by phagocytic cells of the reticuloendothelial system (18, 24).

The influence of GM1 incorporation on circulating vincristine levels over 24 h after i.v. administration is shown in Fig. 2. As expected on the basis of data in Fig. 1, incorporation of GM1 into DSPC/Chol pH 4.0 liposomes containing vincristine resulted in approximately a 3-fold increase in the level of drug remaining in the plasma at 24 h (Fig. 2A). The circulating drug:lipid ratios for these systems are shown in Fig. 2B, which shows that drug release from liposomes in the plasma compartment was not influenced by incorporation of GM1. For these liposomal vincristine formulations, where drug was encapsulated using the pH-gradient loading procedure in liposomes prepared in 300 mM citrate buffer pH 4.0, greater than 90% of the encapsulated drug was released from circulating liposomes over the 24-h time course.

Retention of vincristine entrapped in 100 nm DSPC/Chol liposomes in response to a transmembrane pH gradient can be improved by decreasing the pH of the encapsulated citrate buffer from 4.0 to 2.0 (11). As shown in Fig. 2A, decreasing the pH from 4.0 to 2.0 resulted in a 2.5-fold increase in the circulating vincristine levels achieved at 24 h post i.v. injection. The change in intravesicular pH did not influence the clearance behavior of injected liposomes (data not shown); thus, the increased drug levels result from improved drug retention. At every time point, higher drug:lipid ratios were observed for vincristine encapsulated in liposomes prepared at pH 4.0 (Fig. 2B).

Incorporation of GM1 in combination with the use of an interior pH of 2.0 resulted in an unexpected and dramatic improvement in the vincristine circulation lifetime. As shown in Fig. 2A, plasma vincristine levels were approximately 7.5- and 20-fold higher at 24 h than were achieved with comparable systems prepared in the absence of GM1 using the pH 4.0 buffer. As shown in Fig. 2B, DSPC/Chol/GM1 liposomes prepared at pH 4.0 exhibited less than a 20% decrease in drug:lipid ratio over 24 h in the circulation and exhibited a 12-fold increased circulation half-life as compared to DSPC/Chol pH 4.0 liposomes.
determined following i.v. administration in mice of liposomal vincristine prepared using vincristine circulation lifetimes achieved through the use of GM1 and using at least four mice. DSPC/Chol pH 4.0 ( ), DSPC/Chol/GM1 pH 4.0 ( ), DSPC/Chol pH 2.0 ( ) and DSPC/Chol/GM1 pH 2.0 ( ) liposomes. Vincristine was encapsulated at a drug:lipid ratio displayed significantly reduced weight loss at 4 mg/kg compared to the either free drug or the DSPC/Chol pH, 4.0 liposomal vincristine formu-
lations. Long-term survivors were seen with the DSPC/Chol preparation when administered at 2, 3 and 4 mg/kg, produced long-term survival rates observed which are greater than 50%. No long-term survivors (>70 days) were obtained with either free drug or the DSPC/Chol pH, 4.0 liposomal vincristine formulations. Long-term survivors were seen with the DSPC/Chol preparation at pH 2.0 and the DSPC/Chol/GM1 pH, 4.0 preparation at 4 mg/kg. Both these formulations, however, had significantly lower median survival times than the DSPC/Chol/GM1 pH, 2.0 preparation. Drug-induced weight loss data (% decrease in weight on day 7) shown in Table 1 also suggests a decrease in drug toxicity for the DSPC/Chol/GM1 pH, 2.0 liposomes compared to DSPC/Chol pH 4.0 liposomes. This preparation displayed significantly reduced weight loss at 4 mg/kg compared to the DSPC/Chol pH, 4.0 formulation.

Discussion

Incorporation of lipids such as GM1 and PEG-derivatized phospho-
lipids into liposomes can increase liposome circulation lifetime and therapeutic activity of certain liposomal drugs (12–19, 24–26). This approach has been used for liposomal formulations of doxorubicin and cytosine arabinoside (12, 25). However, in order for GM1 or PEG-PE to improve the drug circulation life-time, the entrapped drug must be retained inside liposomes which reside in the plasma compartment. For example, incorporation of GM1 in previous liposomal vincristine preparations was of questionable value due to the relatively rapid release of drug from liposomes in the circulation (6). The advent of procedures which enhance vincristine retention (11), however, have now made the use of lipids which result in extended liposome circulation lifetimes of interest.

A decrease in the pH of the entrapped citrate buffer for DSPC/Chol liposomes leads to improved vincristine retention in the circulation as evidenced by the 3-fold increase in the circulating drug:lipid ratio of DSPC/Chol pH, 2.0 compared to DSPC/Chol pH, 4.0 preparations 24 h after i.v. administration (Fig. 2A). These results, when combined with the fact that including GM1 into DSPC/Chol liposomes at pH 4.0 results in a 2.5-fold increase in circulating liposomal lipid levels at 24 h, could suggest that plasma vincristine concentrations for GM1 pH, 2.0 liposomes should be approximately 7.5-fold greater than observed for the DSPC/Chol pH, 4.0 systems studied previously (6, 9). The data presented here indicate, however, that lowering the pH of the entrapped buffer to 2.0 and including GM1 in the membrane synergistically combine to dramatically increase the vincristine levels in the plasma. Specifically, 24 h after i.v. administration, circulating drug levels are increased nearly 20-fold when DSPC/Chol/GM1 pH, 2.0 liposomes are used, as compared to DSPC/Chol pH, 4.0 systems.

The mechanism whereby the use of GM1 and pH 2.0 entrapped citrate synergistically stabilize liposomal vincristine preparations is not understood in detail. It is of interest to note that the incorporation of GM1 results in no increase in drug retention for an internal pH of 4.0. However, at pH 2.0, there is a substantial increase in drug retention when GM1 is added. This is likely due to the fact that lowering the pH and adding GM1 act via different mechanisms to increase drug retention. It has been shown previously that vincristine is released from liposomes as the pH gradient across the liposome membrane decays (11). By increasing the initial pH gradient across the membrane, the drug can be retained by the liposome more effectively. The mechanism whereby incorporation of GM1 could decrease drug leakage is less obvious; however, it has been shown that the presence of GM1 decreases the amount of protein to binding to the liposome (26). Such a decrease in plasma protein binding can render liposome membranes less leaky (27). However, at pH 4.0, drug leakage may be sufficiently rapid that any stabilizing effects of GM1 do not significantly influence drug retention in vivo. The enhanced circulation lifetimes observed for the GM1 pH, 2.0 liposomal vincristine preparations translate into substantially improved antitumor activity. This is consistent with previous reports that the antitumor activity of the DSPC/Chol pH, 4.0 formulation of vincristine is significantly higher than observed for free drug or drug encapsulated in leaker liposomes containing egg yolk phosphatidylcholine. The results obtained here extend this initial observation and show that a drug with minimal activity against the murine P388 tumor model can be transformed into one where cures are achieved by improving in vivo drug retention properties. This is particularly evident at drug dosages above 2 mg/kg, where long-term survival rates are observed which are greater than 50%.

It is important to emphasize that the improved therapeutic activity observed here is obtained following a single i.v. dose of encapsulated vincristine. It could be argued that similar results would be achieved with a systemic infusion of free drug. Results from this laboratory (28) and others (12), however, suggest that increased therapeutic activity achieved with liposomal anticancer drugs is due primarily to accumulation of drug-loaded liposomes in the region of tumor cell growth. Furthermore,
recent pharmacological studies\(^4\) indicate that systemic exposure to free vincristine is lower for drug administered in liposomal form compared to the equivalent dosage of freedrug. Therefore, improved drug retention characteristics should result in improved specificity of drug delivery to the tumor and hence, increased therapeutic activity. Comparable drug delivery to a defined region of disease growth would not be expected using a continuous i.v. infusion of free vincristine.

In summary, the results presented here demonstrate that low pH values and incorporation of GM\(_2\) into liposomal membranes results in a synergistic increase in vincristine retention in liposomes in vivo, resulting in turn in a dramatic improvement in the long-term survival rate in a P388 murine leukemia model. It is likely that these procedures will prove useful to increase drug retention of other drugs which can be loaded in response to transmembrane pH gradients to improve the therapeutic profile.

References


\(^4\) L. D. Mayer, unpublished observations.