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Liposome-encapsulated vincristine, vinblastine and vinorelbine: A comparative study of drug loading and retention

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Abstract

A comparative study of the loading and retention properties of three structurally very closely related vinca alkaloids (vincristine, vinorelbine and vinblastine) in liposomal formulations has been performed. All three vinca alkaloids showed high levels of encapsulation when accumulated into egg sphingomyelin/cholesterol vesicles in response to a transmembrane pH gradient generated by the use of the ionophore A23187 and encapsulated $MgSO_4$. However, despite the close similarities of their structures the different vinca drugs exhibited very different release behavior, with vinblastine and vinorelbine being released faster than vincristine both in vitro and in vivo. The differences in loading and retention can be related to the lipophilicity of the drugs tested, where the more hydrophobic drugs are released more rapidly. It was also found that increasing the drug-to-lipid ratio significantly enhanced the retention of vinca alkaloids when the ionophore-based method was used for drug loading. In contrast, drug retention was not dependent on the initial drug-to-lipid ratio for vinca drugs loaded into liposomes containing an acidic citrate buffer. The differences in retention can be explained on the basis of differences in the physical state of the drug inside the liposomes. The drug-to-lipid ratio dependence of retention observed for liposomes loaded with the ionophore technique may provide a way to improve the retention characteristics of liposomal formulations of vinca drugs.
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Keywords: Liposomes; Vincristine; Vinorelbine; Vinblastine; Drug release

1. Introduction

The vinca alkaloids vincristine, vinblastine and vinorelbine represent one of the most widely used classes of antineoplastic agents. Their cytotoxic activity is based on their ability to inhibit microtubule

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dynamics and assembly, arresting cell growth during metaphase [1–3]. Whereas vincristine and vinblastine are derived from the periwinkle plant, vinorelbine (5′-nor-anhydrovinblastine) is a semisynthetic vinca alkaloid that differs from other vincas by a modification of the catharanthine moiety (Fig. 1). As compared to the naturally occurring vincas, vinorelbine exhibits a broader spectrum of antitumor activity and reduced neurotoxicity [4].

Since vinca alkaloids are cell-cycle specific drugs that inhibit cell growth exclusively during metaphase, it is advantageous to expose tumor sites to the drug for prolonged periods of time so that more tumor cells can be exposed to the drug during the sensitive part of their cell cycle. One way to prolong the exposure time is to encapsulate the drug in a slow-release liposomal system. This can result in liposomal formulations of vincristine that exhibit enhanced therapeutic efficacy compared to free drug [5–9].

The antitumor efficacy of liposomally entrapped vincristine is strongly dependent on the rate of drug release from the liposomal carrier in the blood [5,8]. The release rate, in turn, depends on the physico-chemical properties of the drug, in particular lipophilicity, acid–base properties, solubility as well as the capability to form ion-pair complexes or precipitates [10–17]. In the case of vinca alkaloids, the subtle dissimilarities of their chemical structures result in significant differences in their therapeutic indications [18]. The effects of these small structural differences on drug release following encapsulation in liposomes have not been characterized.

In the present work, we compare the retention properties of egg sphingomyelin/cholesterol (ESM/chol) liposomes loaded with vincristine, vinblastine and vinorelbine in response to a magnesium sulfate gradient. It is shown that vinblastine and vinorelbine exhibit faster release in comparison to vincristine both in vitro and in vivo. Furthermore, it is shown that increasing the drug-to-lipid ratio significantly enhances the retention properties of the liposomal systems tested. This provides a possible way to improve the retention characteristics of drugs exhibiting faster release.

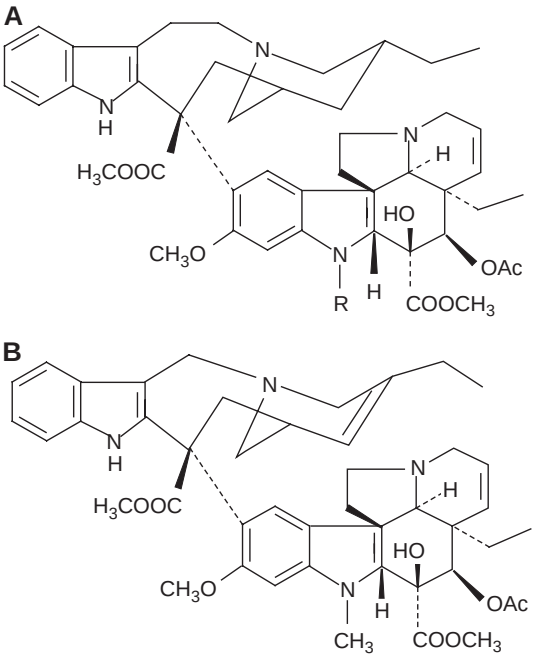

Fig. 1. Chemical structures of (A) vinblastine (R=CH_3) and vincristine (R=CHO), (B) vinorelbine.

2. Materials and methods

2.1. Materials

Female ICR mice were acquired from Harlan (Indianapolis, In). Egg sphingomyelin (ESM) was purchased from Northern Lipids (Vancouver, BC, Canada) and was used without further purification. Cholesterol (Chol), A23187, ammonium sulfate and magnesium sulfate were purchased from Sigma Chemical Company (St. Louis, MO). [^{14}C] cholesteryl hexadecyl ether (CHE) was custom synthesized by DuPont New England Nuclear (Boston, MA). [^{3}H] CHE was obtained from Perkin Elmer Life Sciences (Boston, MA). Vincristine sulfate and vinblastine sulfate were from Fine Chemicals (Australia). Radiolabeled vincristine sulfate (^{14}C) was obtained from Chemsyn Laboratories (Lenexa, KA). Vinorelbine ditartrate was obtained from OmniChem Sa (Louvain-la-Neuve, Belgium). [^{3}H]vinorelbine and [3H]vinblastine were synthesized by Moravek Biochemicals Inc. (Breas, CA). Fetal bovine serum (FBS) was from Invitrogen (Burlington, ON, Canada). All other reagents used in this study were reagent grade.

2.2. Preparation of large unilamellar vesicles exhibiting a transmembrane magnesium sulfate or citrate gradient

ESM/Chol (55:45 mol/mol) lipid mixtures, containing trace amounts of either [^{14}C] CHE or [^{3}H] CHE, were prepared by co-dissolving the lipids in chloroform and drying under a nitrogen stream followed by the removal of residual solvent under high vacuum. Dried lipid films were dissolved in ethanol and the ethanolic lipid solution was then slowly added to the appropriate salt solution (300 mM citrate at pH 4 or 300 mM magnesium sulfate solution) to give 10 vol.% final ethanol concentration. LUVs were generated by extruding the lipid emulsion through two stacked Nuclepore polycarbonate filters with a pore size of 100 nm (10 passes) at 65 °C using an extrusion device obtained from Lipex Biomembranes (Vancouver, BC, Canada) and subsequent removal of ethanol by dialysis. The mean diameter of LUVs was determined by dynamic light scattering using a NICOMP 370 particle sizer (Nicomp Particle Sizing, Santa Barbara, CA) and found to be 115±25 nm. Phospholipid concentrations were determined by the phosphorus assay of Fiske and Subbarow [19].

The magnesium sulfate gradient was formed by dialyzing the dispersion of liposomes against sucrose buffer (20 mM HEPES, 300 mM sucrose, pH 7.4). Subsequent addition of ionophore A23187 to the dispersion of LUVs results in the outward movement of one metal cation in exchange for two protons thus establishing a transmembrane pH gradient [20]. The gradient of citrate was generated through exchange of the external salt solution with 150 mM Hepes-buffered saline (HBS; 150 mM NaCl, 20 mM Hepes, pH 7.4) by gel filtration chromatography using Sephadex G-50 spin columns.

2.3. Drug uptake

For all formulations, the loading was performed at 60 °C. The loading of vinca drugs in citrate-containing LUVs was performed using a standard procedure [21]. For $MgSO_4$-containing LUVs, ionophore A23187 (2 μg/mg lipid), EDTA (15 mM final concentration) and the drug solution containing trace amounts of corresponding radiolabeled drug were added to the liposome preparation (5 mM total lipid) to give the desired initial drug-to-lipid ratio. The presence of external chelator (EDTA) was required to bind metal cations released from the vesicle. Aliquots were taken at different time points for determination of drug uptake by running the aliquots over Sephadex G-50 spin columns. Loaded systems were dialysed for 2 h against sucrose buffer to remove unentrapped drug and traces of ionophore [20]. The lipid and drug concentrations were determined by liquid scintillation counting (Beckman LS 3801) for [^{3}H] and [^{14}C] labels.

2.4. In vitro assay for drug retention

Experiments were carried out in glass test tubes by diluting samples with saline and FBS (0.5 mM final total lipid, 50% FBS), followed by incubation at 37 °C. Under these conditions concentration effects are negligible as the external volume is more than 3 orders of magnitude greater than the intravesicular volume (internal volume of 100 nm liposomes is about 1.5 μl/μmol lipid) [17]. Samples at various time points were taken and concentrations of entrapped drug were determined by dual label liquid scintillation counting following removal of unentrapped drug by size exclusion chromatography using Sepharose CL6B spin columns as described above for drug uptake experiments. The percent of retention was defined as the drug-to-lipid ratio at time t divided by the initial drug-to-lipid ratio.

2.5. In vivo drug release

Liposomal formulations of different vinca alkaloids were injected i.v. into the lateral tail vein of female ICR mice (4 mice per time point) at a lipid dose of 50 mg/kg. At desired time points, mice were anesthetized and whole blood was collected via cardiac puncture into EDTA-containing tubes. Plasma was isolated from the whole blood by centrifugation using a Beckman centrifuge equipped with a GH-3.8 rotor at 1250 rpm for 10 min. The blood samples (50 μl) were mixed with 500 μl of Solvable and left for overnight digestion at room temperature. Then 50 μl of 200 mM EDTA were added followed by 200 μl of H_2O_2 (30 vol.%). The samples were left to decolorize overnight. The next day, 10 μl of 10 N HCl were added before 5 ml of Pico Green 40, and the drug and

lipid concentrations were determined by dual label scintillation counting.

2.6. Cryo-transmission electron microscopy (Cryo-TEM)

The Cryogenic Transmission Microscopy investigations were performed with a Zeiss EM 902A Tranmission Electron Microscope (LEO Electron Microscopy, Oberkochen, Germany). The instrument was operating at 80 kV and in zero loss bright-field mode. Digital images were recorded under low dose conditions with a BioVision Pro-SM Slow Scan CCD camera (Proscan GmbH, Scheuring, Germany) and analySIS software (Soft Imaging System, GmbH, Münster, Germany). An underfocus of 1–2 μm was used to enhance the image contrast.

3. Results

3.1. Drug accumulation in response to magnesium sulfate gradient

It has previously been shown that a transmembrane proton gradient (ΔpH) can be established by the use of the ion translocating properties of ionophores [20]. Briefly, liposomes containing $MgSO_4$ can be incubated with the ionophore A23187, which couples the outward movement of the entrapped Mg^{2+} to the inward movement of protons. The resulting acidification of the vesicle interior can drive the uptake of external drug. In the present study, this approach has been employed to load vincristine, vinblastine and vinorelbine into large unilamellar vesicles (LUVs) composed of egg sphingomyelin/cholesterol (ESM/Chol, 55:45 mol/mol), a lipid composition giving rise to liposomal vincristine formulations exhibiting slow drug release [5,7].

As shown in Fig. 2A–C, all of the drugs used were encapsulated in the liposomes with efficiencies equal to or exceeding 80% following 2 h incubation at 60 °C at a drug-to-lipid ratio of 0.1 wt/wt. For vincristine and vinorelbine, the rate and extent of the drug loading was found to be highly dependent on temperature: at 25 °C, less then 10% of the initially added drug was encapsulated after 2 h incubation (Fig. 2B,C). However, the temperature dependence of

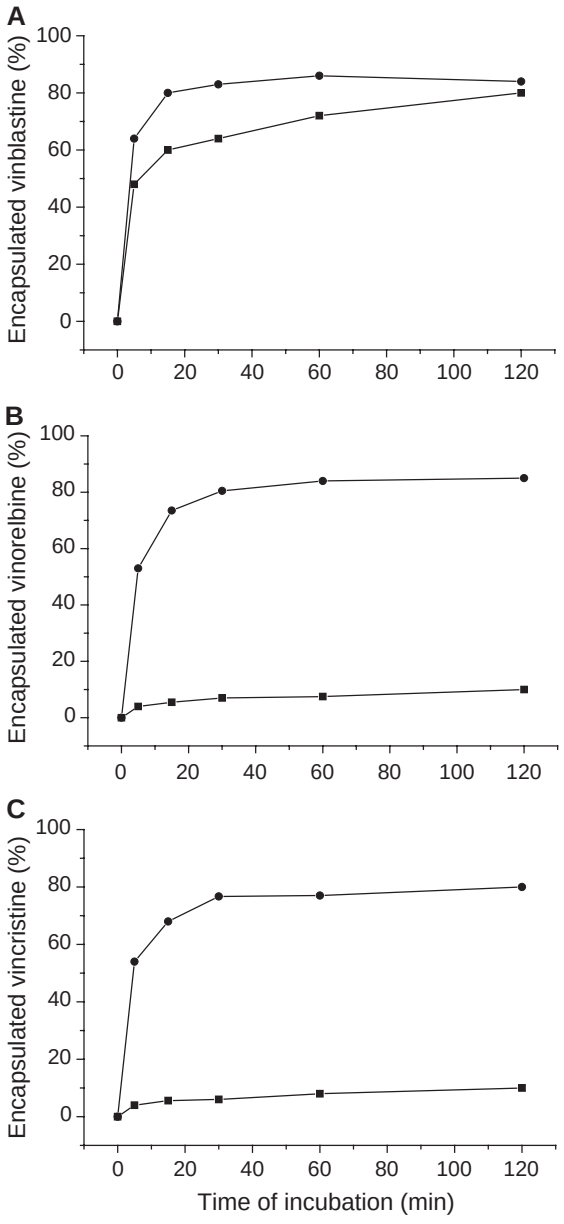

Fig. 2. Effect of temperature on uptake of vinblastine (A), vinorelbine (B) and vincristine (C) into 100 nm ESM/Chol LUVs (55:45 mol/mol) containing 300 mM $MgSO_4$. After addition of ionophore A23187 (2 μg/mg lipid), EDTA (15 mM final concentration) and drug solution to the liposome preparation (5 mM total lipid) to give the drug-to-lipid ratio of 0.1 wt/wt, samples were incubated at 25 (■) and 60 °C (●). Aliquots were taken at different time points for determination of the lipid and drug concentrations by dual label scintillation counting as described in Materials and methods. The percent of drug uptake was defined as the drug-to-lipid ratio at time t divided by 0.1.

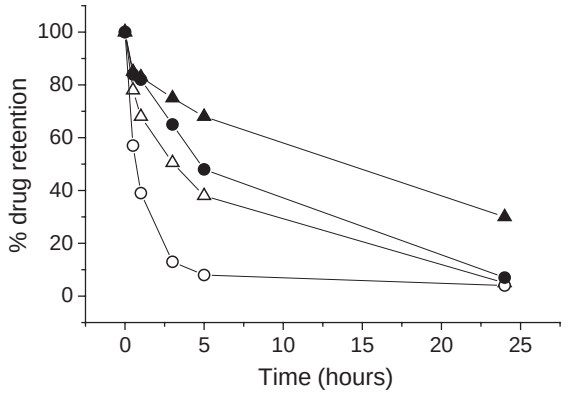

Fig. 3. In vitro release of vinorelbine (circles) and vincristine (triangles) from 100 nm ESM/Chol (55:45 mol/mol) LUVs loaded using magnesium sulfate gradient /ionophore A23187 procedure. The drug-to-lipid ratios were 0.1 wt/wt (open symbols) and 0.3 wt/wt (closed symbols). Release was measured in the presence of 50% FBS at an incubation temperature 37 °C, the lipid concentration was 0.5 mM. Aliquots were taken at different time points for determination of the lipid and drug concentrations by dual label scintillation counting as described in Materials and methods. The percent of retention was defined as the drug-to-lipid ratio at time t divided by the initial drug-to-lipid ratio.

the loading was less profound for vinblastine (Fig. 2A). Even though vinblastine loading was slower at 25 than at 60 °C comparable levels of drug accumulation were achieved after 2 h of incubation at the respective temperatures.

Although the vinca alkaloids used in this study have similar chemical structures, their octanol/water partition coefficients (P) differ significantly, in the order $\log P_{\text{vinblastine}} > \log P_{\text{vinorelbine}} > \log P_{\text{vincristine}}$ (1.89, 1.32 and 1.16, correspondingly) [22]. Thus, the increased uptake of vinblastine may arise due to its higher relative hydrophobicity, which can facilitate its transbilayer movement at lower temperatures. Membrane partition effects may also be involved.

3.2. In vitro release assays: release of vincristine and vinorelbine in response to the fetal bovine serum

The release of vincristine and vinorelbine encapsulated using the $MgSO_4$/ionophore procedure was assayed in an in vitro release test employing incubation of the liposomal samples in 50% fetal bovine serum (FBS, 0.5 mM lipid) at 37 °C. Fig. 3 compares the in vitro release properties of these different vinca alkaloids at two different drug-to-lipid ratios (0.1 and 0.3 mg/mg). Both vincristine and vinorelbine showed better retention at higher drug-to-lipid ratios and vincristine was much better retained than vinorelbine at both drug-to-lipid ratios.

3.3. In vivo pharmacokinetics of liposome-encapsulated vincristine, vinblastine and vinorelbine

The next set of experiments were aimed at determining the retention properties of vincristine-, vinblastine- and vinorelbine-containing liposomes in vivo. The vinca drugs were loaded into ESM/Chol

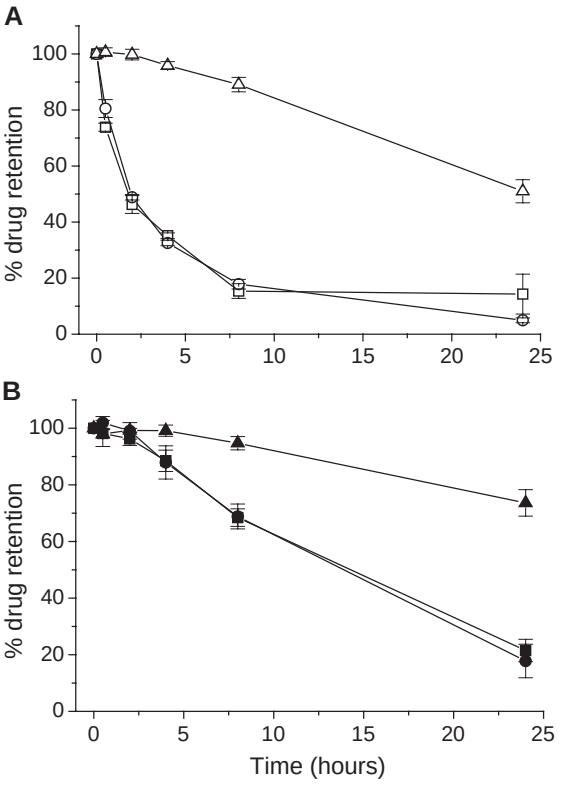

Fig. 4. Comparison of in vivo drug release rates obtained from the liposomal systems loaded at drug-to-lipid ratio of 0.1 wt/wt (A) and 0.3 wt/wt (B) with vinblastine, vinorelbine and vincristine (squares, circles and triangles, correspondingly). Open symbols represent 0.1 wt/wt formulations and closed symbols represent 0.3 wt/wt formulations. Following i.v. injection (lipid dose 50 mg/kg), samples were withdrawn at the indicated times, and the drug-to-lipid ratio of the recovered LUVs was determined. Lipid and drug concentrations were measured as described in Materials and methods. The percent of retention was defined as the drug-to-lipid ratio at time t divided by the initial drug-to-lipid ratio.

LUVs to give final drug-to-lipid ratios of 0.1 and 0.3 wt/wt and injected i.v. into female ICR mice at a lipid dose of 50 mg/kg. Following injection, blood samples were withdrawn at the indicated times, and the drug-to-lipid ratios of the recovered LUVs were determined.

Fig. 4 depicts the in vivo drug release profiles of vincristine, vinorelbine and vinblastine for liposomes loaded with the $MgSO_4$/ionophore technique at different drug-to-lipid ratios. Retention was highly dependent on the drug employed. Vincristine was retained much better than vinblastine and vinorelbine at both high (0.3 mg/mg) and low (0.1 mg/mg) drug-to-lipid ratios. As in the case of drug uptake, the differences of release rates could be directly related to the differences in the octanol–water partition coefficients of the drugs used. It should be noted that the in vitro release assay data were in good agreement with the in vivo release data.

Furthermore, the in vivo retention of all vinca alkaloids was found to be highly dependent on the drug-to-lipid ratio, showing an increase in drug retention with increasing drug-to-lipid ratio. In contrast to this, an increase in drug-to-lipid ratio did not result in improved drug retention when vincristine and vinorelbine were encapsulated in response to a pH gradient formed by the entrapment of an acidic citrate buffer (Fig. 5). Analogous to the ionophore-loaded liposomes vincristine was much better retained than vinorelbine. However, in the case of the citrate loading technique the drug retention was independent of the drug-to-lipid ratio. The observed differences between citrate and ionophore/$MgSO_4$-loaded liposomes could be related to differences in the intravesicular form of the drugs in these two systems. The better retention afforded by the ionophore loading method at higher drug-to-lipid ratios may provide a possible way to improve the retention characteristics of drugs exhibiting faster release rates.

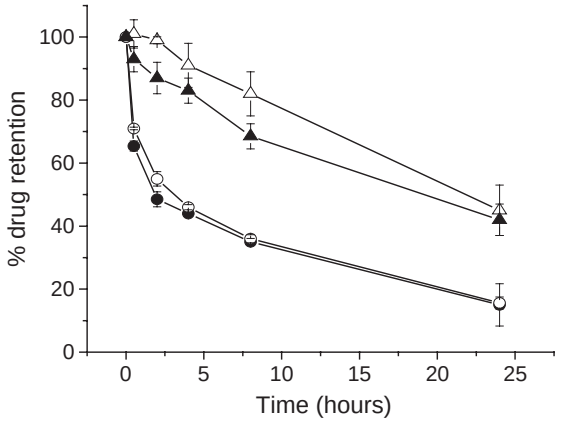

Fig. 5. In vivo retention of vinorelbine (circles) and vincristine (triangles) loaded into 100 nm ESM/Chol (55:45 mol/mol) citrate-containing LUVs. The drug-to-lipid ratios were 0.1 wt/wt (open symbols) and 0.3 wt/wt (closed symbols). The drug release was measured under conditions indicated in the legend to Fig. 4.

4. Discussion

The effectiveness of a liposomal carrier can be limited by its ability to retain drug while in the circulation. Rapid release of the drug will eliminate the benefits of liposomal encapsulation, resulting in efficacy similar to that of an equivalent dose of free drug. Conversely, complete retention will also compromise the therapeutic activity of the encapsulated drug as the drug is not bioavailable. Thus, therapeutic optimum lies somewhere in between these two extreme points. In case of each individual drug, the optimal balance between retention and release must be established experimentally by varying the factors controlling the drug leakage rate.

The fact that clinically used vinca drugs require different intravenous weekly doses for each particular drug suggests that liposomal formulation of those drugs will also require different dosages. On the other hand, the dosage of the liposomal drug may be dependent on its circulation longevity, which can lead to enhanced efficacy. Thus, factors that influence the retention characteristics must be carefully considered in the early stages of the development of a liposomal drug. The objective of this study was to evaluate the loading and retention properties of vinca alkaloids (vincristine, vinorelbine and vinblastine) liposomal formulations as a first step towards their optimization in tumor models.

The results of our studies showed that the uptake and retention properties of the vinca alkaloids vincristine, vinorelbine and vinblastine differed significantly despite the close similarities of their structures. The observed differences correlate with the lipophilicity of the drug. Vinblastine and vinorelbine differ from vincristine at the *R* position in the

vindoline moiety with a methyl group replacing a formyl group (Fig. 1), resulting in a more hydrophobic molecule as indicated by the increase of their octanol/water partition coefficients [22]. Although these values may not accurately reflect the actual membrane/water partition coefficients, they can still be useful when comparing drugs belonging to the same family. Indeed, vinblastine, which has the highest partition coefficient of the vincas tested, can be loaded into ESM/Chol vesicles at room temperature. This is in agreement with studies indicating that vinblastine and vinorelbine exhibit faster rates and higher levels of uptake into cells compared to vincristine [23–25]. However, the higher relative hydrophobicity also results in faster release. As a result, vincristine, the less hydrophobic drug, demonstrates substantially better retention compared to both vinorelbine and vinblastine. This trend was independent of the loading technique despite the differences in drug retention characteristics of the ionophore and citrate loading techniques, demonstrating that the observed effects are a consequence of the physicochemical properties of these drugs.

The ionophore loading technique showed a strong dependence of retention (percent of initially encapsulated drug retained at time t) on the drug-to-lipid ratio or intravesicular drug concentration for that matter (Figs. 3 and 4). An increase in intravesicular drug concentration resulted in improved retention. In the case of the citrate loading technique the retention of the different vinca alkaloids was independent of the drug-to-lipid ratio (Fig. 5). A better understanding of this behavior can be obtained if the argument is carried on in terms of release rates instead of percent retention. The lack of dependency of retention on drug-to-lipid ratio observed for citrate-loaded liposomes means that with increasing drug-to-lipid ratio more drug is released per unit time. In other words, the release rate, i.e. the number of molecules per unit time (dn/dt) crossing the membrane, increases with increasing intravesicular drug concentration (drug-to-lipid ratio). This is in accordance with Fick's law that states that the diffusion rate dn/dt (release rate) through a membrane is directly proportional to the difference in the concentration $\Delta c(t)$ of the material across the membrane. In our case $\Delta c(t)$ is the concentration gradient of the neutral form of the drug as it is the membrane-permeable species. It follows that $dn/dt = -p \times A \times \Delta c(t)$, where p and A are the permeability coefficient and the membrane area, respectively [26]. The release rates observed for the ionophore loading technique at higher drug-to-lipid ratio, on the other hand, are slower than the ones predicted from Fick's law indicating that they are no longer dependent on the total concentration of intravesicular drug (the concentration gradient appears to be smaller than based on the intravesicular drug concentration). This could indicate that part of the drug is precipitated in the liposome interior and the concentration gradient is actually determined by the solubility of the drug in the intravesicular space. It should be noted that the rate of dissolution of the intravesicular precipitate could further modulate the internal drug concentration and even become the rate-limiting step for release.

The interior of ionophore-loaded vinca alkaloid-containing liposomes appears more electron-dense in Cryo-EM micrographs compared with drug-free (unloaded) control liposomes (Fig. 6). This could be a further indication that the drugs are precipitated in the liposome interior. The morphology of the precipitate is different than for other liposomal drugs such as doxorubicin and topotecan that were loaded using the ammonium sulfate or ionophore/magnesium sulfate

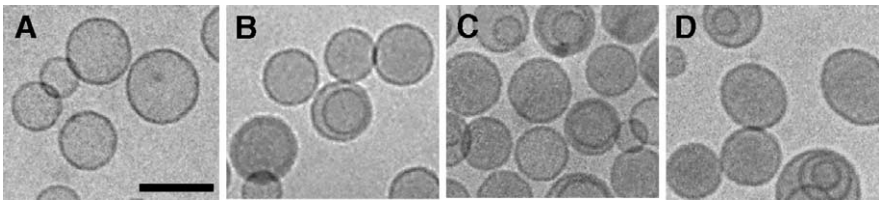

Fig. 6. Cryo-EM micrographs of liposomal formulations of different vinca alkaloids. The drugs were loaded into ESM/chol (55/45) liposomes at a drug-to-lipid ratio of 0.3 mg/mg using the ionophore technique (A23187/$MgSO_4$). (A) Liposomes in the absence of drug, (B) liposomal vinblastine, (C) liposomal vinorelbine, and (D) liposomal vincristine. The size bar represents 100 nm.

technique, where drug crystals could be seen in the liposome interior [10,11,27]. The nature of the precipitate (amorphous versus crystalline) and the extent of precipitation can be affected by factors such as the type of counterion and the intravesicular pH. Further experiments are being conducted to investigate the effect of these variables on release and a more detailed study will be presented elsewhere.

5. Conclusion

Structurally very closely related liposomally encapsulated vinca alkaloids exhibited very different retention properties. Vinblastine and vinorelbine were released much faster than vincristine. The differences in loading and retention of these vinca drugs could be related to the lipophilicity of the drugs, where the more hydrophobic drugs were released more rapidly. Furthermore, increasing the drug-to-lipid ratio significantly enhanced the retention properties of liposomal formulations of these vinca drugs when the ionophore technique was used for loading. This may provide a way to improve the retention characteristics of drugs exhibiting poor retention at low drug-to-lipid ratios.

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