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Ionophore-mediated uptake of ciprofloxacin and vincristine into large unilamellar vesicles exhibiting transmembrane ion gradients

David B. Fenske [a,*], Kim F. Wong [a], Elisabeth Maurer [a], Norbert Maurer [a], Johanna M. Leenhouts [a], Nancy Boman [b], Lawrence Amankwa [b], Pieter R. Cullis [a,b]

[a] *Liposome Research Unit, Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, B.C. V6T 1Z3, Canada*

[b] *Inex Pharmaceuticals Corporation, 100-8900 Glenlyon Parkway, Glenlyon Business Park, Burnaby, B.C. V5J 5J8, Canada*

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Abstract

A new method, based on the ion-translocating properties of the ionophores nigericin and A23187, is described for loading large unilamellar vesicles (LUVs) with the drugs vincristine and ciprofloxacin. LUVs composed of distearoylphosphatidylcholine/cholesterol (DSPC/Chol) (55:45 mol/mol) or sphingomyelin (SPM)/Chol (55:45 mol/mol) exhibiting a transmembrane salt gradient (for example, internal solution 300 mM $MnSO_4$ or K_2SO_4; external solution 300 mM sucrose) are incubated in the presence of drug and, for experiments involving divalent cations, the chelator EDTA. The addition of ionophore couples the outward movement of the entrapped cation to the inward movement of protons, thus acidifying the vesicle interior. External drugs that are weak bases can be taken up in response to this induced transmembrane pH gradient. It is shown that both nigericin and A23187 facilitate the rapid uptake of vincristine and ciprofloxacin, with entrapment levels approaching 100% and excellent retention in vitro. Following drug loading, the ionophores can be removed by gel exclusion chromatography, dialysis, or treatment with biobeads. In vitro leakage assays (addition of 50% mouse serum) and in vivo pharmacokinetic studies (in mice) reveal that the A23187/Mn^{2+} system exhibits superior drug retention over the nigericin/K^+ system, and compares favorably with vesicles loaded by the standard ΔpH or amine methods. The unique features of this methodology and possible benefits are discussed.

Keywords: Drug loading; pH gradient; Ionophore; A23187; Nigericin; Ciprofloxacin; Vincristine; Manganese; Divalent cation

1. Introduction

The ability of transmembrane pH gradients (ΔpH) to influence the equilibrium transmembrane distributions of certain weak acids and weak bases has long been recognized ([1], and Refs. therein). Recent work from this laboratory has demonstrated the transbilayer movement of a wide variety of drugs, biogenic amines, amino acids, peptides, lipids, and ions in large unilamellar vesicles (LUVs) exhibiting a ΔpH (for a review, see [1]). This technology can be employed to load liposomes with drugs such as the anticancer agents doxorubicin and vincristine, resulting in significantly reduced toxicity and equal or increased efficacy for the liposomally encapsulated formulations [2–13].

The pH gradient methodology has distinct advantages over other methods of encapsulation. These

* Corresponding author. Fax: (604) 8224843;
E-mail: fenske@unixg.ubc.ca

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include high trapping efficiencies (90–100%), high drug-to-lipid ratios, and greatly improved drug retention in vivo. The basic pH gradient-loading technique involves formation of the LUVs by extrusion in an appropriate buffer (e.g., 300 mM citrate pH 4.0), followed by formation of the pH gradient on a Sephadex column equilibrated in HEPES/NaCl pH 7.5. Other pH gradient-loading techniques include those involving LUVs exhibiting transmembrane gradients of ammonium sulfate [14–17] or other amines (including methylammonium sulfate, ethylammonium sulfate, propylammonium sulfate, amylammonium sulfate, and several diamines) [18]. The amine gradient gives rise to a secondary pH gradient, which drives drug uptake.

This paper describes a new method for loading LUVs with drugs which exhibit weak base characteristics. We build on an observation first made by Deamer [19] some 25 years ago, that addition of nigericin to SUVs containing potassium salts led to the generation of a pH gradient (interior acidic) of approx. 2 units. We apply this process for ionophores such as nigericin or A23187 to LUVs exhibiting transmembrane gradients of K^+ or $Mn^{2+}(Mg^{2+})$, respectively. The outward movement of the metal ions (down their concentration gradients) results in the acidification of the LUV interior, which drives drug uptake. We show that rapid uptake and excellent retention can be obtained for both vincristine and ciprofloxacin using both ionophores, and that reasonable in vivo clearance characteristics are achieved using the A23187 formulation.

2. Materials and methods

2.1. Materials

DSPC was obtained from Northern Lipids (Vancouver, B.C., Canada). Egg SPM, cholesterol (Chol), nigericin, and A23187 were obtained from Sigma. Vincristine sulfate was obtained from Eli Lilly Canada (Scarborough, Ont., Canada) or from Pharmacia (Mississauga, Ont., Canada), and [^{3}H]vincristine from Amersham (Oakville, Ont., Canada). Ciprofloxacin and [^{14}C]ciprofloxacin were obtained from Inex Pharmaceuticals. [^{3}H]Cholesterol hexadecyl ether (CHE) and [^{14}C]CHE were obtained from Dupont New England Nuclear. Normal mouse serum was obtained from Cedar Lane Laboratories (Hornby, Ont., Canada). All other chemicals were reagent grade.

2.2. Preparation of large unilamellar vesicles

DSPC/Chol and SPM/Chol (55:45 mol/mol) lipid mixtures, containing a trace of either [^{14}C]CHE or [^{3}H]CHE, were prepared by lyophilization from *t*-butanol. The lipid film was hydrated in the salt of choice at 60°C and subjected to five cycles of freeze-thawing using liquid nitrogen and water at 60°C, with vigorous vortexing of the lipid between each thaw and freeze cycle. LUVs were prepared by extruding the lipid emulsion through polycarbonate filters with a 0.1 μm pore size under high pressure (300–400 psi) at 60°C [20]. Lipids were hydrated with a variety of salt solutions, including K_2SO_4, KH_2PO_4, K_2HPO_4, K-tartrate, $CaCl_2$, $MnSO_4$, and $MgSO_4$. The salt concentrations were 300 mM or 600 mM; for some experiments the pH was adjusted, usually in the range of 6–7.5.

2.3. Formation of salt gradient

LUVs exhibiting a transmembrane salt gradient were prepared by solvent exchange using columns of Sephadex G-50 (1.5×10 cm) pre-equilibrated with 300 mM sucrose. Salt gradients were also established by gel filtration chromatography using spin columns [21], in which the equilibrated G-50 gel is packed into a 1 ml disposable syringe by centrifugation to 760×*g*. To establish the gradient, 50–100 μl aliquots of the LUVs are applied to each spin column and centrifuged at 760×*g* on a desktop centrifuge for 2 min. In a few cases, the gradient was formed by dialysis.

2.4. Uptake of ciprofloxacin and vincristine

For both drugs, uptake was performed at 60°C at a total lipid concentration of 5 mM (1 ml). For ciprofloxacin, the initial drug-to-lipid ratio was either 0.2 or 0.3 (mol/mol), and for vincristine the initial drug-to-lipid ratio was 0.03 (mol/mol). For uptake via nigericin in response to K^+ gradients, the LUVs and drug were combined and incubated for a period

of 15 min at 60°C. An aliquot (100 μl) was removed in order to determine the initial drug-to-lipid ratio, and a further aliquot (50–100 μl) was passed down a spin column to assess any uptake prior to addition of the ionophore. The nigericin (dissolved in EtOH) was then added (approx. 5 μl volume) to the suspension to give the desired concentration (ranging from < 1 ng/μmol lipid to 1 μg/μmol lipid). At various times, aliquots of the suspension were removed and applied to spin columns, with the eluant analyzed by dual label counting to monitor uptake of the drug (see below). The procedure for A23187-dependent drug uptake in response to gradients of divalent cations was essentially the same, except that in most cases EDTA was added to the external buffer to give a final concentration of 3–15 mM.

2.5. Drug leakage in response to mouse serum

An in vitro assay was chosen to give a qualitative comparison of the leakage of different drugs from the ionophore systems. Essentially, equal volumes of the liposomal drug formulation and of mouse serum were combined in a test tube and incubated at 37°C. Leakage of the drug from the LUVs was assayed by removal of aliquots for spin column analysis.

2.6. Drug and lipid assays

Ciprofloxacin concentrations were determined by measuring the absorbance at 275 nm following disruption of the vesicles and solubilization of the drug by a modified Bligh and Dyer extraction procedure [20]. Vincristine concentrations were determined by measuring the absorbance in 80% ethanol at 295 nm [22]. [^{14}C]Ciprofloxacin was diluted with cold ciprofloxacin and the specific activity determined by liquid scintillation counting and absorbance spectroscopy. The specific activity of [^{3}H]vincristine was determined in the same manner. For experiments involving [^{14}C]ciprofloxacin, the lipid mixtures were labeled using trace amounts of [^{3}H]CHE. For [^{3}H]vincristine uptake the lipid was labeled with [^{14}C]CHE. Lipid specific activities were determined by liquid scintillation counting and by quantification of phospholipid via phosphate assays [23]. For all uptake experiments, drug-to-lipid ratios were determined by dual label liquid scintillation counting. These were compared with drug-to-lipid ratios obtained by chemical and spectrophotometric assays and found to be identical.

2.7. Fluorimetric assay for A23187

The quantity of A23187 in SPM/Chol LUVs was determined by measuring the fluorescence intensity of the ionophore at an emission wavelength of 437 nm following solubilization of the liposomal formulation and complexation of the released divalent cations. Briefly, an aliquot of LUV/A23187 (corresponding to 5 μmol total lipid) was combined with a 333 mM EDTA solution (3 μl) and the volume was made up to 1 ml with ethanol:methanol (70:30 v/v). The sample was vortexed until clear, and the fluorescence intensity measured. Calibration standards were prepared by the addition of known aliquots of A23187 to a 1 ml solution consisting of ethanol:methanol (70:30 v/v) and containing 5 mM total lipid.

2.8. In vivo pharmacokinetics

SPM/Chol LUVs (100 nm diameter) were used (55:45 mol/mol). The vesicles were prepared in a 300 mM $MnSO_4$ solution and the external medium was exchanged with 300 mM sucrose by overnight dialysis. Ciprofloxacin was loaded at a drug-to-lipid ratio of 0.2 (mol/mol) and vincristine was loaded at a drug-to-lipid ratio of 0.03 (mol/mol). Drug uptake was accomplished by addition of 0.1 μg A23187/μmol lipid, followed by an aliquot of EDTA pH 7 (to give a final concentration of 30 mM) and the appropriate amount of drug. The resulting solution was heated at 65°C for 30 min for ciprofloxacin or 15 min for vincristine.

Each sample was passed down a Sephadex G-50 column (to remove external drug and ionophore) and diluted with 300 mM sucrose to allow for a drug dose in mice of 15 mg/kg for ciprofloxacin or 2 mg/kg for vincristine. Each mouse was injected via a lateral tail vein with 200 μl total volume. ICR mice were used for the ciprofloxacin studies and BDF-1 mice were used for the vincristine studies. At varying time points, mice were anesthetized (ketamine, xylazine) and blood was collected via cardiac puncture. Blood was immediately centrifuged at 500×g and

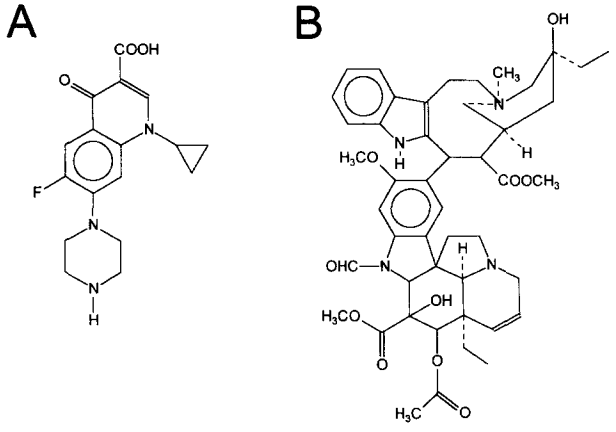

Fig. 1. Chemical structures of ciprofloxacin ($pK_1 = 6.0$, $pK_2 = 8.8$) (A) and vincristine ($pK_1 = 5.0$, $pK_2 = 7.4$) (B).

plasma was collected for lipid and drug determination by dual label scintillation counting.

3. Results

The chemical structures of ciprofloxacin and vincristine are shown in Fig. 1; their pK values are given in the figure legend. Ciprofloxacin is a quinolone antibiotic widely used in the treatment of respiratory and urinary tract infections. Recent work indicates that liposomal formulations are more effective in protecting mice from infection and provide greater efficacy in treating infected mice than the free drug [24]. Vincristine, a Vinca alkaloid derived from the

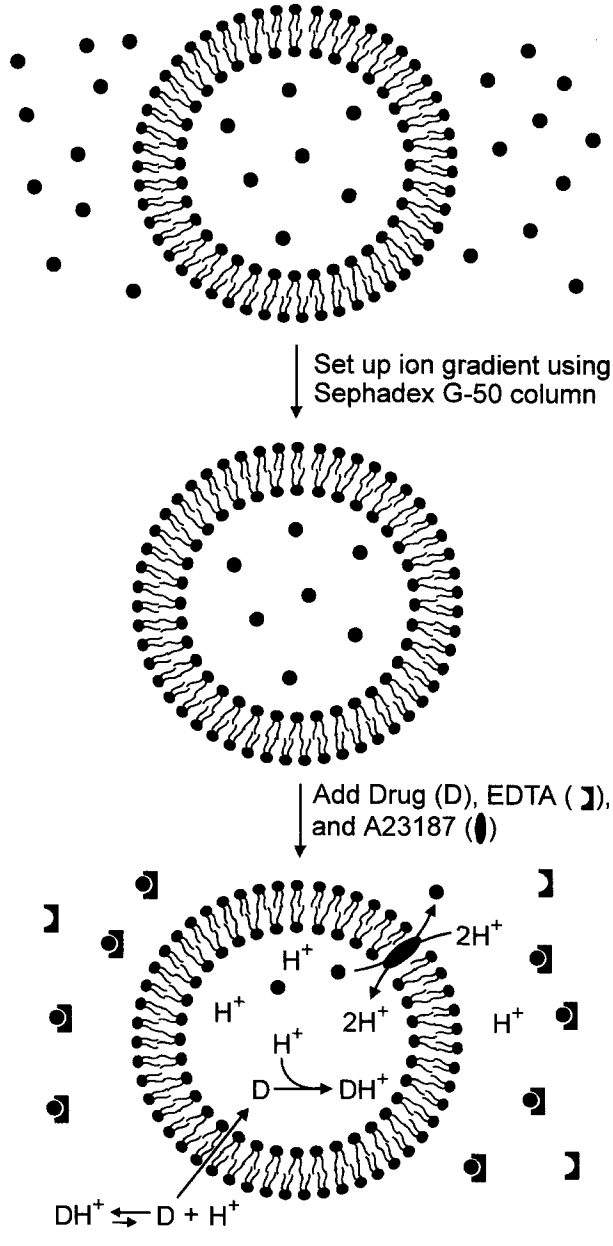

Fig. 2. Diagrammatic representation of ionophore-dependent drug loading, exemplified for the Mn^{2+}/A23187 system. LUVs are hydrated and subsequently extruded in 300 mM $MnSO_4$, following which Mn^{2+} ions (represented as ●) are present in roughly equal concentrations within the vesicles and in the external medium (top). A Mn^{2+} ion gradient is established by passage of the LUVs down a column of Sephadex G-50 hydrated in 300 mM sucrose (which may be buffered) (center). Gradient formation can also be accomplished using spin columns or by dialysis. The remote loading process involves addition of drug, EDTA, and A23187 to the LUVs exhibiting the ion gradient. The drug and EDTA can be added prior to the ionophore or they can be added simultaneously (see text for more details). A23187 is an electroneutral transporter, which couples the external transport of one Mn^{2+} ion (down its concentration gradient) to the internal transport of two protons. This process results in acidification of the vesicle interior, creating a ΔpH of 3 units. The external drug exists in neutral and protonated forms, and it is the former which can diffuse across the membrane (down its concentration gradient). Once inside the vesicle, they are protonated and trapped, resulting in an extremely low internal concentration of neutral drug, which therefore continues to drive uptake. In order for this process to occur, an external chelator such as EDTA is required to bind Mn^{2+} ions as they are transported out of the vesicle, thereby maintaining a sufficient gradient of the free ion. The process is similar for the ionophore nigericin, which catalyzes the electroneutral transport of one K^+ for one H^+, except that a K^+ salt is required, and an external chelator is not required to achieve good uptake of drug. For clarity, solutes such as sulfate anions, sucrose, and buffers are not shown. In addition, the A23187 transport mechanism is not shown in detail. For more details, see the text and [24].

periwinkle plant, is an important anticancer drug effective against a wide variety of neoplasms [25,26], which exhibits significantly enhanced efficacy in liposomal form [6–8,27]. Both drugs present challenges with respect to the development of liposomal formulations. Ciprofloxacin is a zwitterionic compound that is nearly insoluble in its net neutral or uncharged forms, which occur in the range near physiological pH (pH 6–8), and is poorly retained in vesicles [14]. Vincristine has two pK values, and exhibits poor retention within vesicles unless the internal pH is very low (pH 2) [6–8].

The general principles behind ionophore-dependent drug uptake are illustrated in Fig. 2. LUVs are formed which entrap a high concentration of a metal ion such as K^+ or Mn^{2+}, and an ion gradient is established by removal of the external metal ion (Fig. 2, top and center). Drug is added to the external medium, and uptake is initiated by addition of an ionophore which couples the outward transport of the metal ion to the inward movement of H^+. This creates a pH gradient (inside acidic) which results in uptake of compounds with weak base characteristics, such as the drugs ciprofloxacin and vincristine. The ionophore nigericin catalyzes a one-for-one exchange of K^+ for H^+, whereas A23187 transports $2H^+$ for every M^{2+} (where M = Ca, Mn, or Mg) [28–30]. Both processes are electroneutral [29,30]. As described below, both ionophores are capable of generating pH gradients of 2–3 units.

Although the general principle is straightforward, a number of parameters need to be varied in order to achieve optimal drug uptake. These include lipid composition, internal salt concentration, the ionophore to lipid ratio, and the pH of the internal and external solutions. In addition, systems containing divalent cations may require an external chelator to drive uptake to completion (Fig. 2, bottom). Examples involving the ionophores nigericin and A23187 are described below, with the optimized conditions for uptake summarized in Tables 1 and 2.

3.1. Nigericin-dependent uptake of ciprofloxacin

The uptake of ciprofloxacin in DSPC/Chol (55:45) LUVs containing 300 mM K_2SO_4 is illustrated in Fig. 3A. The external medium was 300 mM sucrose. Little or no drug uptake occurred in response to the

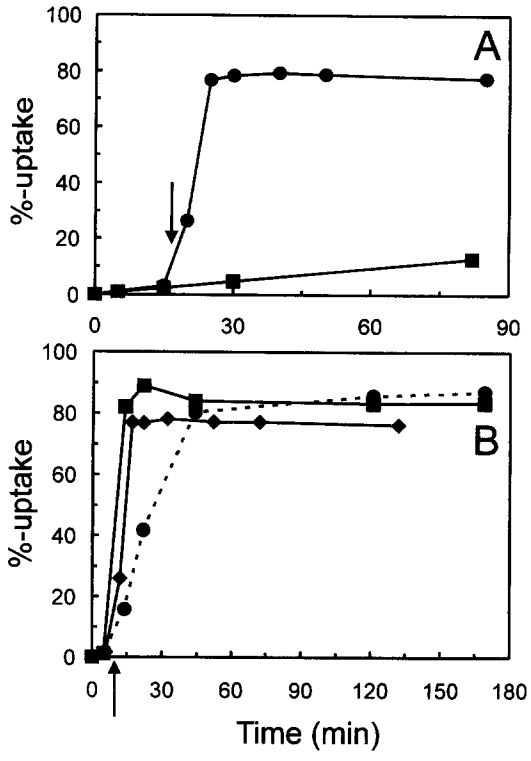

Fig. 3. (A) Effect of nigericin concentration on the uptake of ciprofloxacin in 100 nm DSPC/Chol LUVs containing 300 mM K_2SO_4. The nigericin was present at 1 μg/μmol lipid (●) or at 0.01 ng/μmol lipid (■). No uptake of drug occurred prior to the addition of nigericin, which is indicated by the arrow. The initial drug-to-lipid ratio was 0.3, and the uptake temperature was 60°C. (B) Effect of nigericin concentration on the uptake of ciprofloxacin in 100 nm DSPC/Chol LUVs containing 600 mM K_2SO_4. The nigericin was present at 0.1 μg/μmol lipid (♦), at 1 ng/μmol lipid (■), or at 0.1 ng/μmol lipid (●; dotted line). No uptake of drug occurred prior to the addition of nigericin, which is indicated by the arrow. The initial drug-to-lipid ratio was 0.3–0.37, and the uptake temperature was 60°C.

K^+ gradient alone, whereas within 5 min of addition of nigericin (indicated by the arrow) at a concentration of 1 μg/μmol lipid, 80% uptake was observed. The process was extremely rapid, giving high entrapment levels within minutes, with excellent retention observed over 3 h at 60°C. The observed uptake was not influenced by increasing the internal salt concentration to 600 mM (Fig. 3B).

The rapid uptake observed in Fig. 3A suggested that lower ionophore levels would be equally as effective. For LUVs containing 300 mM K_2SO_4, reducing the nigericin concentration by a factor of 10^5 (to 0.01 ng nigericin/μmol lipid) resulted in

Table 1
Optimized[a] conditions for drug loading experiments using nigericin/K^+ systems

Lipid composition	Internal salt[b]	D/L_i^c	I/L^d	% uptake	T (°C)	External solution: 300 mM sucrose+
(A) Ciprofloxacin						
DSPC/Chol	K_2SO_4	0.3	1000	80	60	
DSPC/Chol	600 mM K_2SO_4	0.3	100	80	60	
DSPC/Chol	600 mM K_2SO_4	0.3	1	80	60	
DSPC/Chol	600 mM K_2SO_4	0.37	0.1	85	60	
SPM/Chol	K_2SO_4 pH 7.4	0.2	1	80	60	20 mM HEPES pH 7.0
SPM/Chol	K_2SO_4 pH 6.1	0.2	1	90	60	20 mM HEPES pH 7.0
SPM/Chol	K_2SO_4 pH 6.1	0.2	1	100	60	20 mM HEPES pH 6.2
SPM/Chol	K_2SO_4 pH 6.1	0.2	0.5	100	70	20 mM HEPES pH 5.5
(B) Vincristine						
DSPC/Chol	K_2-tartrate pH 7.4	0.05	1	85–90	60	20 mM HEPES pH 5.3–6.3
SPM/Chol	K_2SO_4 pH 7.4	0.05	1	92	60	20 mM HEPES or MES pH 6.0
SPM/Chol	KH_2PO_4	0.05	1	80	60	

[a]Defined as ≥ 80% uptake, with leakage of < 5% over 2 h.
[b]300 mM unless otherwise indicated.
[c]Initial drug-to-lipid ratio, given in (mol/mol) for ciprofloxacin, and (w/w) for vincristine.
[d]Ionophore/lipid ratio (ng nigericin/μmol lipid).

only 12% uptake over 80 min (Fig. 3A). For LUVs containing 600 mM K_2SO_4, no change in uptake was observed following reduction of the ionophore concentration by a factor of 10^3 (to 1 ng nigericin/μmol lipid) (Fig. 3B). However, a 10^4-fold reduction resulted in a significant decrease in the rate of uptake of ciprofloxacin (Fig. 3B), with at least 45 min required to obtain uptake levels of 80% or greater. Thus a nigericin concentration of 1 ng/μmol lipid was chosen to obtain optimal loading levels within a reasonable time span. It will be seen later that under appropriate conditions this can be reduced to 0.5 ng nigericin/μmol lipid.

The initial experiments described above were performed using DSPC/Chol LUVs, a composition giving rise to stable, highly ordered (and therefore relatively impermeable) vesicles, which have been utilized in formulations of vincristine and doxorubicin [2,4,6,7]. Recently, a superior vincristine formulation was achieved using SPM/Chol (55:45) LUVs in conjunction with the standard pH gradient-loading technique [8,27]. In order to assess the use of

Table 2
Optimized[a] conditions for drug loading experiments using A23187 systems

Lipid composition	Internal salt[b]	D/L_i^c	I/L^d	% uptake	T (°C)	External solution: 300 mM sucrose+
(A) Ciprofloxacin						
DSPC/Chol	$MnSO_4$	0.27–0.35	0.1	95	60	3 mM EDTA
SPM/Chol	$MnSO_4$	0.2	0.1	70–80	60	15 mM EDTA pH 4.4
SPM/Chol	$MnSO_4$	0.2	0.1	98	60	15 mM EDTA pH 5.9
SPM/Chol	$MgSO_4$ pH 6.5	0.2	0.1	80	60	15 mM EDTA pH 7.0
SPM/Chol	$MgSO_4$ pH 6.5	0.2	0.1	80	60	20 mM HEPES 15 mM EDTA pH 7.0
SPM/Chol	$MgSO_4$ pH 6.5	0.2	0.1	86	60	20 mM HEPES 15 mM EDTA pH 6.0
SPM/Chol	$MgSO_4$ pH 6.5	0.2	0.5	97	60	20 mM HEPES 15 mM EDTA pH 6.0
(B) Vincristine						
SPM/Chol	$MnSO_4$	0.05	0.1	> 95	60	20 mM HEPES 3 mM EDTA pH 7.5

[a]Defined as ≥ 80% uptake, with leakage of < 5% over 2 h.
[b]300 mM unless otherwise indicated.
[c]Initial drug-to-lipid ratio.
[d]Ionophore/lipid ratio (μg A23187/μmol lipid).

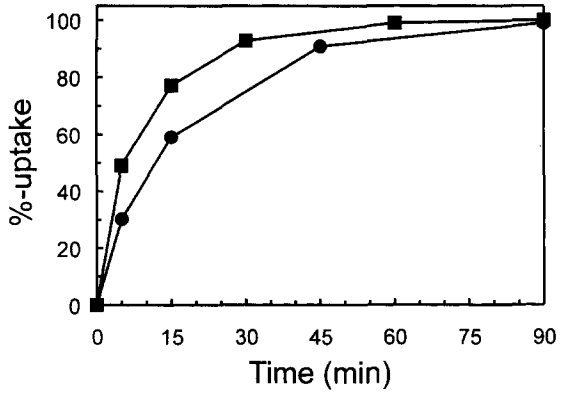

Fig. 4. Optimized conditions for the uptake of ciprofloxacin in 100 nm SPM/Chol LUVs containing 300 mM K_2SO_4 pH 6.1. For the uptake at 60°C, the nigericin was present at 1 ng/μmol lipid, and the external medium was 300 mM sucrose 15 mM EDTA pH 6.2 (■). For the uptake at 70°C, the nigericin was present at 0.5 ng/μmol lipid, and the external medium was 300 mM sucrose 15 mM EDTA pH 5.5 (●). The nigericin was added at time 0. The initial drug-to-lipid ratio was 0.2.

SPM/Chol LUVs in the present study, we have examined the uptake of ciprofloxacin (and vincristine) using both nigericin and A23187 (below). To standardize our experiments, we chose a basic system consisting of 100 nm SPM/Chol (55:45) LUVs containing 300 mM K_2SO_4, an initial drug-to-lipid ratio of 0.2, and an ionophore concentration of 1 ng nigericin/μmol lipid.

Optimum uptake was obtained for an (initial) internal pH of 6.1 and an external pH of 6.2 (HEPES-buffered sucrose), with 100% uptake achieved after 60 min at 60°C (Fig. 4). The same result was obtained at 70°C when the ionophore concentration was reduced to 0.5 ng nigericin/μmol lipid (for an external pH of 5.5), although 90 min were required to achieve 100% uptake (Fig. 4). Raising either the external or internal pH reduced the uptake (e.g., only 80% uptake for internal and external pH values near 7.0). Thus, the SPM-containing vesicles are equivalent to or better than the DSPC-containing vesicles in terms of uptake levels, but require longer loading times. Whereas the DSPC/Chol LUVs are fully loaded within 5 min, the SPM/Chol LUVs achieve maximum uptake only after 30–60 min.

3.2. *Nigericin-dependent uptake of vincristine*

Using the same basic system described above, the uptake of vincristine (drug-to-lipid ratio = 0.03 mol/mol) was determined as a function of internal and external pH. High uptake levels (93–95% uptake) were obtained for $pH_i = 7.4$ and $pH_o = 6$ (see Fig. 7B). For $pH_o = 7.5$, the uptake was low (only 60%), with poor retention over 75 min (not shown). Reasonable encapsulation of vincristine (80%) was also observed for LUVs containing 300 mM KH_2PO_4 (not shown).

Uptake of vincristine in response to transmembrane gradients of K_2-tartrate has also been investi-

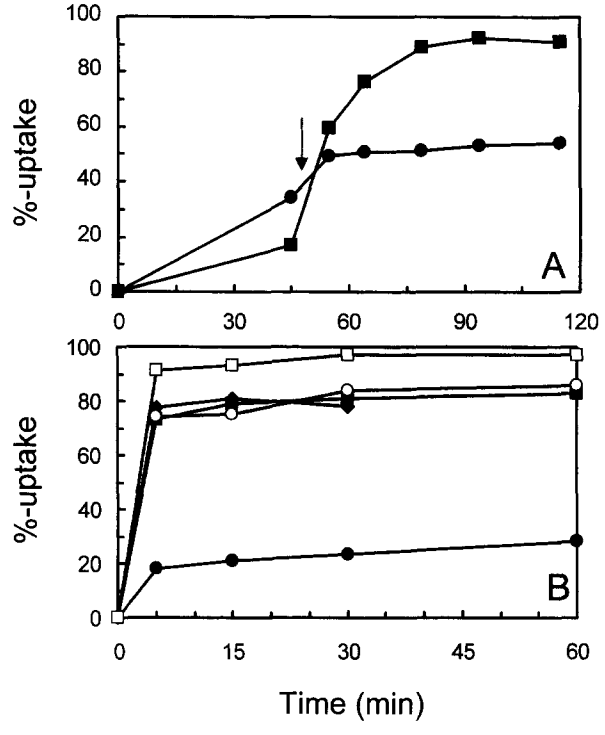

Fig. 5. (A) Effect of external EDTA on the uptake of ciprofloxacin in 100 nm DSPC/Chol LUVs containing 300 mM $MnSO_4$. The external medium was 300 mM sucrose 3 mM EDTA (■) or 300 mM sucrose (●). The addition of A23187 (0.1 μg/μmol lipid) is indicated by the arrow. The uptake temperature was 60°C, and the initial drug-to-lipid ratio was 0.35 (mol/mol). (B) Effect of external pH and ionophore concentration on the uptake of ciprofloxacin in 100 nm SPM/Chol LUVs containing 300 mM $MgSO_4$ pH 6.5. For 0.1 μg A23187/μmol lipid, the external medium was 20 mM HEPES 300 mM sucrose 15 mM EDTA pH 7.0 (■), 300 mM sucrose 15 mM EDTA pH 7.0 (♦), 300 mM sucrose 15 mM EDTA pH 4.4 (●), or 20 mM HEPES 300 mM sucrose 15 mM EDTA pH 6.0 (○). For 0.5 μg A23187/μmol lipid, the external medium was 20 mM HEPES 300 mM sucrose 15 mM EDTA pH 6.0 (□). The samples were placed in a water bath at 60°C at $t = -5$ min, and the A23187 was added at time 0. The initial drug-to-lipid ratio was 0.2 (mol/mol).

gated in DSPC/Chol LUVs (Table 1), with values of 85–90% obtained within 15 min at 60°C using 300 mM K_2-tartrate pH 7.4 as the internal salt, and HEPES-buffered sucrose pH 5.3–6.3 as the external medium. Under these conditions, a ΔpH of 2.3, measured using [^{14}C]methylamine [31], was present following drug uptake. This is in agreement with early data of Deamer and coworkers, who found that nigericin could induce a pH gradient of 2.2 units across sonicated vesicle membranes [19]. For purposes of comparison, vincristine uptake was also performed using LUVs containing 300 mM ammonium sulfate [16], with all other conditions the same (data not shown). The results were identical, indicating that the ionophore and ammonium sulfate methods give equivalent results as far as drug uptake is concerned.

Vincristine uptake in response to K_2-tartrate was also examined in SPM/Chol LUVs, but only 70% uptake was achieved (data not shown).

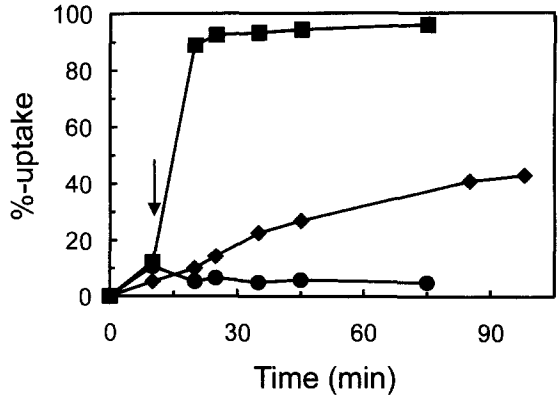

Fig. 6. Effect of external pH and EDTA on the uptake of vincristine in 100 nm SPM/Chol LUVs containing 300 mM $MnSO_4$. The external medium was 20 mM HEPES 300 mM sucrose 3 mM EDTA pH 7.5 (■), 300 mM sucrose 3 mM EDTA pH 6 (◆), or 20 mM HEPES 300 mM sucrose pH 7.5 (●). The addition of A23187 (0.1 μg/μmol lipid) is indicated by the arrow. The uptake temperature was 60°C, and the initial drug-to-lipid ratio was 0.03 (mol/mol).

3.3. *A23187-dependent uptake of ciprofloxacin*

The carboxylic ionophore A23187 transports divalent cations across membranes with specificity $Mn^{2+} > Ca^{2+} > Mg^{2+}$ and relative binding affinities of 210:2.6:1, respectively [28]. Calcium salts tend to be relatively insoluble, and the major exception ($CaCl_2$) suffers from the high membrane solubility of neutral HCl, which contributes to the loss of any induced pH gradient. Preliminary ciprofloxacin uptake experiments using DSPC/Chol LUVs containing 300 mM $CaCl_2$ gave low encapsulation levels (40%) and poor retention (not shown). Therefore we turned our attention to salts of Mn^{2+} and Mg^{2+}, with particular attention to the former in light of its significantly higher relative binding affinity for A23187.

Fig. 5A details the uptake of ciprofloxacin in response to gradients of 300 mM $MnSO_4$. The effect of both ionophore and external EDTA are shown for uptake of ciprofloxacin (initial drug-to-lipid ratio = 0.35) into DSPC/Chol (55:45) LUVs at 60°C. At time $t = 0$ min, the LUVs and ciprofloxacin were combined and placed in a water bath at 60°C. As $MnSO_4$ solutions are acidic (pH 3–4), it was expected that some uptake would be observed prior to the addition of the A23187, and this was in fact observed. At 45 min, about 35% uptake was observed in the sample without EDTA, and about 18% in the EDTA-containing sample. In the latter case, the EDTA lowers the external pH and therefore ΔpH, which accounts for the lower uptake (the uptake is also pH dependent, as discussed below). These values are close to the maximum uptake observed without ionophore. At $t = 47$ min, A23187 was added to give a final concentration of 0.1 μg/μmol lipid (arrow). In the absence of external EDTA, a further small uptake to about 55% occurred, which was stable over a period of 3 h. In the presence of external EDTA, further uptake of ciprofloxacin, to a final level > 90%, occurred over a period of 45 min. This was increased to 95% by reducing the initial drug-to-lipid ratio to 0.27 (Table 2). Thus, even with an acidic internal salt solution, the ionophore is necessary to obtain high uptake values. Furthermore, the presence of an external chelator such as EDTA is essential to drive this process. This requirement for EDTA can be understood from a simple equilibrium model for drug uptake (see below). Under the conditions described here, the ionophore A23187 in the presence of external EDTA will generate a ΔpH of 3 units (B. Cheung, unpublished results).

The uptake of ciprofloxacin into SPM/Chol (55:45) LUVs was also investigated (data not

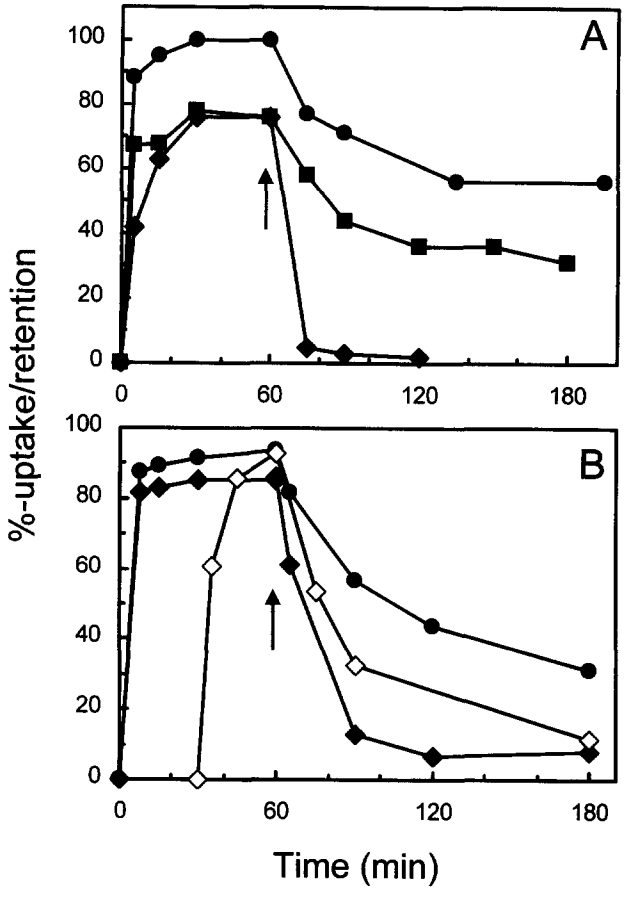

Fig. 7. (A) Uptake of ciprofloxacin in 100 nm SPM/Chol LUVs at 60°C and its retention in the presence of 50% mouse serum at 37°C. Uptake experiments were performed for the following combinations of ionophore/internal salt: A23187/$MnSO_4$ (●), A23187/$MgSO_4$ (■), nigericin/K_2SO_4 (♦). The arrow indicates the addition of mouse serum (to a final concentration of 50%) and transfer of the sample to 37°C. (B) Uptake of vincristine in 100 nm SPM/Chol LUVs at 60°C and its retention in the presence of 50% mouse serum at 37°C. Uptake experiments were performed for the following combinations of ionophore/internal salt: A23187/$MnSO_4$ (●), nigericin/K_2SO_4 (♦), nigericin/K_2SO_4 ($pH_i = 7.4$, $pH_o = 6.0$) (◇). For the latter two preparations, both the internal and external pH values (pH_i and pH_o, respectively) were adjusted prior to uptake, and the external solution was 20 mM MES 300 mM sucrose pH 6.0. All internal salts were present at 300 mM. The arrow indicates the addition of mouse serum (to a final concentration of 50%) and transfer of the sample to 37°C.

shown), and as above, low uptake (30%) was observed in the absence of EDTA. In the presence of EDTA, the final entrapment levels were dependent on the external pH. Only 80% uptake was observed at $pH_o = 4.4$, but this was increased to 98–100% at $pH_o = 5.9$. The pH optimum for the uptake of ciprofloxacin, via nigericin and A23187, appears to be around 6.

Excellent encapsulation of ciprofloxacin can also be achieved using gradients of $MgSO_4$ (internal salt = 300 mM $MgSO_4$ pH 6.5, external medium = 300 mM sucrose 15 mM EDTA), as shown

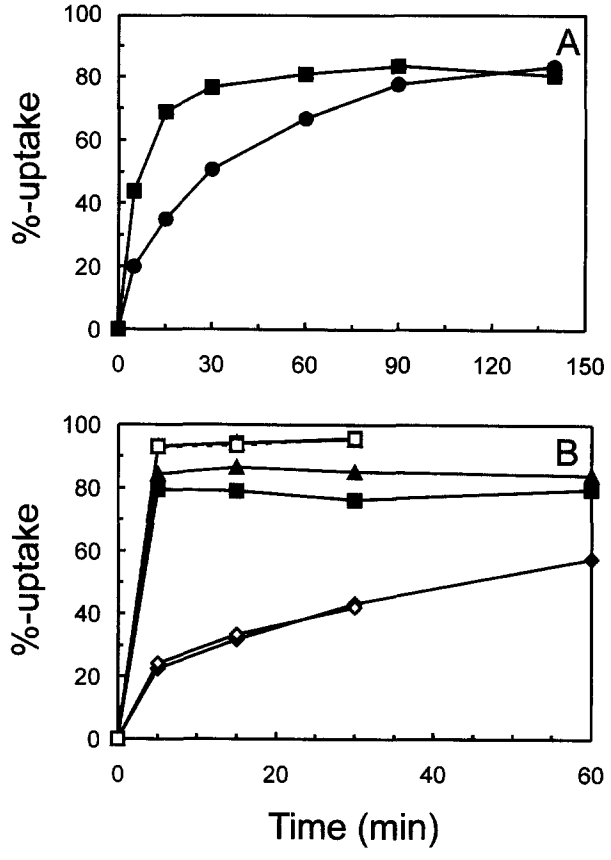

Fig. 8. Removal of nigericin from SPM/Chol LUVs as assayed by drug uptake. (A) Effect of spin columns on uptake of ciprofloxacin at 60°C. Uptake was monitored for LUVs containing 300 mM K_2SO_4 at a nigericin concentration of 1 ng/μmol lipid (■), and for LUVs which were preincubated with nigericin for 5 min at 60°C, and then passed down a spin column prior to addition of the drug (●). (B) Effect of dialysis on uptake of vincristine by ionophore and amine methods at 60 and 65°C. LUVs (1–2 ml) containing 300 mM KH_2PO_4 (♦,◇), 300 mM ethylammonium sulfate (■,□), or 300 mM amylammonium sulfate (▲,△) were dialyzed exhaustively (3×2 l) overnight at room temperature against 300 mM sucrose. Vincristine was then added and uptake assessed. The closed symbols refer to uptake at 60°C and the open symbols to uptake at 65°C. For a comparison, the ion gradient of one LUV sample containing EAS was formed using a Sephadex G-50 column (□ and dotted line). The experiments with EAS and AAS were performed to ensure that there was no loss of vesicle contents during the dialysis period.

in Fig. 5B. The lipid composition and uptake temperature are given in the figure legend. For A23187 concentrations of 0.1 μg/μmol lipid, maximal uptake values (86%) were observed at $pH_o = 6.0$ (20 mM HEPES+sucrose-EDTA). For these conditions, increasing the A23187 concentration 5-fold (to 0.5 μg/μmol lipid) resulted in 97% entrapment. This is equivalent to the $MnSO_4$ results, aside from the necessity of using higher ionophore concentrations. A summary of these results is given in Table 2.

3.4. A23187-dependent uptake of vincristine

Excellent uptake and retention of vincristine can also be obtained using Mn^{2+}/A23187 with SPM/Chol LUVs (Fig. 6). Less than 5% uptake was observed when EDTA was lacking in the external medium of HEPES-buffered sucrose pH 7.5. In the presence of 3 mM EDTA (pH_o=7.5), over 95% uptake was observed within an hour. A summary of these results is given in Table 2.

3.5. In vitro leakage assays: release of ciprofloxacin and vincristine from LUVs in response to mouse serum

A simple in vitro leakage assay employing mouse serum was developed to assess the relative retentive properties of the various liposomal systems (see Section 2). The release of ciprofloxacin from SPM/Chol LUVs resulting from incubation in 50% mouse serum at 37°C is shown in Fig. 7A. The best retention of drug was observed for the Mn^{2+}/A23187 system (60% remaining after 2 h), followed by the Mg^{2+}/A23187 system (35%) and finally by the K^+/nigericin system. The much more rapid loss of material from LUVs containing nigericin may result from the presence of K^+ in serum, which would cause reverse transport and reduce or collapse the pH gradient.

The release of vincristine from SPM/Chol LUVs resulting from incubation in 50% mouse serum at 37°C is shown in Fig. 7B. As with ciprofloxacin, better retention of drug was observed for the Mn^{2+}/A23187 system than for the K^+/nigericin system. In the latter case, better retention was observed using phosphate or tartrate salts (not shown), or using sulfate salts with an external pH of 6. The retention observed with the Mn^{2+}/A23187 system was similar to that observed for LUVs loaded using methylammonium sulfate (not shown).

3.6. Removal of ionophores from LUVs

Following drug uptake and in vivo administration, the presence of ionophores in LUVs is disadvantageous for two reasons. First, high serum concentrations of ions such as Na^+ and K^+ may cause reverse transport with loss of the induced pH gradient. Second, ionophores are potentially toxic compounds, and therefore methods for reducing their concentration are important. As ionophores will exchange between vesicles [29], their removal should be relatively straightforward. We have developed a fluorimetric assay for determination of A23187 concentrations in formulations of SPM/Chol, which has allowed us to quantify the amount of ionophore before and after various treatments (see below). The potential for reducing nigericin concentrations has been assessed by examining the uptake of ciprofloxacin following various treatments of an LUV sample.

If a vesicle sample containing nigericin was passed down a spin column prior to loading with ciprofloxacin, the rate of uptake was significantly reduced, and the time to achieve similar uptake levels was

Table 3
Fluorimetric determination of A23187 in a liposomal formulation

Sample No.	A23187 removal method	Initial amount of A23187 (ng)	Amount of A23187 measured (ng)
S1	None	146	123
S2	Sephadex G-50 column	109	n.d.[a]
S3	Dialysis (1 h)	500	n.d.[a]
S4	SM-2 biobeads	500	n.d.[a]
S4+45 ng A23187	None	45	41
S4+90 ng A23187	None	90	94

[a]Not determined (number is at background levels (≤ 15 ng)).

increased by a factor of 4 (Fig. 8A). This strongly suggests the removal of a significant proportion of the nigericin. If the LUVs containing nigericin were first subjected to dialysis (3 h), or if they were passed down a second spin column, similar uptake rates were observed, suggesting little or no further removal of nigericin. Interestingly, the in vitro leakage rates of ciprofloxacin from normal and nigericin-reduced LUVs were found to be identical (data not shown). When LUVs containing nigericin were loaded with vincristine following exhaustive dialysis (room temperature for 20 h) and spin column treatment, the uptake levels at 1 h (60%) (Fig. 8B) were comparable to that obtained for ciprofloxacin using a single spin column (Fig. 8A). Taken together, these results suggest that nigericin can be partially removed from LUVs, but complete removal may not be feasible.

The concentration of A23187 in LUVs can be quantitated using a fluorimetric assay (see Section 2), that allows determination of the amount remaining following various purification procedures. The results are given in Table 3 for SPM/Chol LUVs initially containing A23187 at a concentration of 0.1 μg/μmol lipid. For sample 1 (S1), 146 ng of A23187 was added to the LUVs, and 123 ng was measured by the assay. Following passage of the LUVs down a Sephadex G-50 column eluted with 300 mM sucrose (S2), the remaining levels were below background. Likewise, dialysis against 300 mM sucrose (S3), or treatment with SM-2 biobeads (S4) reduced the A23187 concentration below background levels. The addition of known aliquots of ionophore to S4 resulted in expected concentrations in the LUVs. The results demonstrate the surprising result that A23187 can be effectively removed from LUVs, in contrast to the incomplete removal observed with nigericin.

3.7. *In vivo pharmacokinetics of liposomal ciprofloxacin and vincristine*

The pharmacokinetics of ciprofloxacin and vincristine loaded into SPM/Chol LUVs following injection into mice are shown in Fig. 9. Ciprofloxacin was loaded by the ionophore and methylammonium sulfate methods (Fig. 9A), while vincristine was loaded using the standard pH gradient and ionophore methods (Fig. 9B). Following injection into mice, samples were withdrawn at the indicated times, and the drug-to-lipid ratio of the recovered LUVs was determined. For ciprofloxacin (Fig. 9A), the A23187 system exhibited much superior retention than the K_2SO_4/nigericin system, and was similar to LUVs containing methylammonium sulfate. For vincristine (Fig. 9B), the retention observed for LUVs loaded using A23187 was comparable to LUVs loaded using 300 mM citrate pH 4.0 [6–8]. This suggests that the efficacy achievable with LUVs loaded with drugs employing the ionophore system should be comparable to either of the existing methods.

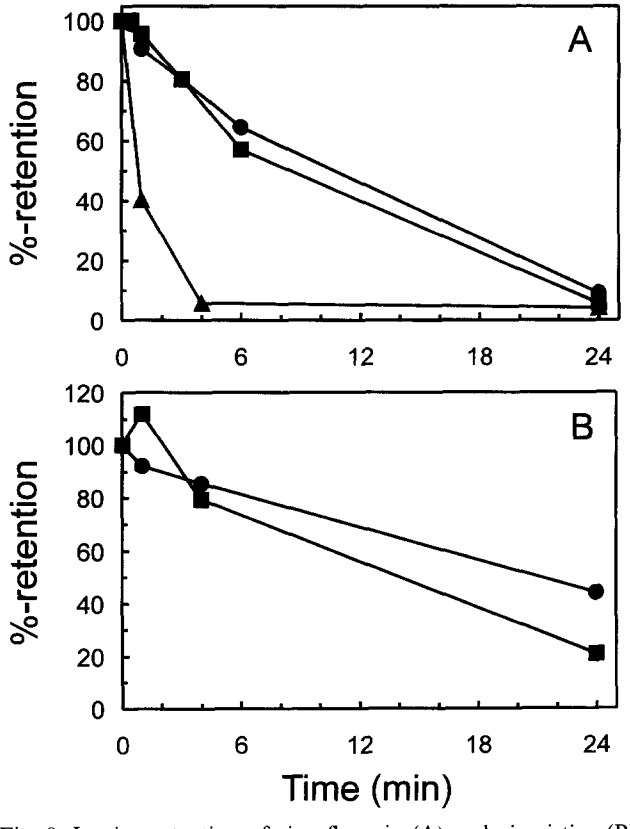

Fig. 9. In vivo retention of ciprofloxacin (A) and vincristine (B) in 100 nm SPM/Chol (55:45) LUVs. Drug-to-lipid ratios were determined following i.v. administration in mice of liposomal ciprofloxacin (A) containing 300 mM methylammonium sulfate (●), 300 mM $MnSO_4$/A23187 (■), or 300 mM K_2SO_4/nigericin (▲), or of liposomal vincristine (B) containing 300 mM citrate pH 4.0 (●) or 300 mM $MnSO_4$/A23187 (■). Vincristine was encapsulated at a drug-to-lipid ratio of 0.05, and measured using [^{3}H]vincristine as a tracer. Lipid was measured using [^{14}C]cholesteryl hexadecyl ether as a tracer. The % retention is defined as the drug-to-lipid ratio at time t divided by the initial measured drug-to-lipid ratio.

4. Discussion

This paper describes a new method for loading weakly basic amines (a category which includes many pharmaceutically important drugs) into LUVs via transmembrane ion gradients. The method relies on the ionophore-mediated generation of a transmembrane pH gradient of 2–3 units (inside acidic) in response to a transmembrane ion gradient, and is based on the inward movement of protons which are coupled to the outward movement of metal ions such as K^+ or Mn^{2+}.

The original ΔpH method, developed for loading doxorubicin, involves formation of the LUVs by extrusion in a citrate buffer (pH 4.0), and establishment of a pH gradient by passage down a column of Sephadex G-50 equilibrated in HEPES-buffered saline (pH 7.5) [5]. The pH gradient (interior acidic) is formed prior to addition of the drug, and requires well-buffered internal and external solutions in order to achieve significant encapsulation and retention. One of the limitations of this method is revealed for drugs such as ciprofloxacin, which has very low solubility near neutral pH (<0.4 mM at room temperature) [14].

An alternative method for ΔpH-dependent drug loading involves drug uptake in response to a secondary pH gradient established by a primary transmembrane gradient of ammonium sulfate [15–17] or other amines [18]. This method has an advantage over the conventional method described above. As solutions are generally unbuffered, slightly acidic drugs such as ciprofloxacin can reduce the external pH to a value near or below 6, where solubility is higher. This method results in excellent uptake of ciprofloxacin and other drugs such as doxorubicin [15–17].

In the present paper, we have examined drug uptake using two well-characterized ionophores, nigericin and A23187. The method allows for rapid (5–15 min) uptake of drug and excellent in vitro retention. Essentially complete uptake of ciprofloxacin and vincristine was achieved for both ionophores. One difference between the two ionophores is the optimal external pH range for maximum uptake. For nigericin, the highest uptake levels were achieved for $pH_o = 5.5$–6.0, whereas for A23187, the optimal range was $pH_o = 6$–7.5. Interestingly, the optimal pH for A23187-mediated transport of Ca^{2+} and Mn^{2+} is in the range of 6.7–7 [32,33], essentially identical to the pH maxima obtained for the transport of K^+ by nigericin [34].

A second difference between nigericin and A23187 is the necessity for an external chelator such as EDTA to achieve maximum uptake using A23187. Whereas uptake levels in the range of 80–100% are obtained with nigericin, only 20–50% uptake is observed for A23187 in the absence of EDTA compared with 95–100% in its presence. Insight into the differences between the two ionophores, specifically the requirement for a chelator in the case of A23187, can be gained using an elementary equilibrium analysis of the transmembrane distributions of ionophore and ions. This analysis requires knowledge of the stoichiometry of the ionophore:divalent cation complex. While some studies support an A23187:Ca^{2+} stoichiometry of 2:1 [29,32], others support a 1:1 complex [35–40]. Furthermore, A23187:Mn^{2+} complexes appear to exist as mixtures of 2:1 and 1:1 [32]. Both the 2:1 and 1:1 complexes have been observed in solution for several divalent cations [41]. In Appendix A, we treat both the 2:1 and 1:1 cases, and observe that the result is independent of the assumed complex stoichiometry, depending instead on the stoichiometry of Mn^{2+}/H^+ transported. The key result, given in Eqs. A4 and A7 of Appendix A, details the relationship between the equilibrium pH and ion gradients. Thus, for nigericin (Eq. A4):

$$[H^+]_i/[H^+]_o = [K^+]_i/[K^+]_o$$

and for A23187 (Eq. A7):

$$[H^+]_i/[H^+]_o = ([Mn^{2+}]_i/[Mn^{2+}]_o)^{1/2}$$

This analysis reveals a direct relationship between the equilibrium pH gradient and equilibrium K^+ gradient generated by nigericin, i.e., in order to produce a ΔpH of 3 units (inside acidic), a K^+ gradient of 1000:1 (inside:outside) must be generated. In contrast, a square-root relationship exists between the equilibrium pH gradient and equilibrium Mn^{2+} gradient generated by A23187, i.e., to achieve $\Delta pH = 3$ (inside acidic), a Mn^{2+} gradient of 10^6:1 (inside:outside) must be generated. In other words, a much higher inside-outside concentration gradient of Mn^{2+} must be maintained to maintain the same ΔpH. As a result, as Mn^{2+} is transported out during

the drug loading process, the equilibrium ΔpH is rapidly reduced as the external Mn^{2+} concentration rises. This compromises equilibrium drug loading levels, which for a drug containing a single amino function obeys the relation $[Drug]_i/[Drug]_o = [H^+]_i/[H^+]_o$ [1]. The presence of external EDTA alleviates this problem by binding external Mn^{2+}, thus maintaining extremely high Mn^{2+} transmembrane concentration gradients.

The requirement for EDTA may be further accentuated by retention of externally transported divalent cations in the vicinity of the membrane surface, due to the high affinities of divalent cations for membranes. For PC membranes, the affinity of cations follows the sequence $M^{3+} > M^{2+} > M^+$, and whereas sizeable binding constants have been measured for Ca^{2+}, Mg^{2+} and some lanthanides, the alkaline monovalent cations bind very weakly if at all [42,43]. A higher 'effective' ion concentration at the vesicle surface would further reduce the inside:outside Mn^{2+} gradient. Thus, EDTA is required to remove Mn^{2+} from the vesicle surface so as to maintain an ion gradient sufficiently large to generate the requisite pH gradient.

While both ionophores are capable of facilitating essentially complete uptake of ciprofloxacin and vincristine, and the loaded LUVs exhibit excellent retention characteristics in buffer, the superiority of A23187-dependent loading is revealed by in vitro leakage assays (Fig. 7), and from the in vivo pharmacokinetics (Fig. 9). With respect to the in vitro studies, the Mn^{2+}/A23187 system exhibits superior retention of ciprofloxacin (Fig. 7A) and vincristine (Fig. 7B).

The in vivo pharmacokinetics of ciprofloxacin (Fig. 9A) reveal similar behavior as observed with the in vitro assay. The retention is dramatically better for the Mn^{2+}/A23187 system than for K^+/nigericin, and is seen to be similar to LUVs loaded using methylammonium sulfate. For vincristine (Fig. 9B), the Mn^{2+}/A23187 and standard ΔpH (internal citrate) [7,27] methods displayed comparable retention.

As ionophores are potentially toxic compounds, we have examined means of removing them from vesicles. Using a fluorimetric assay, we have been able to show that essentially all of the A23187 can be removed from SPM/Chol LUVs by several methods (Table 3), including gel exclusion chromatography, dialysis, and treatment with biobeads. Further, the results of uptake experiments strongly suggest that a portion of nigericin can also be removed. However, even if *none* of the A23187 was removed from the LUVs, the amounts used (approx. 20 μg/kg) would correspond to doses approx. 2 orders of magnitude below established toxic levels (LD_{50} values range from 4500 μg/kg i.v. [44] to 9200 μg/kg i.p. [45]). Furthermore, the actual residual levels of ionophore (following removal) are more than 3 orders of magnitude below the lowest-observed-effect level of 11–15 μg/kg [45,46], where observed effects are mild.

While toxicity concerns could also be raised for Mn^{2+}, they appear to be unwarranted at present. Most deleterious effects involve long term exposures [47–49]. Furthermore, in anticancer applications, the toxicity of Mn^{2+} will be insignificant compared to that of the drug. Finally, several Mn-containing MR contrast agents are under development and appear to pose no safety risk at doses up to 40 μmol/kg [50–53], which is in the same range as the ionophore system. If it becomes clear that Mn^{2+} toxicity *is* a problem in some situations, the ion could be changed to Mg^{2+} with no decrease in drug loading efficiency. Other ions, such as Fe^{2+} or Ba^{2+}, may also be possible candidates for drug loading.

Potential benefits of the ionophore loading method include, first, superior uptake and retention over the standard pH gradient technique (especially for drugs such as ciprofloxacin, which has low solubility near neutral pH). Second, the ionophore method may allow higher absolute amounts of drug to be accumulated per mole of lipid. This is important, as it has been shown for drugs such as doxorubicin that the toxicity decreases at higher drug-to-lipid ratios [54]. Third, drug uptake, retention and efficacy may be improved by exploiting the tendency of many drugs to form complexes with divalent or trivalent cations. Drugs in this category include doxorubicin [55–59], mitoxantrone [60], norfloxacin [61], ciprofloxacin [62], and daunorubicin [63]. The formation of drug-metal complexes can lead to alterations in the biological activity of the drug, which may increase cytotoxicity. This has been demonstrated for complexes of doxorubicin with Fe^{3+} and Cu^{2+} [55–59,64–66]. The ionophore method can, therefore, potentially allow selection of divalent cations that

could function both in drug uptake and biological activity.

In summary, we have described a new method for loading of LUVs via pH gradients induced by ionophore-dependent cation transport. For both vincristine and ciprofloxacin, trapping efficiencies approaching 100% are obtained, and the in vitro and in vivo retention is comparable to that observed using other loading methods. Further work is required to determine whether higher drug-to-lipid ratios and improved retention can be achieved, and to determine how the in vivo efficacy compares with LUVs loaded using the standard ΔpH and amine methods.

Acknowledgements

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Appendix

The relationship between equilibrium metal ion and pH gradients generated by ionophores: an elementary equilibrium analysis

For nigericin-mediated K^+ transport, our initial conditions consist of an LUV containing K_2SO_4. A nigericin molecule (N^-) present on the inner monolayer can bind a single K^+ ion, forming the membrane permeable neutral form NK, diffuse to the opposite side, and release the ion. The dissociation constant of the ionophore-metal complex, K_m, is given by:

$$K_m = [N^-][K^+]/[NK] \quad \text{(A1)}$$

Potassium transport will continue until the concentrations of the ionophore-K^+ complex on the internal and external membrane surface are equal, i.e., until $[NK]_i = [NK]_o$. Assuming that $K_{m,i} = K_{m,o}$ it follows that

$$[N^-]_o/[N^-]_i = [K^+]_i/[K^+]_o \quad \text{(A2)}$$

An analogous treatment for the transport of H^+ ions gives

$$[N^-]_o/[N^-]_i = [H^+]_i/[H^+]_o \quad \text{(A3)}$$

Therefore

$$[H^+]_i/[H^+]_o = [K^+]_i/[K^+]_o \quad \text{(A4)}$$

For A23187-mediated Mn^{2+} transport, our initial conditions consist of an LUV containing $MnSO_4$. For a 2:1 complex stoichiometry, two A23187 molecules (A^-) present on the inner monolayer can bind a single Mn^{2+} ion, forming the membrane permeable neutral form MnA_2, diffuse to the opposite side, and release the ion. The dissociation constant of the ionophore-metal complex, K_m, is thus given by:

$$K_m = [Mn^{2+}][A^-]^2/[MnA_2] \quad \text{(A5)}$$

Manganese transport will continue until the concentrations of the ionophore-Mn^{2+} complex on the internal and external membrane surface are equal, i.e., until $[MnA_2]_i = [MnA_2]_o$. Assuming that $K_{m,i} = K_{m,o}$ it follows that

$$[A^-]_o/[A^-]_i = ([Mn^{2+}]_i/[Mn^{2+}]_o)^{1/2} \quad \text{(A6)}$$

As the equilibrium H^+ distributions are given by Eq. A3, it follows that

$$[H^+]_i/[H^+]_o = ([Mn^{2+}]_i/[Mn^{2+}]_o)^{1/2} \quad \text{(A7)}$$

For the case of 1:1 complex stoichiometry, it is thought that the 2:1 complex forms initially and dissociates to the 1:1 complex with the addition of further ion [41]. As the resulting MnA^+ species carries a +1 charge, it must co-transport an anion to give a neutral membrane-permeable species [32,41]. A likely candidate in the present case is OH^-, as complexes of the form MA·OH have been observed in solution [41]. In this scenario, an A23187 molecule (A^-) present on the inner monolayer can bind a single Mn^{2+} ion, forming MnA^+, which then interacts with a hydroxyl group to give the neutral complex MnA·OH, which diffuses to the opposite side, releasing the ion. The ionophore then binds a single proton and returns to the vesicle interior. The external movement of the hydroxyl group is equivalent to the internal movement of a proton, and thus two H^+ are transported per Ca^{2+}, thereby preserving the observed electroneutrality [29]. The equilibrium can be written as:

$$A^- + Mn^{2+} + H_2O \leftrightarrows MnA{\cdot}OH + H^+ \quad \text{(A8)}$$

The dissociation constant of the ionophore-metal

complex, K_m, is thus given by:

$$K_m = [Mn^{2+}][A^-]/[MnA{\cdot}OH][H^+] \quad (A9)$$

Manganese transport will continue until the concentrations of the ionophore-Mn^{2+} complex on the internal and external membrane surface are equal, i.e., until $[MnA{\cdot}OH]_i = [MnA{\cdot}OH]_o$. Assuming that $K_{m,i}=K_{m,o}$ it follows that

$$[A^-]_o/[A^-]_i = [Mn^{2+}]_i[H^+]_o/[Mn^{2+}]_o[H^+]_i \quad (A10)$$

It is straightforward to show that combining Eqs. A3 and A10 yields Eq. A7. Thus the relationship between the equilibrium transmembrane pH and ion gradients is the same regardless of the stoichiometry of the A23187-Mn^{2+} complex.

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