Boronic Acids Facilitate the Transport of Ribonucleosides through Lipid Bilayers†

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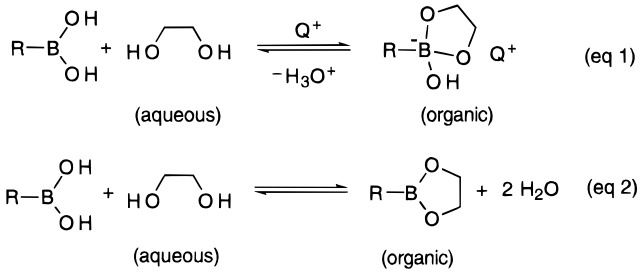
Abstract □ Boronic acids were found to facilitate the transport of various ribonucleosides in and out of liposomes (artificial cells). Most experiments were conducted with large unilamellar vesicles composed of dipalmitoylphosphatidyl choline, cholesterol, and phosphatidic acid in the ratio 20:15:2. Rates of nucleoside efflux were determined by a UV assay, whereas the influx experiments used a radiotracer method. Five boronic acids were studied: 3,5-bis(trifluoromethyl)phenylboronic acid (**1**), 3,5-dichlorophenylboronic acid (**2**), 4-*tert*-butylphenylboronic acid (**3**), 1-butylboronic acid (**4**), and 4-carboxyphenylboronic acid (**5**). The order of carrier effectiveness was found to be **1** > **3** > **2** >> **4** >> **5**. Efflux and influx experiments with mixtures of nucleosides and sucrose showed virtually complete transport selectivity in favor of the nucleosides. Mechanistic studies indicate that the nucleoside transport mechanism involves transient formation of a lipophilic, trigonal boronate ester.

Introduction

The ability of nucleoside drugs to traverse cell membranes is an important factor affecting their biological activity. Except for very lipophilic derivatives, such as 3′-azido-3′-deoxythymidine (AZT), passive membrane diffusion is a minor transport pathway.[1] Usually, nucleoside drugs enter cells via membrane-bound nucleoside transporters.[2] Subsequent intracellular phosphorylation leads to biologically active nucleotides. It has been shown that inhibition of nucleoside membrane transport can protect cultured cells against nucleoside drug action.[3] In addition, mutant murine lymphoma cells lacking a functional nucleoside transporter were found to be nucleoside drug resistant.[4] It is possible that, under certain circumstances, increasing nucleoside membrane transport via prodrug strategies,[5] or using artificial carriers,[6] may improve drug efficacy. This report concerns facilitated nucleoside transport through a lipid bilayer using an artificial carrier.

Boronic acids have been shown to improve the transport of reducing monosaccharides and aryl glycopyranosides through bulk, liquid organic membranes.[7] The hydrophilic diol-containing compounds combine reversibly with the boronic acid (association constants usually range from 10 to 10^4 M^{-1}) to form lipophilic covalent complexes which are extracted into the liquid organic membrane.[7] Depending on the experimental conditions, transport can be achieved via eq 1 or 2, or a mixture of both. In the case of eq 1, a phase-transfer cation, Q^+, needs to be present. The potential of this transport system was recently extended with the discovery that boronic acids can transport glucose through lipid bilayer membranes.[8]

Boronic acids are also able to transport ribonucleosides through liquid organic membranes; however, transport only occurs via eq 1, *i.e., simple boronic acids alone do not induce liquid membrane transport*.[9] The aim of this study was to determine if simple boronic acids can transport nucleosides



across lipid bilayer membranes. Although the eventual goal of this work is to determine if boronic acids can carry nucleoside drugs into cells, the study described here has focused on the experimentally easier problem of determining if boronic acids can facilitate nucleoside transport in and out of liposomes (artificial cells). Since liposomes themselves are being considered as drug delivery vehicles,[10] the development of methods to trigger liposome leakage is an active research area.[11] From this perspective, the use of boronic acids as "chemical triggers" to release encapsulated nucleoside drugs represents a novel approach to improving liposomal drug delivery.

Experimental Section

Reagents were obtained from the following sources: all lipids (Avanti Polar Lipids) except phosphatidic acid, Sigma; all enzymes, Sigma; 1,2-di[1-^{14}C]palmitoylphosphatidylcholine ([^{14}C]DPPC), Amersham; sucrose, ([1-^{3}H(N)]fructose, and [5-^{3}H]uridine, Sigma; phenylboronic acid and Triton X-100, Aldrich; 3,5-dichlorophenylboronic acid and 3,5-bis(trifluoromethyl)phenylboronic acid, Lancaster; Sephadex G-50, Pharmacia; dialysis tubing, Spectrum; and Fluorodyne scintillation fluid, Life Sciences. 4-*tert*-Butylphenylboronic acid was synthesized from 4-bromo-*tert*-butylbenzene as described previously.[8] UV spectra were obtained on a Perkin Elmer Lambda 2 instrument.

Liposome Preparation[12]—A chloroform solution of DPPC (2 μmol), cholesterol (C, 1.5 μmol), and egg phosphatidic acid (PA, 0.2 μmol) was evaporated using a rotary evaporator (<30 °C) and the lipid film dried under vacuum for 1 h. The liposomes were dispersed in 200 μL of marker solution (also containing 35 mM sodium phosphate, 75 mM NaCl, pH 7.4) with the aid of a Vortex mixer. Pyrex beads were added before vortexing to facilitate removal of the lipid film from the sides of the flask. The resulting opaque dispersion was frozen in an ethanol/dry ice bath and then allowed to thaw in a water bath at 37 °C. This freeze−thaw cycle was repeated 10 times. The resulting mixture was extruded, at room temperature, 29 times through a 19 mm polycarbonate filter (Nucleopore) with 100 nm diameter pores using a hand-held Basic LiposoFast device purchased from Avestin, Inc., Ottawa, Canada.[13] The extrusion device consisted of a machined housing that secured the polycarbonate filter between two 0.5 mL syringes. The liposomal mixture was forced back and forth from one syringe to the other. An odd number of passages ensured that the mixture always finished up in the receiving syringe. This method has been shown to produce LUVs with a mean diameter of 80 nm.[13,14] To separate untrapped marker, the mixture was dialyzed against NaCl solution (0.15 M) for at least 2 h using dialysis tubing of 15 000 MW cutoff. When dialysis could not be used due to high rates of liposome leakage, Sephadex G-50 mini spun columns were used to rapidly separate untrapped nucleoside from the liposomes.[12] A typical nucleoside encapsulation volume was 0.8 μL/μmol of lipid.

† Molecular recognition with boron acids, part 10. For part 9, see ref 7e.

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UV Assay for Nucleoside Efflux—A 3 mL cuvette, filled with buffer solution (35 mM sodium phosphate, 75 mM NaCl, pH 7.4), was stirred magnetically by a small "spectral" stir bar (Fisher) located at the bottom. A dialysis bag, formed by tying both ends of a piece of 90 x 4 mm dialysis tubing (12 000−14 000 MW cutoff), was charged with 200 μL of dialyzed liposome solution (100 mM of entrapped nucleoside) and suspended inside the cuvette. The entire system (see Figure 1) was allowed to equilibrate for 1 h before an aliquot of boronic acid solution (150 μL of buffer solution for water-soluble boronic acids or DMSO for organic-soluble boronic acids) was added to the external cuvette solution. Every 20 min, a 1 mL aliquot of the external solution was removed, its optical density at the nucleoside λ_{max} was determined (λ_{max} values for each nucleoside are provided in Table 1), and then the aliquot was returned to the cuvette. At the end of the run, one drop of 50% reduced Triton X-100 solution was added to the dialysis bag via a syringe. After a further 45 min, or until no change in absorbance was seen, a final absorbance reading was taken to determine the total amount of nucleoside encapsulated.

Enzyme Assay for Sucrose Efflux—The rate of sucrose efflux was determined using the enzymatic assay described by Outlaw.[15] Aliquots of sucrose-filled liposome preparation were added to two cuvettes. One cuvette contained the complete assay solution of 35 mM sodium phosphate, pH 7.5; 75 mM NaCl; 2 mM $MgCl_2$; 2.5 units of hexokinase/glucose-6-phosphate dehydrogenase; 1.5 units of invertase; 1.5 units of phosphoglucoisomerase; 1 mM ATP; and 0.5 mM NADP. The other cuvette, containing the same reagents except for the NADP, was a control solution used to correct for background absorption at 340 nm. Aliquots of the appropriate boronic acid were added to each cuvette, and the absorbance at 340 nm was monitored over time. The total amount of sucrose encapsulated inside the liposomes was determined by lysing the liposomes with 10% Triton X-100 solution.

Radiotracer Assay for Uridine Influx in the Presence of Sucrose—A stock solution containing sucrose (5 mM) and uridine (5 mM, doped with 8 μCi/mL of 15.2 Ci/mmol [^{3}H]uridine) was prepared. Empty liposomes containing 70 mM sodium phosphate at pH 7.5 were prepared as described above, except the phospholipid lipid layer was doped with [^{14}C]DPPC (112 mCi/mmol) at a ratio of 0.25 μCi/10 μmol of DPPC. The uridine influx experiment was initiated when 475 μL of liposome solution, 475 μL of stock solution, and 50 μL of boronic acid in DMSO were added together at 295 K. Every 10 min, a 100 μL sample was withdrawn, and spun down through a 1 mL Sephdex G-50 column. The liposome fraction was added to 10 mL of scintillation fluid and the ^{3}H and ^{14}C activity levels were counted using a scintillation counter. Each influx experiment was repeated at least twice with independent solutions.

Results and Discussion

Nucleoside Transport out of Liposomes—Various nucleosides were encapsulated inside large unilamellar vesicles (100 mM nucleoside inside 80 nm diameter LUVs, encapsulation volume 0.8 μL/μmol lipid) and their rates of efflux determined in the presence and absence of different boronic acids. Typically, the liposomes were composed of dipalmitoylphosphatidyl DPPC, C, and PA in the ratio 20:15:2. Compared to unsaturated phospholipids, saturated DPPC forms liposomes that are less permeable to small hydrophilic molecules.[12,14] In addition, the presence of large amounts of cholesterol within the membrane is also known to decrease permeability.[14] Thus, the unilamellar vesicle system used in this study was one of the least permeable that could be prepared.

The liposomes were prepared using the membrane extrusion method.[13] The efflux experiment is described in Figure 1. A small dialysis bag was constructed by tying a short length of dialysis tubing at both ends. The bag, containing 200 μL of nucleoside-filled liposome solution, was suspended inside a 3 mL cuvette containing an isomolar buffer solution. The pore size of the bag was wide enough to allow rapid passage of small molecules, but narrow enough to retain the much larger liposomes. Thus, a boronic acid added to the external solution in the cuvette rapidly enters the dialysis bag, diffuses into a liposome, binds reversibly with a nucleoside and transports it out (Figure 1). After escaping from a liposome, the nucleoside rapidly exits the dialysis bag and enters the external solution. Control experiments showed that the rate of appearance of marker compounds in the external solution equaled the rate of efflux from the liposome. For example, rates of glucose efflux, determined via an enzymatic assay,[8] and rates of uridine efflux, determined by the radiotracer assay described below, were found to be essentially the same in the presence or absence of the dialysis bag described in Figure 1.

Rates of nucleoside efflux were determined from the change in UV absorption of the external solution after addition of boronic acid. Five boronic acids were investigated: 3,5-bis-(trifluoromethyl)phenylboronic acid (**1**), 3,5-dichlorophenylboronic acid (**2**), 4-*tert*-butylphenylboronic acid (**3**), 1-butylboronic acid (**4**), and 4-carboxyphenylboronic acid (**5**). Figure 2 shows typical efflux plots for uridine, **6**, in the presence and absence of boronic acids. The relative efflux enhancements for nucleosides **6**−**10** are recorded in Table 1. Efflux rates for the uridine derivatives **6**−**10** were facilitated by four of

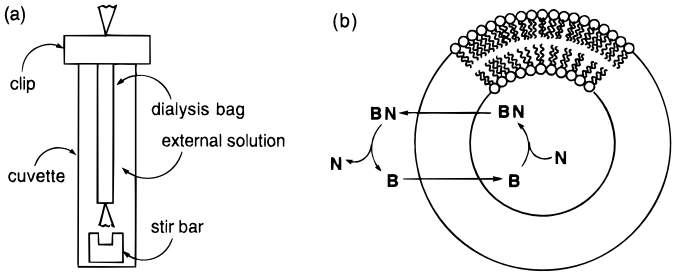

Figure 1—(a) Experimental setup used to determine nucleoside efflux from liposomes. (b) The efflux mechanism; boronic acid (B) transports nucleoside (N) through liposome bilayer via a nucleoside–boronate complex (BN).

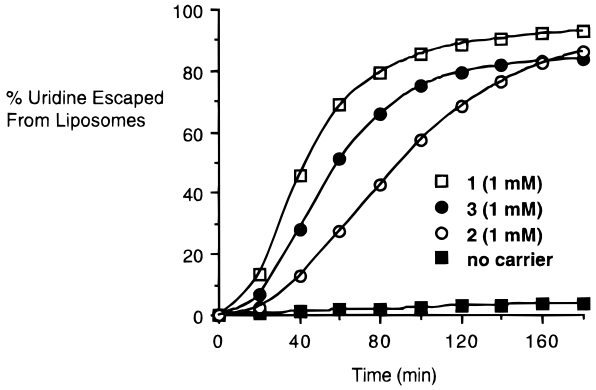

Figure 2—Percent uridine, **6**, escaped from liposomes versus time, pH 7.4. Identities and concentrations of the boronic acid carriers are provided in the legend. Initially, the liposomes contained 100 mM uridine; after lysis the external solution contained 0.1 mM nucleoside.

Table 1—Nucleoside λ_{max} Values, Nucleoside Efflux Enhancements, and Boronic Acid pK_a's

		Nucleoside Efflux Enhancements with Different Boronic Acids (±20%)[a]				
Nucleoside	λ_{max} (nm)[b]	**1** pK_a = 7.2	**2** pK_a = 7.4	**3** pK_a = 9.3	**4** pK_a = 10.4	**5** pK_a = 8.4
Uridine, **6**	261	50	25	40	2	1
5-Methyluridine, **7**	267	40		20	2	1
5-Fluorouridine, **8**	268	50		20	2	1
Inosine, **9**	260	10		2	1	1
Cytidine, **10**	269	30		10	2	1

[a] Enhancement, over initial background leakage rates, produced by 1 mM of carrier at pH 7.4. [b] Wavelength used to monitor nucleoside leakage.

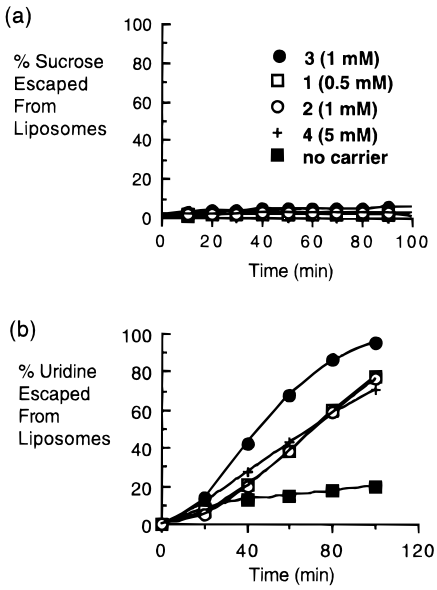

Figure 3—Percent (a) Sucrose and (b) Uridine, **6**, escaped from liposomes versus time, pH 7.4. Identities and concentrations of the boronic acid carriers are provided in the legend. Initially, the liposomes contained 100 mM uridine and 100 mM sucrose.

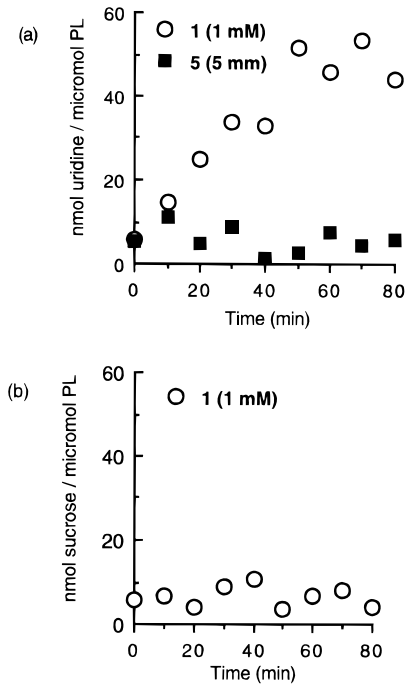

Figure 4—Incubation of empty liposomes (18 μmol phospholipid/mL) with a solution of uridine/sucrose (5 mM each) and boronic acids **1** or **5**. (a) Ratio of uridine to phospholipid in liposomal fraction as detemined by tracer methods. (b) Ratio of sucrose to phospholipid in liposomal fraction as detemined by tracer methods.

the boronic acid carriers, with the order of carrier effectiveness being **1** > **3** > **2** >> **4**. The extremely hydrophilic boronic acid **5** produced no measurable transport enhancement. As discussed previously, boronic acid transport ability appears to increase with both lipophilicity and acidity.[8] In all cases, efflux rates exhibited an approximate first-order dependence on boronic acid concentration. Certain ribonucleosides such as adenosine could not be studied by this method due to their poor solubility. As well, the lipophilic 2'-deoxynucleosides could not be studied because their background leakage rates were too high.

The results of a number of control experiments provided strong evidence that transport was due to a selective complexation event and not due to a general change in lipid bilayer permeability. Previous leakage experiments with encapsulated anionic dyes, such as calcein and carboxyfluoroscein, as well as the anionic carbohydrate derivatives glucose 6-phosphate and isocitrate, showed no evidence for leakage upon treatment of the liposomes with boronic acids.[8] Using the methodology described in Figure 1, no efflux was also observed with the anionic 5′-uridine monophosphate. An even more striking example was found when sucrose was used as the entrapped marker. In addition to sucrose being a disaccharide, its diol stereochemistry is such that it hardly binds to boronic acids in aqueous solution.[16] Therefore, we were not surprised to find that boronic acids were unable to facilitate sucrose transport. To demonstrate the selectivity of the transport method, liposomes were prepared with a mixture of 100 mM nucleoside and 100 mM sucrose encapsulated inside. As shown in Figure 3 treatment of liposomes containing uridine and sucrose with boronic acids **1**–**4** produced uridine efflux enhancements that were similar to those described in Table 1, whereas an enzymatic assay showed zero sucrose efflux. The same results were obtained with nucleosides **7**–**10** (data not shown). Thus, the boronic acids were able to enter the liposomes, selectively bind to the nucleosides, and transport them out in the presence of large amounts of sucrose.

Nucleoside Transport into Liposomes—Having shown that boronic acids can transport nucleosides out of liposomes, the next step was to demonstrate nucleoside transport into liposomes. The UV-based assay used for the efflux experiments was not sensitive enough to detect liposome influx. Therefore, radiotracers were used as monitors of solute concentrations. Liposomes containing only buffer solution were prepared with trace [^{14}C]DPPC incorporated in the phospholipid membrane. These "empty" liposomes (18 μmol phospholipid/mL) were incubated with solutions containing equimolar amounts of uridine and sucrose (5 mM each), in the presence and absence of boronic acid. Each transport experiment involved two concurrent incubations; in one case the uridine included trace [^{3}H]uridine, and in the other case the sucrose contained trace [^{3}H]sucrose. Every 10 min a small aliquot of each incubation mixture was withdrawn and passed through Sephadex which separated the liposomal component from the external solution. The ratio of ^{3}H to ^{14}C associated with the liposomal fraction, determined from scintillation counting, was considered to be a measure of the amount of uridine or sucrose incorporated into the liposomes.

As shown in Figure 4, when the liposome/uridine/sucrose incubation mixture was treated with boronic acid **1** (1 mM) the amount of uridine associated with the liposomes increased over time. On the other hand, the amount of sucrose incorporated into the liposomes remained low and invariant. When the nontransporting boronic acid **5** was used, or when boronic acid was omitted, association of both uridine and sucrose with the liposomes was found to be negligible. These results are consistent with the selective delivery of uridine into the liposomes in the presence of noncomplexing sucrose. The rate of facilitated uridine influx was quite slow; passive equilibrium between the liposome internal phase and external solution was reached after about 1 h. This is in qualitative agreement with previously observed liposome permeability fluxes obtained with similar bilayer compositions.[14c,d]

Membrane Transport Mechanism—Since there is little doubt that ribonucleoside transport is mediated by covalent complexation between the boronic acid and the cis-2′,3′-diol on the ribose ring, the question of the bilayer transport mechanism reduces to whether the transported species is the ion-pair shown in eq 1 or the neutral trigonal boronate ester in

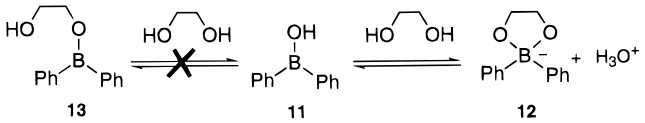

Figure 5—Covalent complexation between a diol and **11** in aqueous solution can only produce the bidentate tetrahedral anion **12** and not the monodentate trigonal ester **13**.

eq 2.[7–9] The results of two control experiments strongly suggest the latter. (i) Uridine influx and efflux rates determined at neutral pH were found to be insensitive to cation lipophilicity. Addition of 5 mM sodium, tetramethylammonium, or tetrabutylammonium chloride had essentially no effect on observed transport rates. Transport as an ion pair (eq 1) would be expected to be highly sensitive to changes in cation lipophilicity. (ii) Diphenylborinic acid (**11**) was found to be incapable of inducing uridine influx or efflux. Because **11** contains two B–C bonds and is relatively acidic (pK_a = 6.2), it can only form the anionic tetrahedral complex **12** when it combines with a diol in aqueous solution (Figure 5). The monodentate borinate ester **13** is hydrolytically very unstable and does not form. Previous work with liquid organic membranes has shown that under conditions where eq 1 is operating, the highly lipophilic **12** is easily formed and readily transported.[7b] The observation that **11** has no nucleoside transport ability with liposomes is strong evidence against the transport mechanism of eq 1 and for the transport mechanism of eq 2.

Conclusion

It has been shown for the first time that boronic acids can be used as carriers to facilitate the transport of nucleosides through a lipid bilayer membrane. The evidence is strongly in favor of eq 2 as the membrane transport mechanism. This finding raises the possibility of using boronic acids as nucleoside drug adjuvants to enhance therapeutic efficacies by increasing nucleoside membrane transport. Future work will focus on improving the transport efficiency in terms of rates and selectivities. Although the facilitated transport of nucleosides has clinical potential, it is the facilitated transport of nucleotides that would be of extraordinary benefit. It is generally believed that direct introduction of nucleotides into cells would circumvent the problem of drug ineffectiveness with cells that are unable to convert nucleoside analogues into bioactive nucleotides.[17] The development of nucleotide carriers to transport clinically important nucleotides into cells is the aim of a number of research groups.[18] We are hopeful that conjugation of a boronic acid with an appropriate phosphate binding group will produce an efficient artificial nucleotide membrane carrier.

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