Boronate-Mediated Biologic Delivery

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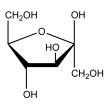
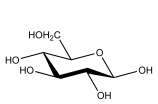
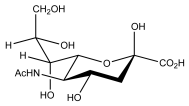
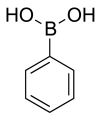
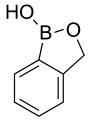
Table S1: Literature values of K_a (M^{-1}) for boronic acids and saccharides.

	 D-fructose	 D-glucose	 Neu5Ac	Method	Reference
	128 ± 20	5 ± 1	13 ± 1	^{1}H NMR in H_2O containing D_2O (2% v/v) [a]	This work
	160	4.6	21	Competition with alizarin red S [a]	1
	—	—	11.6 ± 1.9	^{11}B NMR in H_2O/D_2O/MeOH mixture	2
	79	0	—	^{1}H NMR in D_2O (100% v/v) [a]	3
	160	6	21	Competition with alizarin red S [a]	4
	336 ± 43	28 ± 4	43 ± 5	^{1}H NMR in H_2O containing D_2O (2% v/v) [a]	This work
	606	17	—	^{1}H NMR in D_2O (100% v/v) [a]	3
	—	31	—	Competition with alizarin red S [a]	5
	664	21	160	Competition with alizarin red S [a]	4

[a] Values were determined in 0.10 M sodium phosphate buffer, pH 7.4.

Materials

N-Acetylneuraminic acid was from Carbosynth (Berkshire, UK). Phenylboronic acid, 2-hydroxymethylphenylboronic acid, and 5-amino-2-hydroxymethylphenylboronic acid were from Combi-Blocks (San Diego, CA). BODIPY® FL, STP ester was from Molecular Probes (Eugene, OR). *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and wild-type RNase A (Type III-A) were from Sigma–Aldrich (St. Louis, MO) and used without further purification. MES buffer was from Sigma–Aldrich and purified by anion-exchange chromatography to remove trace amounts of oligomeric vinylsulfonic acid.[6] Spectra/Por® dialysis bags (3500 MWCO) were from Fisher Scientific (Thermo Fisher Scientific, Walham, MA). *Escherichia coli* BL21(DE3) cells were from Novagen (Madison, WI). [*methyl*-^{3}H]Thymidine (6.7 Ci/mmol) was from Perkin–Elmer (Boston, MA). Columns of HiTrap Heparin HP resin for protein purification and analytical resins were from GE Biosciences (Piscataway, NJ). Ribonuclease substrate 6-FAM–dArUdAdA–6-TAMRA was from Integrated DNA Technologies (Coralville, IA). Non-binding surface (NBS) 96-well plates were from Corning (Corning, NY). Terrific Broth (TB) was from Research Products International Corp (Mt. Prospect, IL). Bovine Serum Albumin (BSA) was from Thermo Scientific (Rockfield, IL). SDS–PAGE gels were from Bio-Rad Laboratories (Hercules, CA). GD3 Ganglioside (bovine milk; ammonium salt), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and an extruder were from Avanti Polar Lipids (Alabaster, Alabama). Cell culture medium and supplements were from Invitrogen (Carlsbad, CA). Phosphate-buffered saline was either Dulbecco's PBS (DPBS) from Invitrogen or the same solution made in the laboratory (PBS), containing (in 1.0 L): 0.2 g KCl, 0.2 g KH_2PO_4, 8 g NaCl, and 2.16 g $Na_2HPO_4 \cdot 7H_2O$ at pH 7.4. All other chemicals used were of commercial reagent grade or better, and were used without further purification.

Instrumentation and Statistics

^{1}H NMR spectra were acquired at the National Magnetic Resonance Facility at Madison at 298 K on an Avance III 500 MHz spectrometer with a TCI 500 H-C/N-D cryogenic probe from Bruker AXS (Madison, WI, ^{1}H, 500 MHz). Protein absorbance values were measured on a Varian Cary 50 UV–Vis Spectrometer (Agilent Technologies, Santa Clara, CA) and/or a NanoVue spectrometer (GE Healthcare, Piscataway, NJ). Confocal microscopy was carried out using an Eclipse C1 laser scanning confocal microscope from Nikon (Melville, NY). Flow cytometry was done using a LSRII (BD Biosciences, San Jose, CA) at the University of Wisconsin–Madison Carbone Cancer Center Flow Cytometry Facility. The mass of RNase A and boronated RNase A conjugates were confirmed at the University of Wisconsin–Madison Biophysics Instrumentation Facility by matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectrometry with a Voyager-DE-PRO Biospectrometry Workstation from Applied Biosystems (Foster City, CA). [*methyl*-^{3}H]Thymidine incorporation into K-562 genomic DNA was quantified by scintillation counting using a Microbeta TriLux liquid scintillation and luminescence counter from Perkin–Elmer. Fluorescence measurements were made with an infinite M1000 plate reader from Tecan (Männedorf, Switzerland). Calculations for statistical significance were performed with GraphPad Prism version 5.02 software from GraphPad Software (La Jolla, CA), and a value of $p < 0.05$ was considered to be significant.

Determination of K_a Values by ^{1}H NMR Spectroscopy

Methodology to determine the values of K_a for boronic acids and saccharides was adapted from work by Hall and coworkers.[3,5] A boronic acid (B) and a saccharide (S) were assumed to bind in one modality, B·S:

$$B + S \rightleftarrows B \cdot S$$

$$K_a = \frac{[B \cdot S]}{[B][S]}$$

The [B·S]/[B] ratio was determined by the integration of aryl protons of the boronic acid·saccharide complex and the free boronic acid. The individual [B], [B·S], and [S] can be calculated from eq 1–3.

$$[B \cdot S] + [B] = [B_T]$$

$$\frac{[B \cdot S]}{[B]} + 1 = \frac{[B_T]}{[B]}$$

eq 1:

$$[B] = \frac{[B_T]}{\frac{[B \cdot S]}{[B]} + 1}$$

eq 2:

$$[B \cdot S] = \frac{[B \cdot S]}{[B]}[B], \text{ where } [B] \text{ is calculated from eq 1}$$

$$[B \cdot S] + [S] = [S_T]$$

eq 3:

$$[S] = [S_T] - [B \cdot S], \text{ where } -[B \cdot S] \text{ is calculated from eq 2}$$

Each value of K_a arose from at least two independent experiments with freshly prepared solutions, and each experiment consisted of a titration with 6–9 different concentrations. All NMR spectra were analyzed with Topspin 3.0 software from Bruker AXS. NMR experiments were done in a 0.10 M NaH_2PO_4 buffer, pH 7.4, containing D_2O (2% v/v). ^{1}H NMR experiments consisted of the first increment of a 2D NOESY with gradients for improved water suppression.

Representative Procedure for Making a Boronic Acid Solution

NaH_2PO_4 (3.0 g, 25 mmol) and PBA (458 mg, 3.75 mmol) were dissolved in distilled, deionized water in a volumetric flask (~200 mL H_2O, 5 mL D_2O). The pH was adjusted carefully to 7.4 using 10 M NaOH, and additional water was added for a final volume of 250 mL. Final solutions were 15 mM boronic acid (PBA = solution 1; benzoxaborole = solution 2) in 0.10 M sodium phosphate monobasic buffer, pH 7.4, containing D_2O (2% v/v).

Determination of the Value of K_a for PBA and Fructose

To a 25-mL volumetric flask, D-fructose (674 mg, 3.75 mmol) and ~20 mL of solution 1 was added. The solution was adjusted carefully to pH 7.4 by the addition of 10 M NaOH. (The volume of added NaOH was used in the calculation of the boronic acid concentration.) The volume was then increased to 25 mL by adding solution 1. This procedure resulted in a pH 7.4 solution of PBA (15 mM), D-fructose (150 mM), NaH_2PO_4 (0.10 M), and D_2O (2% v/v) (solution A). Mixing various volumes of solution 1 and solution A generated D-fructose concentrations in the range of 4–14 mM. The [B·S]/[B] ratio was determined for every concentration as follows.

Representative Procedure for Determining the Chemical Shifts of Aryl Protons in Bound and Free Boronic Acids

A ^{1}H NMR spectra of solution 1 (Figure S1A) and solution A (Figure S1B) were acquired. The two spectra were overlaid to determine which peaks belonged to the bound boronic acid and free boronic acid (Figure S1C). This analysis was used to interpret the spectra from the titrations with sugars (Figure S1D).

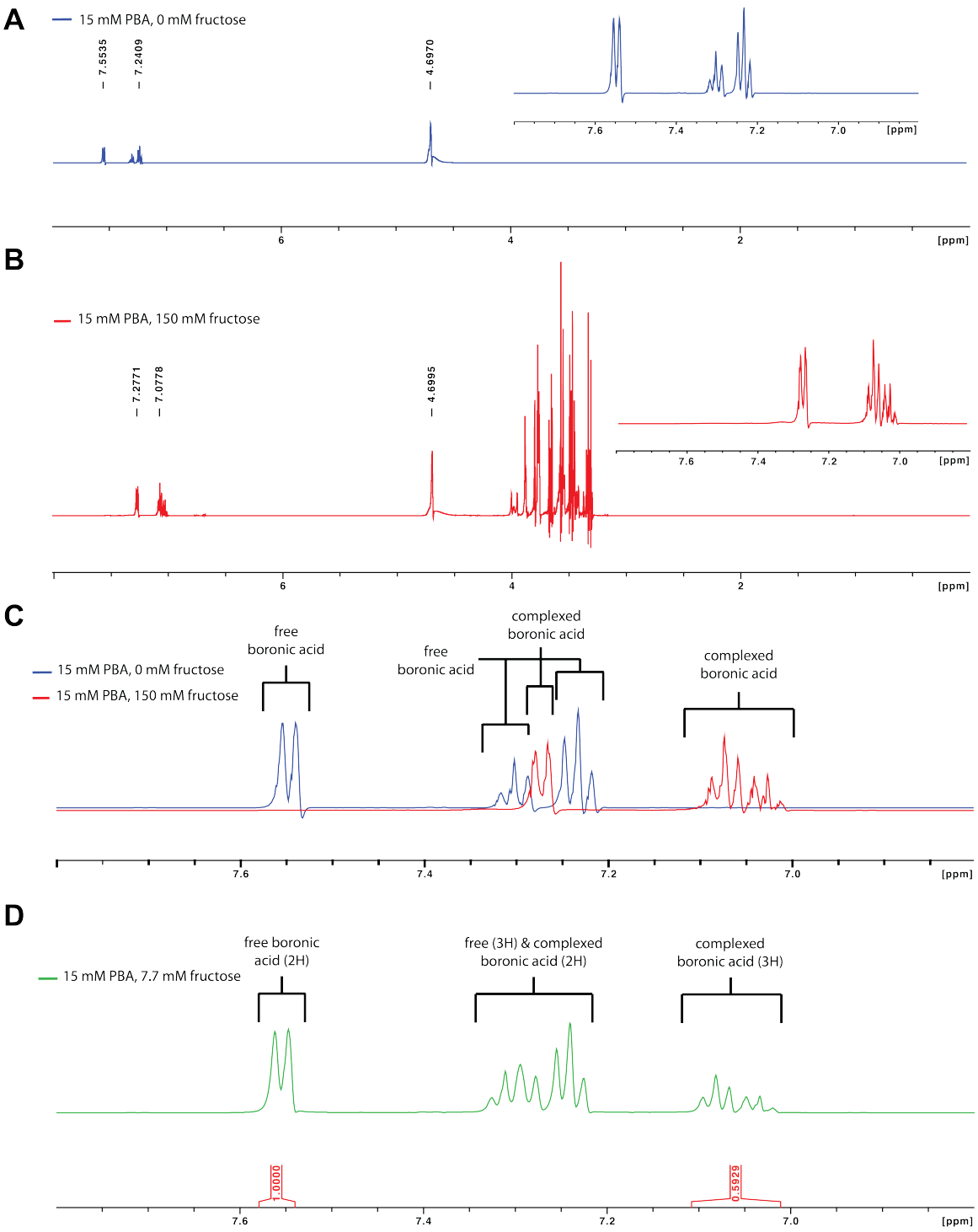


Figure S1. Determination of the peaks corresponding to the aryl protons in bound and free boronic acid. (A) ^{1}H NMR spectrum of solution 1. (B) ^{1}H NMR spectrum of solution A. (C) Overlay of aromatic region of spectra from panels A and B. (D) Example of a spectrum that was interpreted using the overlay from panel C, and used to determine the value of K_a for fructose with PBA. The [B·S]/[B] ratio was calculated from the isolated peaks for the complex (3H, 7.01–7.11 ppm) and the isolated peaks for the free boronic acid (2H, 7.54–7.58 ppm).

Determination of the Value of K_a for Benzoxaborole and Fructose

To a 25-mL volumetric flask, D-fructose (674 mg, 3.75 mmol) and ~20 mL of solution 2 was added. The solution was adjusted carefully to pH 7.4 by the addition of 10 M NaOH. (The volume of added NaOH was used in the calculation of the boronic acid concentration.) The volume was then increased to 25 mL by adding solution 2. This procedure resulted in a pH 7.4 solution of benzoxaborole (15 mM), D-fructose (150 mM), NaH_2PO_4 (0.10 M), and D_2O (2% v/v) (solution B). Mixing various volumes of solution 2 and solution B generated fructose concentrations in the range of 4–14 mM. The [B·S]/[B] ratio was determined as depicted in Figure Ss. A value for K_a was calculated for every concentration as described above.

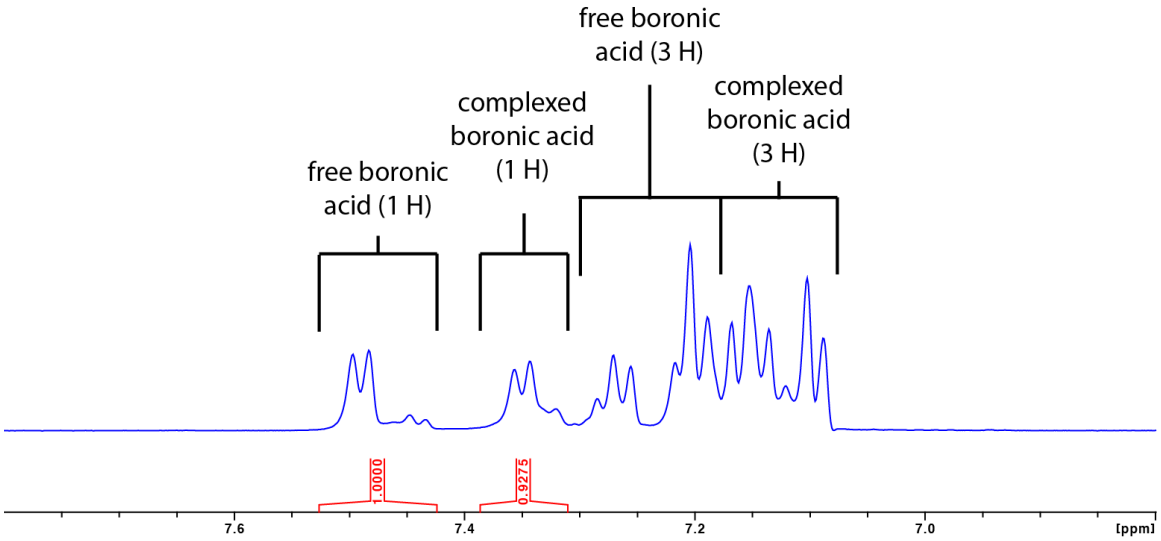


Figure S2. Example of a ^{1}H NMR spectrum that was used to determine the K_a value for benzoxaborole and D-fructose (10.3 mM). Peaks corresponding to the aryl protons in bound and free boronic acid were determined a described above. The [B·S]/[B] ratio was calculated from the isolated peaks for the complex (1H, 7.31–7.38 ppm, a mixture of isomeric species) and the isolated peaks for the free boronic acid (1H, 7.43–7.53 ppm, a mixture of isomeric species). Additional saccharide decreased the integration of the small peak at 7.44 ppm equally with that at 7.49 ppm, which arise from free boronic acid; and the shoulder peak at 7.31 ppm increased equally with that at 7.35 ppm, which arises from the complex.

Determination of the Value of K_a for PBA and Glucose

To a 25-mL volumetric flask, D-glucose (2.25 g, 12.5 mmol) and ~20 mL of solution 1 was added. The solution was adjusted carefully to pH 7.4 by the addition of 10 M NaOH. (The volume of added NaOH was used in the calculation of the boronic acid concentration.) The volume was then increased to 25 mL by adding solution 1. This procedure resulted in pH 7.4 solution of PBA (15 mM), D-glucose (500 mM), 0.1 M NaH_2PO_4 (0.10 M), and D_2O (2% v/v) (solution C). Mixing various volumes of solution 1 and solution C generated D-glucose

concentrations in the range of 20–70 mM. The [B·S]/[B] ratio was determined as depicted in Figure S3. A value for K_a was calculated for every concentration as described above.

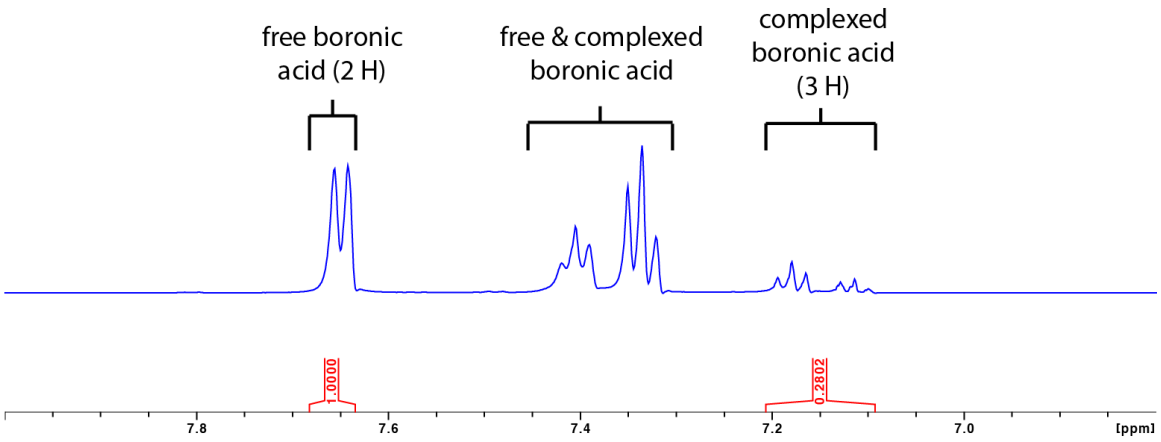


Figure S3. Example of a ^{1}H NMR spectrum that was used to determine the K_a value for PBA and D-glucose (44.9 mM). Peaks corresponding to the aryl protons in bound and free boronic acid were determined a described above. The [B·S]/[B] ratio was calculated from the isolated peaks for the complex (3H, 7.09–7.21 ppm) and the peaks for the free boronic acid (2H, 7.64–7.68 ppm).

Determination of the Value of K_a for Benzoxaborole and Glucose

To a 25-mL volumetric flask, D-glucose (2.25 g, 12.5 mmol) and ~20 mL of solution 2 was added. The solution was adjusted carefully to pH 7.4 by the addition of 10 M NaOH. (The volume of added NaOH was used in the calculation of the boronic acid concentration.) The volume was then increased to 25 mL by adding solution 2. This procedure resulted in a pH 7.4 solution of benzoxaborole (15 mM), D-glucose (500 mM), NaH_2PO_4 (0.10 M), and D_2O (2% v/v) (solution D). Mixing various volumes of solution 2 and solution D generated D-glucose concentrations in the range of 20–70 mM. The [B·S]/[B] ratio was determined as depicted in Figure S4. A value for K_a was calculated for every concentration as described above.

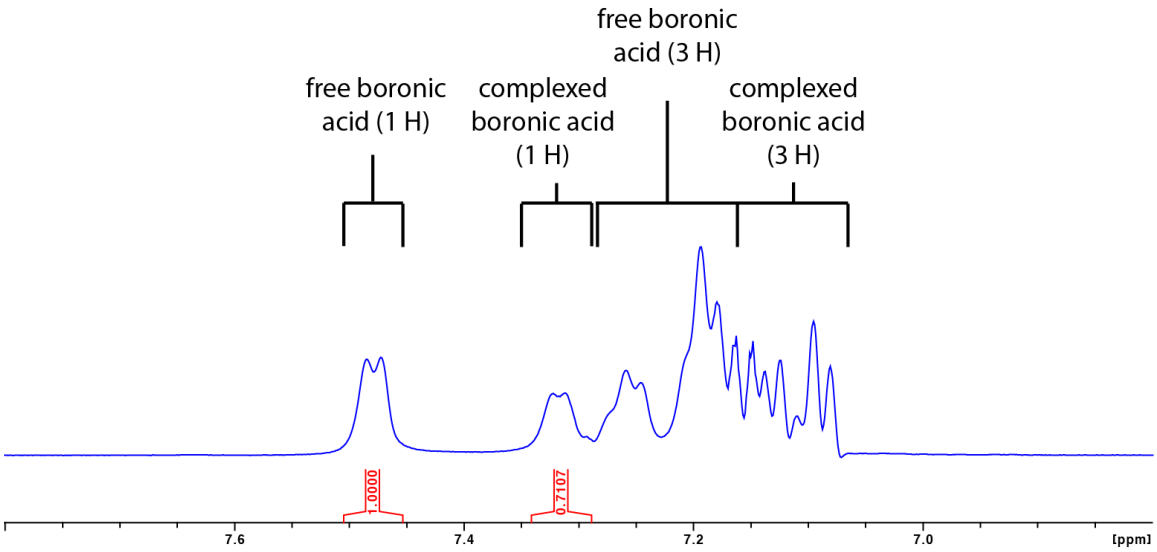


Figure S4. Example of a ^{1}H NMR spectrum that was used to determine the K_a value for benzoxaborole and D-glucose (32.9 mM). Peaks corresponding to the aryl protons in bound and free boronic acid were determined a described above. The [B·S]/[B] ratio was calculated from the isolated peaks for the complex (^{1}H, 7.29–7.34 ppm) and the isolated peaks for the free boronic acid (^{1}H, 7.45–7.51 ppm). Note the broadening of the aryl protons, which had been reported for NMR spectra of boronic acids in the presence of pyranose sugars.[5]

Determination of the Value of K_a for PBA and Neu5Ac

To a 10-mL volumetric flask, Neu5Ac (1.53 g, 5.0 mmol) and ~20 mL of solution 1 was added. The solution was adjusted carefully to pH 7.4 by the addition of 10 M NaOH. (The volume of added NaOH was used in the calculation of the boronic acid concentration.) The volume was then increased to 25 mL by adding solution 1. This procedure resulted in a pH 7.4 solution of PBA (14.2 mM), Neu5Ac (500 mM), NaH_2PO_4 (0.10 M), and D_2O (2% v/v) (solution E). Mixing various volumes of solution 1 and solution E, generated Neu5Ac concentrations in the range of 7–65 mM. The [B·S]/[B] ratio was determined as depicted in Figure S5. A value for K_a was calculated for every concentration as described above.

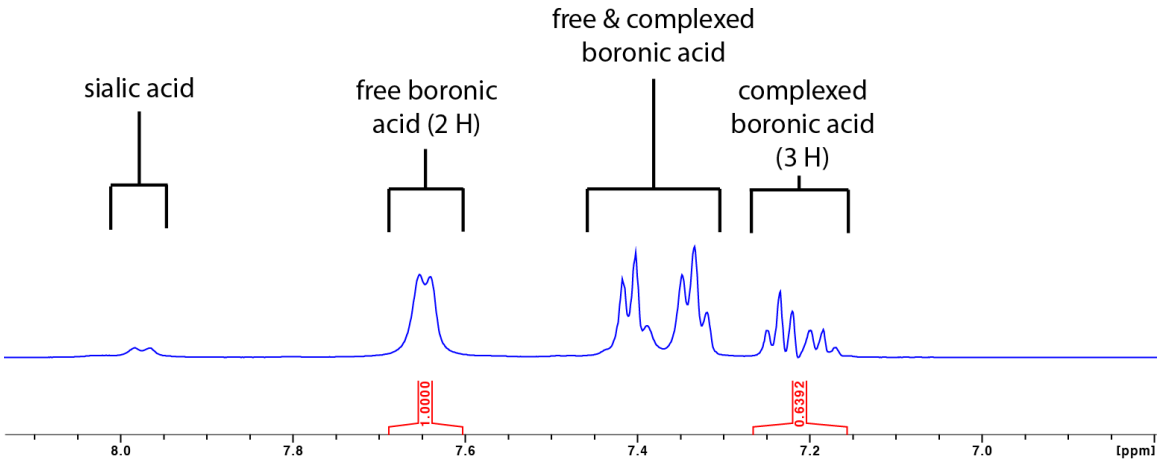


Figure S5. Example of a ^{1}H spectrum that was used to determine the K_a value for PBA with Neu5Ac (35.4 mM). Peaks corresponding to the aryl protons in bound and free boronic acid were determined a described above. The [B·S]/[B] ratio was calculated from the isolated peaks for the complex (3H, 7.15–7.26 ppm) and the isolated peaks for the free boronic acid (2H, 7.6–7.69 ppm). Note that the aryl peaks have been broadened by the addition of the saccharide.

Determination of the Value of K_a for Benzoxaborole and Neu5Ac

To a 10-mL volumetric flask, Neu5Ac (1.56 g, 5.0 mM) and ~20 mL of solution 2 was added. The solution was adjusted carefully to pH 7.4 by the addition of 10 M NaOH. (The volume of added NaOH was used in the calculation of the boronic acid concentration.) The volume was then increased to 25 mL by adding solution 2. This procedure resulted in a pH 7.4 solution of benzoxaborole (14.2 mM), Neu5Ac (500 mM), NaH_2PO_4 (0.10 M), and D_2O (2% v/v) (solution F). Mixing various volumes of solution 2 and solution F generated Neu5Ac concentrations in the range of 7–65 mM. The K_a value was calculated for every concentration as previously described. F The [B·S]/[B] ratio was determined as depicted in Figures S6 and S7. A value for K_a was calculated for every concentration as described above.

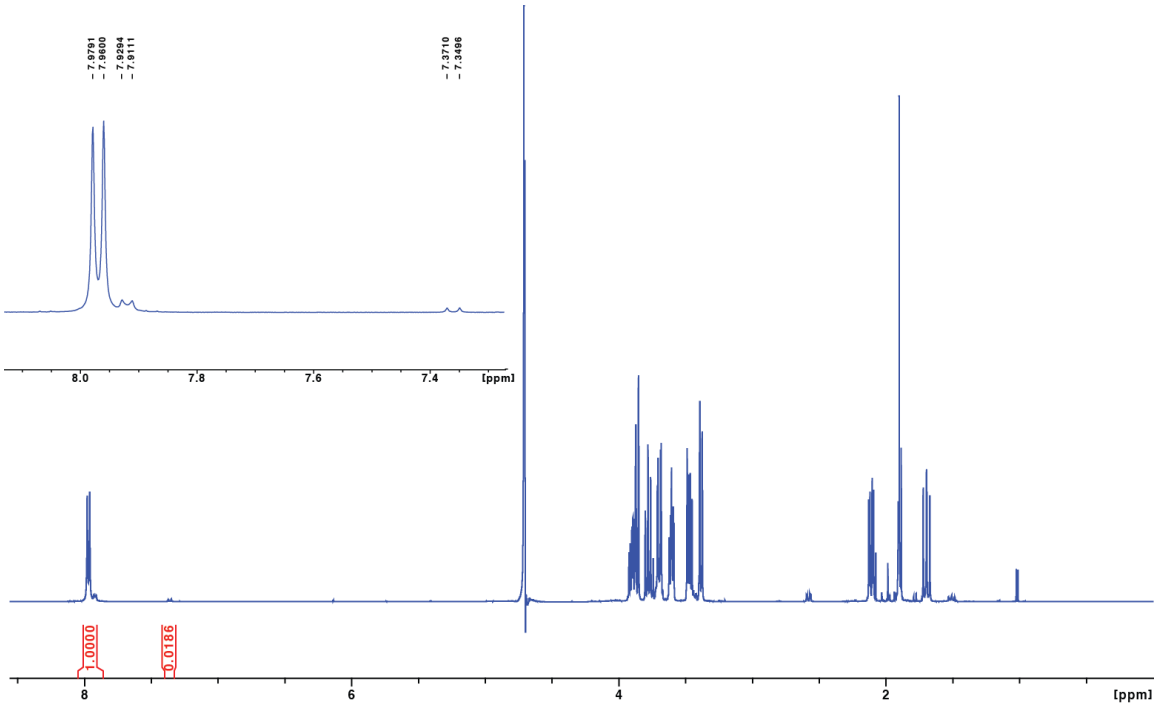


Figure S6. Apparent in the ^{1}H NMR spectrum of Neu5Ac is a small peak that overlapped with the aromatic regions of the boronic acids. This peak was subtracted out of all NMR spectra used to evaluate the interaction of benzoxaborole and Neu5Ac.

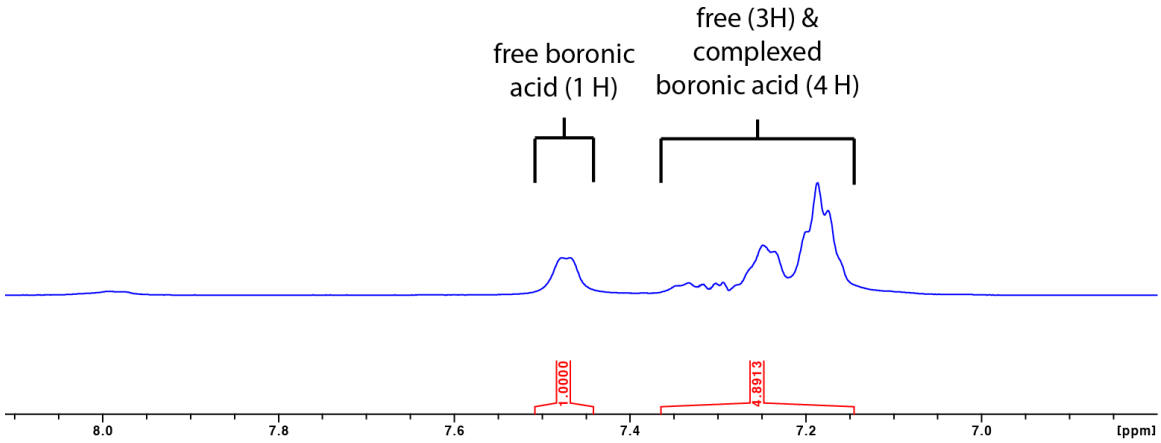


Figure S7. Example of a ^{1}H spectrum that was used to determine the K_a value for benzoxaborole with Neu5Ac (14.4 mM). Peaks corresponding to the aryl protons in bound and free boronic acid were determined a described above. The [B·S]/[B] ratio was determined from the isolated peaks for the free boronic acid (1H, 7.44–7.51 ppm) and the remainder of the aromatic region (7.15–7.36 ppm), which represented 3H from the free boronic acid and all 4 aromatic protons from the complex. Unlike D-fructose and D-glucose, the single isolated proton of the complexed species (7.33 ppm) was too broad to integrate accurately, and the entire region was used instead.

Preparation of Boronated RNase A

5-Amino-2-hydroxymethylphenylboronic acid (320 mg, 1.70 mmol) was added to 30 mL of distilled, deionized H_2O, and the resulting solution was adjusted to pH 5.0 with NaOH. To this solution was added RNase A (200 mg, 15 μmol), followed by EDC (640 mg, 3.30 mmol), and the pH was adjusted again to 5.0 with NaOH. The reaction mixture was incubated at ambient temperature overnight on a nutating mixer by BD (Franklin Lakes, NJ). Additional EDC (360 mg, 1.9 mmol) was added, and the solution was incubated at the same conditions for 3.5 h (24 h total). The solution was then subjected to centrifugation (5 min at 1000 rpm, and 5 min at 5000 rpm) to remove insoluble boronic acid, and dialyzed (3500 molecular weight cutoff) against distilled, deionized H_2O for 3 d at 4 °C, with daily water exchanges. The solution was then passed through a 0.45-μm filter and loaded onto a 5-mL column of HiTrap Heparin HP resin. To prepare a high-salt buffer, NaCl (58.4 g, 1.00 mol) was added to 100 mL of a 10× stock solution of PBS. This solution was diluted with distilled, deionized H_2O to a final volume of 1 L, and adjusted to pH 7.4, making a buffer of PBS plus an additional 1 M NaCl. The resin was washed with 75 mL of PBS buffer, and protein was eluted with a linear gradient of 225 mL of additional NaCl (0.0–1.0 M) in PBS buffer. Fractions were collected, pooled, concentrated, stored at 4 °C, and analyzed by MALDI–TOF mass spectrometry. The mass spectrum between 13–16 kDa was fitted to a Gaussian curve with GraphPad Prism version 5.02 software to determine the average mass. Conjugation to 5-amino-2-hydroxymethylboronic acid was confirmed by trypsin-digest mass spectrometry, which revealed the additional mass (113 Da, corresponding to dehydrated 5-amino-2-hydroxymethylboronic acid) of carboxylic acid-containing peptide fragments. The ribonucleolytic activity of boronated RNase A ($k_{cat}/K_M = 1.6 \pm 0.2 \times 10^7$ $M^{-1}\cdot s^{-1}$) was 17% that of unmodified RNase A ($k_{cat}/K_M = 9.6 \pm 0.7 \times 10^7$ $M^{-1}\cdot s^{-1}$).

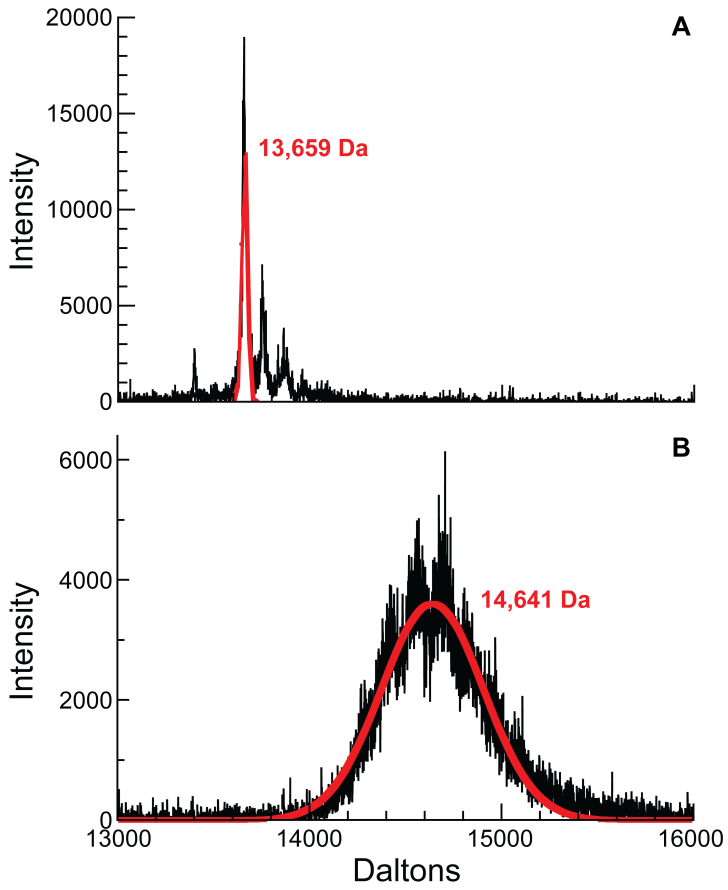


Figure S8. MALDI–TOF spectra of (A) unmodified RNase A and (B) boronated RNase A. Data were fitted to a Gaussian curve (red line). The observed molecular mass of unmodified RNase A (13,659 Da) was subtracted from the observed molecular mass of boronated RNase A (14,641 Da) to give 982 Da. This value was divided by the molecular mass of 5-amino-2-hydroxymethylphenylboronic acid after correcting for the water lost during conjugation (148.95 Da – 18.02 Da = 130.93 Da) to give 7.5 ± 2.0 boronic acids conjugated to RNase A, where SD = 2.0 arises from the SD of the Gaussian fit, 265.2 Da, divided by 130.93 Da.

Preparation of Inactivated, Boronated RNase A

RNase A (38 mg, 2.8 μmol) was dissolved in 575.5 μL of 0.10 M sodium acetate buffer, pH 4.9. In a separate solution, 2-bromoacetic acid (123 mg, 883 μmol) was dissolved in 9.2 mL of 0.10 M sodium acetate buffer and the resulting solution was adjusted to pH 5.2. An aliquot (288 μL) of the 2-bromoacetic acid solution was added to the RNase A solution to generate a final concentration of 32 mM 2-bromoacetic acid and 3.2 mM RNase A. The reaction mixture was incubated at ambient temperature for 24 h on a nutating mixer, after which the reaction was dialyzed overnight against distilled, deionized H_2O. The inactivated RNase A was then loaded onto a column of Mono S HR 16/10 cation-exchange resin from Pharmacia. The resin was washed with a 40-mL linear gradient of NaCl (0.00–0.05 M) in 10 mM sodium phosphate buffer, pH 6.0, and eluted with a 603-mL linear gradient of NaCl (0.05–0.40 M) in 10 mM sodium phosphate buffer, pH 6.0. Fractions were collected, pooled, and dialyzed overnight at 4 °C against 50 mM sodium acetate buffer, pH 5. Inactivated RNase A was then loaded onto a 5-mL

column of HiTrap Heparin HP resin. The resin was washed with 10 mL of 50 mM sodium acetate buffer, pH 5.0, and eluted with a 200-mL linear gradient of NaCl (0.0–0.4 M) in 50 mM sodium acetate buffer, pH 5.0. Fractions were collected, and analyzed by MALDI–TOF mass spectrometry. Fractions with a molecular mass greater than that of wild-type RNase A were pooled and dialyzed extensively with distilled, deionized H_2O at 4 °C. 5-Amino-2-hydroxymethylphenylboronic acid was then conjugated as described above. Briefly, to 0.5 mL of chemically inactivated RNase A (6 mg, 400 nmol) was added 5-amino-2-hydroxymethylphenylboronic acid (10 mg, 50 μmol), and adjusted to pH 5. EDC was then added (19 mg, 100 μmol), and the resulting solution was adjusted to pH 5. The reaction mixture was incubated at ambient temperature for 20.5 h on a nutating mixer before adding additional EDC (11 mg, 56 μmol), and then incubated for an additional 3.5 h. Inactivated, boronated RNase A was dialyzed against distilled, deionized H_2O and purified on a 1-mL column of HiTrap Heparin HP resin as described for boronated RNase A, but scaled for the 1-mL column. The ribonucleolytic activity of inactivated, boronated RNase A (k_{cat}/K_M = 3.0 ± 0.2 × 10^5 $M^{-1}\cdot s^{-1}$) was 2% that of boronated RNase A (k_{cat}/K_M = 1.6 ± 0.2 × 10^7 $M^{-1}\cdot s^{-1}$).

Preparation of BODIPY FL-Labeled Ribonucleases

Both unmodified and boronated RNase A were labeled with BODIPY FL. An aliquot (3.83 mL) of a solution of ribonuclease (120 μM) was adjusted to pH 8.3. BODIPY FL STP ester (5 mg; 9 μmol) was dissolved in 0.5 mL of DMF. To the solution of ribonuclease was added 125 μL of the BODIPY FL STP ester solution. The reaction mixture was incubated at ambient temperature on a nutating mixer for 4–6 h, and then incubated at 4 °C on a nutating mixer overnight. Labeled ribonuclease was loaded onto a 1-mL column of HiTrap Heparin HP resin. The resin was washed with 30 mL of 10 mM sodium phosphate buffer, pH 6.0. The protein was eluted with a 60-mL linear gradient of NaCl (0.0–1.5 M) and pH (6.0–7.4) in 10 mM sodium phosphate buffer, pH 7.4. Fractions were collected, pooled, concentrated, and analyzed by SDS–PAGE and MALDI–TOF mass spectrometry.

Labeled ribonucleases were dissolved in at least a 10× volume of DPBS, passed through a 0.45-μm syringe filter from Whatman (Piscataway, NJ), and re-concentrated before being used in assays. In this manner, the proteins were dissolved in solution that was largely DPBS. Concentrations of proteins were determined by UV spectroscopy using the extinction coefficient of RNase A at 280 nm (ε = 0.72 $(mg\cdot mL^{-1})^{-1}\cdot cm^{-1}$)[7] but on a molar basis (ε = 9.9 $mM^{-1}\cdot cm^{-1}$). The absorbance of benzoxaborole was found to be negligible, contributing <6% to the $A_{280\ nm}$ of the boronated ribonuclease. The concentration of labeled ribonucleases was corrected for fluorophore absorbance by using the manufacturer's protocol (http://tools.invitrogen.com/content/sfs/manuals/mp00143.pdf). Percent labeling was determined by UV spectroscopy at 504 nm using the extinction coefficient of BODIPY FL (ε = 68,000 $M^{-1}\cdot cm^{-1}$) as per the manufacturer's protocol.

Heparin-Affinity Assays

The affinity of unmodified and boronated RNase A for heparin was assessed by retention on a 1.0-mL column of HiTrap Heparin HP resin (GE Healthcare, Piscataway, NJ). Unmodified and boronated RNase A were mixed in a 1:1 ratio (~146 nmol each) in DPBS, and the resulting solution was loaded onto the resin. The resin was washed with 5 mL of PBS, followed by elution

with 45 mL of a linear gradient of NaCl (0.0–1.0 M) in PBS. Elution was monitored by absorbance at 280 nm, and eluted proteins were identified by mass spectrometry. A small amount of unmodified RNase A was apparent in peak B (Figure 1). We hypothesize that boronated RNase A was able to complex to a small amount of unmodified RNase A and extend its elution time. The same assay was then repeated with 100 mM D-fructose in both buffers. To make D-fructose–supplemented buffers, fructose (18 g, 100 mmol) was added to 100 mL of a 10× stock solution of PBS, either no additional NaCl or NaCl (58.4 g, 1.00 mol) was added, and both buffers were diluted to a final volume of 1 L and adjusted to pH 7.4.

Fluorescence Polarization Assays

Liposomes were formed by transferring DOPC (dissolved in chloroform solution) and GD3 gangliosides (dissolved in 63:35:5 chloroform/methanol/water) to glass tubes and drying them under Ar(g) and then under vacuum. Lipids were re-suspended in 25 mM HEPES buffer, pH 7.0, containing NaCl (75 mM). The solution of lipids was mixed by vortexing for 2 min, and incubated at 37 °C for 1 h. For DOPC liposomes, DOPC was resuspended at a concentration of 5 mM. For GD3 ganglioside-labeled liposomes, DOPC and GD3 gangliosides were mixed at 3 and 2 mM concentrations, respectively. Large unilammelar vesicles were formed by extrusion through a 0.1-μm polycarbonate filter from Whatman (GE Healthcare, Piscataway, NJ). This process produces a population of vesicles of near uniform size (~100–150 nm diameter as measured by dynamic light scattering). A portion of the DOPC lipids before extrusion were aliquoted as a control.

Fluorescence polarization assays were performed using 50 nM BODIPY FL-labeled unmodified and boronated RNase A in black NBS 96-well plates (Corning Costar, Lowell, MA). Ribonucleases were incubated with DOPC liposomes (625 μM total lipid) or GD3 ganglioside-labeled liposomes (375 μM DOPC, 250 μM GD3 ganglioside = 625 μM total lipid) in 25 mM HEPES buffer, pH 7.0, containing NaCl (75 mM) in the absence or presence of D-fructose (10 mM). In control wells, ribonucleases were incubated with non-extruded DOPC lipids. Fluorescence polarization at 470/535 nm with a *G*-factor of 1.257 was recorded after shaking the plate briefly and incubating at ambient temperature for 1 h. Control well polarization was subtracted from experimental well polarization for each ribonuclease. The assay was performed in triplicate.

The affinity of boronated RNase A for GD3 ganglioside-labeled liposomes was assessed by using serially diluted liposomes. GD3 ganglioside-labeled liposomes were serially diluted in 25 mM HEPES, pH 7.0, containing NaCl (75 mM) with dilutions of 62.5 nM–1250 μM total lipid. Because the composition of these liposomes was 3:2 DOPC/GD3 ganglioside, this dilution resulted in solutions containing GD3 ganglioside at 25 nM–500 μM. Control DOPC liposomes (with no GD3 ganglioside) were likewise diluted in the same buffer, producing solutions of 62.5 nM–1250 μM total lipid. Liposomes were then incubated with 50 nM BODIPY FL-labeled boronated RNase A in the same buffer in a black NBS 96-well plate. Fluorescence polarization was recorded after shaking the plate briefly and incubating at ambient temperature for 35 min. Fluorescence polarization from GD3 ganglioside-labeled liposomes was subtracted from that of DOPC-only liposomes, thereby correcting for binding to DOPC and for changes in solution viscosity. The assay was performed in duplicate. The fraction of labeled ribonuclease bound for each sample well was calculated by dividing its polarization from the polarization of ribonucleases incubated with the highest concentration of GD3 ganglioside (set at 100% bound).

The value of K_d was calculated by plotting the fraction bound against the concentration of GD3 ganglioside and fitting the data to a binding isotherm.[8]

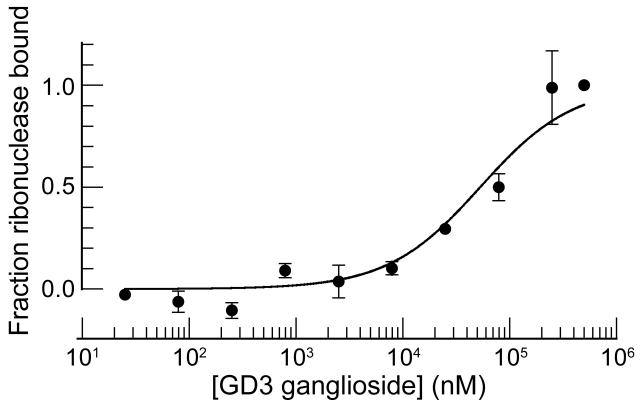


Figure S9. Fluorescence polarization data for the binding of boronated RNase A to GD3 ganglioside in liposomes. BODIPY FL-labeled boronated RNase A was incubated with liposomes containing GD3 ganglioside in 25 mM HEPES buffer, pH 7.0, containing NaCl (75 mM). Data points represent the mean (± SE) of duplicate experiments. Data were fitted to a binding isotherm[8] to give K_d = (53 ± 11) μM.

Cell Culture

Cell lines were obtained from American Type Culture Collection (Manassas, VA) and were maintained according to the recommended procedures. Cells were grown in a cell culture incubator at 37 °C under CO_2 (5% v/v) in flat-bottomed culture flasks. Cell medium was supplemented with GIBCO fetal bovine serum (FBS) (10% v/v), penicillin (100 units/mL), and streptomycin (100 μg/mL) in the appropriate cellular medium as follows: Pro-5, MEM α + ribonucleosides + deoxyribonucleosides; Lec-2, MEM α – ribonucleosides – deoxyribonucleosides; and K562, RPMI 1640. Cells were counted by hemocytometry for dispensing into 12-well plates (Corning Costar, Lowell, MA) or 8-well chambered coverglass slides (Nuc Lab-Tek II, Thermo Scientific).

Flow Cytometry Assays

BODIPY-FL was excited with a 488 nm solid-state laser and the emission was collected with a 530/30 bandpass filter. To collect the most reproducible data, for every flow cytometry experiment, the sensitivity (voltage) of the photomultiplier tube was set for all data collections using mid-range Rainbow beads from Spherotech (Lake Forest, IL) to a predetermined fluorescence target value. At least 10,000 cellular events were acquired for each sample. Data were analyzed using FlowJo 8.1.3 (Treestar, Ashland, Oregon).

The day prior to an experiment, Pro-5 and Lec-2 cells were plated in 12-well plates at 1×10^5 cells/well. The day of the experiment, the appropriate amount of D-fructose was dissolved into the cellular medium to obtain a final D-fructose concentration of 250 mM, and the medium was passed through a 0.45-μm syringe filter from Whatman. Non–fructose-containing medium was filtered likewise. Stock solutions of fluorescently labeled ribonucleases were diluted into the cell culture to a final concentration of 5 μM. Ribonucleases were incubated with cells for 4 h. Then, the cells were rinsed with PBS (2 × 400 μL), removed from the cell culture plate with trypsin

(400 μL, 0.05% (1×) with EDTA; Invitrogen, Carlsbad, California), placed in flow cytometry tubes containing 80 μL of FBS, and incubated on ice until analyzed by flow cytometry. Final fluorescence values were divided by the percent fluorophore labeling of the ribonuclease to determine the corrected value of fluorescence. Experiments were run twice in triplicate.

Confocal Microscopy

Pro-5 cells were plated on Nunc Lab-tek II 8-well chambered coverglass 24 h before use and grown to 80% confluency. Cells were incubated with 5 μM BODIPY FL-labeled ribonucleases for 4 h. Cell nuclei were stained with Hoechst 33342 (Invitrogen, 2 μg/mL) for the final 15 min of incubation. Cells were then washed twice with PBS, suspended in PBS, and examined using a Nikon Eclipse C1 laser scanning confocal microscope.

Ribonucleolytic Activity Assays

The ribonucleolytic activities of RNase A, boronated RNase A, and inactivated, boronated RNase A were determined by quantifying their ability to cleave 6-FAM–dArUdAdA–6-TAMRA, as described previously.[9] Assays were carried out at ambient temperature in 2 mL of 0.10 M MES–NaOH buffer, pH 6.0, containing NaCl (0.10 M). Fluorescence data were fitted to the equation: $k_{cat}/K_M = (\Delta I/\Delta t) / (I_f - I_0)[E]$, in which $\Delta I/\Delta t$ is the initial reaction velocity, I_0 is the fluorescence intensity before addition of ribonuclease, I_f is the fluorescence intensity after complete substrate hydrolysis, and [E] is the total ribonuclease concentration. The assay was performed in triplicate.

Cell-Proliferation Assays

The effect of unmodified and boronated RNase A on the proliferation of K-562 cells was assayed as described previously.[10] For assays, 5 μL of a solution of ribonuclease or PBS (control) was added to 95 μL of cells (5.0×10^4 cells/mL). For co-treatment assays with D-fructose, ribonucleases were first serially diluted at 2× concentration, followed by addition of an equal volume of 2 M D-fructose in PBS to each ribonuclease dilution, resulting in a 1× ribonuclease dilution as before but now containing 1 M D-fructose. Then, 5 μL of each dilution was added to cells as above, including a control of PBS containing 1 M D-fructose. Because 5 μL of samples were added to 95 μL of cells, the final concentration of D-fructose in each well was 50 mM. After a 44-h incubation, K-562 cells were treated with [*methyl*-^{3}H]thymidine for 4 h, and the incorporation of radioactive thymidine into cellular DNA was quantitated by liquid scintillation counting. The results are shown as the percentage of [*methyl*-^{3}H]thymidine incorporated relative to control cells treated with PBS. Data are the average of three measurements for each concentration, and the entire experiment was repeated in triplicate. Values for IC_{50} were calculated by fitting the curves by nonlinear regression to the equation: $y = 100\% / (1 + 10^{(\log(IC_{50}) - \log[\text{ribonuclease}])h})$, in which y is the total DNA synthesis following the [*methyl*-^{3}H]thymidine pulse and h is the slope of the curve.

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