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Boronate-Mediated Biologic Delivery

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Supporting Information

ABSTRACT: Inefficient cellular delivery limits the landscape of macromolecular drugs. Boronic acids readily form boronate esters with the 1,2- and 1,3-diols of saccharides, such as those that coat the surface of mammalian cells. Here pendant boronic acids are shown to enhance the cytosolic delivery of a protein toxin. Thus, boronates are a noncationic carrier that can deliver a polar macromolecule into mammalian cells.

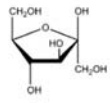
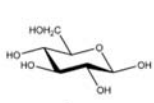
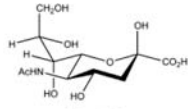
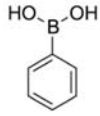
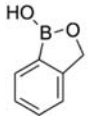
The utility of many biologic drugs is limited by inefficient cellular delivery.[1] Previous efforts to overcome this limitation have focused largely on the use of cationic domains—peptidic (e.g., HIV–TAT, penetratin, and nonaarginine) or nonpeptidic (e.g., PAMAM dendrimers and polyethylenimine)—to enhance the attraction between a chemotherapeutic agent and the anionic cell surface.[2] Natural ligands (e.g., folic acid, substance P, and the RGD tripeptide) have also been used to facilitate cellular delivery by targeting agents to specific cell-surface receptors.[3] Although these methods have achieved some success, additional delivery strategies are desirable.

The cell surface is coated with a dense forest of polysaccharides known as the glycocalyx.[4] We anticipated that targeting therapeutic agents to the glycocalyx would enhance their cellular delivery, as has been demonstrated with lectin conjugates.[5] Boronic acids readily form boronate esters with the 1,2- and 1,3-diols of saccharides,[6] including those in the glycocalyx.[7] In addition, boronate groups are compatible with human physiology, appearing in chemotherapeutic agents and other remedies.[8] Further, pendant boronic acids conjugated to polyethylenimine have been shown to enhance DNA transfection.[9] Here we demonstrate the use of pendant boronic acids to mediate the delivery of a protein into the cytosol of mammalian cells.

Bovine pancreatic ribonuclease (RNase A) is a small, well-characterized enzyme that has been the object of much seminal work in protein chemistry.[10] If this ribonuclease can gain access to the RNA that resides in the cytosol, then its prodigious catalytic activity can lead to cell death.[11] Hence, RNase A can serve as an ideal model for assessing the delivery of a protein into the cytosol (rather than an endosome) because success can be discerned with assays of cytotoxic activity.

Initially, we quantified the affinity of simple boronic acids to relevant saccharides. Sialic acid is of particular interest because of its abundance in the glycocalyx of cancer cells.[12] Phenylboronic acid (PBA) binds with higher affinity to sialic acid than to other pyranose saccharides,[13] suggesting that simple boronic acids could target chemotherapeutic agents selectively to tumors. 2-Hydroxymethylphenylboronic acid (benzoxaborole[14]) has the highest reported affinity for pyranose saccharides,[13,15] which are abundant in the glycocalyx; hence, we reasoned that benzoxaborole could be an ideal boronate for drug delivery. We used ^{1}H NMR spectroscopy to evaluate directly the affinity of PBA and benzoxaborole for fructose, glucose, and *N*-acetylneuraminic acid (Neu5Ac), which contains a sialic acid moiety, under physiological conditions. Our K_a values (Table 1) are in accord with values determined by other workers using competition and other assays (Table S1 in the Supporting Information).[13a,c,15] We found that benzoxaborole has a greater affinity than PBA for each saccharide in our panel and that benzoxaborole, like PBA, has a greater affinity for Neu5Ac than for glucose. Accordingly, we chose benzoxaborole for our boronate-mediated delivery studies.

Table 1. Values of K_a (M^{-1}) for Boronic Acids and Saccharides[a]

	 D-fructose	 D-glucose	 Neu5Ac
	128 ± 20	5 ± 1	13 ± 1
	336 ± 43	28 ± 4	43 ± 5

[a]Each value is the mean ± standard deviation (SD) for ≥15 measurements in 0.10 M sodium phosphate buffer (pH 7.4) containing 2% (v/v) D_2O.

To display benzoxaborole moieties on RNase A, we conjugated 5-amino-2-hydroxymethylphenylboronic acid (**1**) to protein carboxyl groups by condensation using a carbodiimide (Figure 1). Of the 11 carboxyl groups of RNase A, 7.5 ± 2.0 were condensed with boronate **1**, as determined by mass spectrometry.

Boronation should increase the affinity of a protein for oligosaccharides. To test this hypothesis qualitatively, we

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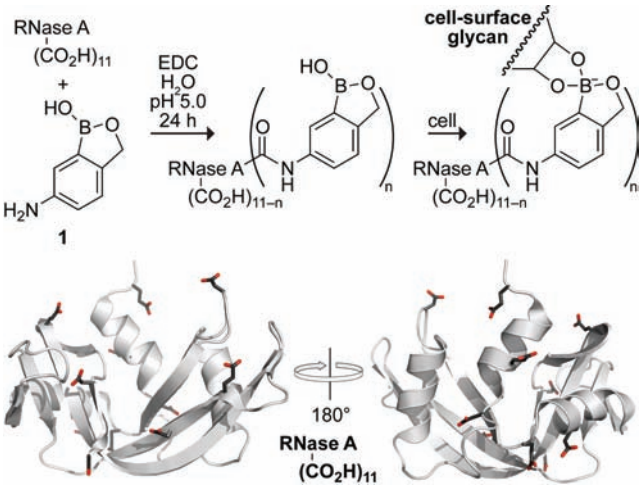

Figure 1. Boronation of RNase A and its putative mechanism for expediting cellular delivery. The location of each carboxyl group of RNase A is depicted in the ribbon diagram (PDB entry 7rsa[16]).

measured the retention of boronated and unmodified RNase A on a column of heparin, a common physiological polysaccharide. Boronated RNase A was indeed retained longer on the column (Figure 2). If the prolonged retention were due

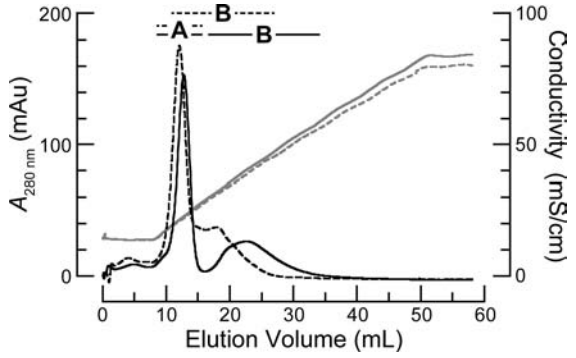

Figure 2. Elution profile of a mixture of unmodified RNase A (eluting in region A) and boronated RNase A (eluting in region B) from a column of immobilized heparin in the absence (solid lines) or presence (dashed lines) of fructose (0.10 M). Black lines, $A_{280\,nm}$; gray lines, conductivity.

to boron–saccharide complexation, then fructose in the buffer should compete with immobilized heparin for boron complexation. When these conditions were employed, the retention of boronated RNase A was indeed diminished (Figure 2).

To evaluate the enhanced affinity of boronated RNase A for oligosaccharides, we measured its affinity for ganglioside GD3 within a 1,2-dioleoyl-*sn*-glycero-3-phosphocholine liposome. This ganglioside has two sialic acid residues and is overexpressed on the surface of cancer cells.[17] By using fluorescence polarization to analyze binding, we found that boronation increased the affinity of the protein for the ganglioside, an effect that was abrogated by fructose (Figure 3). The K_d value of boronated protein for GD3 ganglioside liposomes was (53 ± 11) μM. This affinity is ~440-fold greater than that for the binding of a single benzoxaborole to Neu5Ac (Table 1), consistent with a multivalent interaction between the boronated protein and the ganglioside.

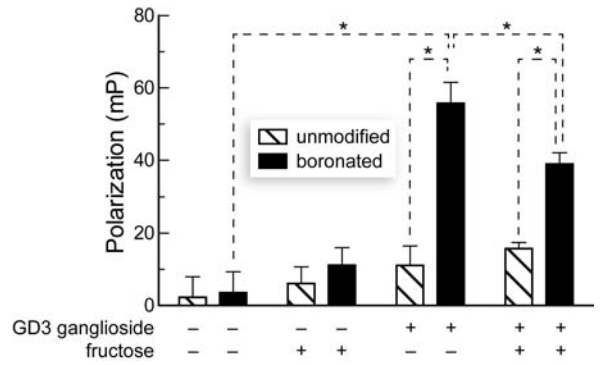

Figure 3. Fluorescence polarization assay of ribonucleases binding ganglioside-labeled liposomes in the presence or absence of 10 mM fructose. Data were normalized to the polarization of each ribonuclease incubated with nonextruded DOPC lipids. Each data point represents the mean ± SD for triplicate experiments. Asterisks indicate values with $p < 0.05$.

Encouraged by the enhanced affinity of the boronated protein for oligosaccharides in vitro, we sought to test our hypothesis that boronate conjugation increases cellular uptake. To quantify cellular internalization, we used a fluorophore-labeled protein and flow cytometry. To determine concurrently whether the pendant boronates would elicit selectivity for cells with higher quantities of cell-surface sialic acid, we employed a line of Chinese hamster ovary cells (Lec-2) that have lower levels of sialic acid in their glycocalyx than their progenitor line (Pro-5).[18] We found that boronation of RNase A increased its cellular uptake by 4–5-fold (Figure 4). This enhancement was

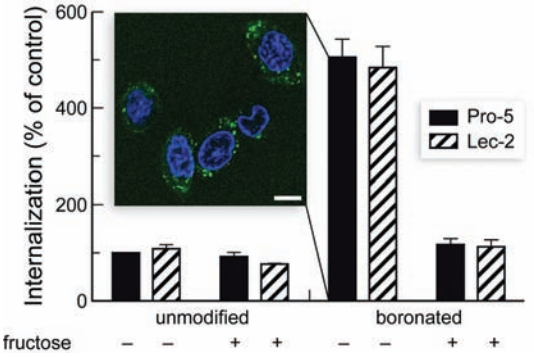

Figure 4. Internalization of unmodified and boronated RNase A into Pro-5 and Lec-2 cells in the absence or presence of fructose (0.25 M). Flow cytometry data were normalized to the internalization of unmodified RNase A into Pro-5 cells. Error bars represent SDs. Inset: Confocal microscopy image of live Pro-5 cells incubated for 4 h with boronated RNase A (5 μM) that had been labeled covalently with a green fluorophore. Nuclei were stained blue with Hoechst 33322 (2 μg/mL). Scale bar: 10 μm.

eliminated by fructose. Cell-surface sialic acid content did not affect uptake significantly, consistent with the modest (1.5-fold) increase in the K_a value for benzoxaborole with sialic acid versus glucose (Table 1). Confocal microscopy of the boronated protein revealed punctate staining (Figure 4 inset), which is consistent with uptake by endocytosis following complexation with cell-surface saccharides.

Although flow cytometry can quantify protein internalization into a cell, it does not differentiate between proteins in endosomes versus those in the cytosol. Delivery into the

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cytosol is essential for the efficacy of numerous putative chemotherapeutic agents. Boronated RNase A retained 17% of its ribonucleolytic activity.[19] Accordingly, boronated RNase A has the potential to be cytotoxic if it can gain entry to the cytosol. We found that boronated RNase A inhibited the proliferation of human erythroleukemia cells (Figure 5). The

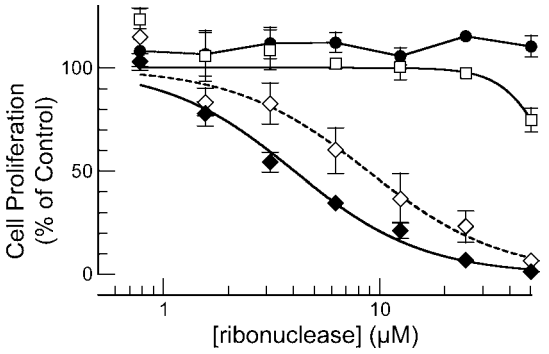

Figure 5. Inhibition of K-562 cell proliferation by unmodified and boronated RNase A: (●) unmodified RNase A (IC_{50} >50 μM); (◆) boronated RNase A (IC_{50} 4.1 ± 0.4 μM); (◇) boronated RNase A in the presence of fructose (50 mM) (IC_{50} 9 ± 1 μM); (□) boronated RNase A alkylated with 2-bromoacetate (IC_{50} >50 μM). The proliferation of K-562 cells was measured by the incorporation of [*methyl*-^{3}H]thymidine. Each data point represents the mean ± standard error of the mean for three separate experiments performed in triplicate.

addition of fructose diminished the cytotoxic activity, presumably by decreasing the overall internalization. Chemically inactivated boronated RNase A was much less cytotoxic, indicating that the ribonucleolytic activity induced toxicity, not the pendant boronates. We conclude that boronation not only facilitates cellular uptake of a protein but also enhances its delivery to the cytosol.

Boronates have attributes that make them attractive as mediators of drug delivery. First, endosomes become more acidic as they mature. In synergy, the affinity of boronates for saccharides decreases with decreasing pH.[13a] Moreover, the ensuing loss of complexation causes boronates to become more hydrophobic.[20] These attributes could facilitate translocation to the cytosol. Second, boronates are not cationic,[21] averting the nonspecific Coulombic interactions elicited by cationic domains,[2] which can lead to high rates of glomerular filtration and opsonization in vivo.[22] Finally, we note that numerous diseases are associated with changes in cell-surface glycosylation,[12,23] and we anticipate that boronic acids with specificity for particular glycans could serve as the basis for targeted delivery strategies.[24]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Analytical data and experimental protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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