Potentiation of ribonuclease cytotoxicity by a poly(amidoamine) dendrimer

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Materials. *Escherichia coli* strain BL21(DE3) cells were from Novagen (Madison, WI). K-562 cells, which were derived from a continuous human chronic myelogenous leukemia line, were from the American Type Culture Collection (Manassas, VA). Cell culture medium and supplements, as well as Dulbecco's phosphate-buffered saline (DPBS) were from Invitrogen (Carlsbad, CA). [*methyl*-^{3}H]Thymidine (6.7 Ci/mmol) was from Perkin–Elmer (Boston, MA). Protein purification columns were from GE Biosciences (Piscataway, NJ). Wild-type RNase A, PAMAM Generation 2 dendrimer, and 2-(*N*-morpholino)ethanesulfonic acid (MES) were from Sigma–Aldrich (St. Louis, MO). Dendrimers in methanol were placed under reduced pressure to remove methanol, then dissolved in DPBS; the pH of the resulting solution was adjusted to ~7.4. MES buffer was purified by anion-exchange chromatography to remove trace amounts of oligomeric vinylsulfonic acid.[1] 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). All other chemicals used were of commercial reagent grade or better, and were used without further purification. Terrific Broth (TB) was from Research Products International (Mt. Prospect, IL), or was made by dissolving Tryptone (12 g), yeast extract (24 g), glycerol (4 mL), KH_2PO_4 (2.31 g), and K_2HPO_4 (12.54 g) in H_2O (1.00 L).

Analytical Instruments and Statistical Calculations. The incorporation of [*methyl*-^{3}H]thymidine into the genomic DNA of K-562 cells was quantified by scintillation counting using a Microbeta TriLux liquid scintillation and luminescence counter (Perkin–Elmer, Wellesley, MA). Fluorescence measurements were made with an Infinite M1000 plate reader (Tecan, Switzerland). Thermal denaturation data were collected with a Cary 3 double-beam spectrophotometer equipped with a Cary temperature controller (Varian, Palo Alto, CA). Molecular mass was determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectrometry using a Voyager-DE-PRO Biospectrometry Workstation (Applied Biosystems, Foster City, CA) at the campus Biophysics Instrumentation Facility. Flow cytometry data were collected by using a FACSCalibur flow cytometer equipped with a 488-nm argon-ion laser (Becton Dickinson, Franklin Lakes, NJ). Calculations for statistical significance were performed using the Student's *t*-test, and a value of $p < 0.05$ was considered significant.

Protein Production. RNase A variants were produced as described previously,[2] but with the following exceptions. Phenylmethanesulfonyl fluoride (PMSF) was added during some preparations to minimize protein degradation. Ribonucleases were refolded for 24–72 h at room temperature or 4 °C. Following purification, proteins used in cytotoxicity assays were dialyzed against PBS. Protein concentration was determined by UV spectroscopy using an extinction coefficient at 278 nm of 0.72 $(mg/ml)^{-1} \cdot cm^{-1}$ for RNase A and its variants. Variants with a cysteine residue at position 19 were protected by reaction with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) to form a mixed disulfide.[3,4] Immediately prior to conjugation with a latent fluorophore or 2′,7′-diethylfluorescein (DEF),[3,5] protected ribonucleases were treated with a 4-fold molar excess of dithiothreitol (DTT) and desalted by chromatography on a PD-10 desalting column (GE Biosciences, Piscataway, NJ). The deprotected ribonucleases were reacted for 2–4 h at 25 °C with a 10-fold molar excess of a thiol-reactive fluorophore.[3,5] Conjugates were purified by chromatography using a HiTrap SP HP column. The concentration of labeled proteins was determined by using a bicinchoninic acid assay kit from Pierce (Rockford, IL) with wild-type RNase A as a standard. Human ribonuclease inhibitor (RI) was prepared in *E. coli* as described previously.[6] Following their purification, the purity and molecular mass of the ribonucleases and RI were verified with SDS–PAGE.

Conformational Stability Assay. The conformational stability of wild-type RNase A was determined by following the change in UV absorbance at 287 nm with increasing temperature, as described previously.[11] Briefly, the temperature of PBS containing RNase A (25 μM) with 1 or 10 μM dendrimer, or without dendrimer (control), was heated from 25–80 °C at 0.15 °C/min. The A_{287} was followed at 1-°C intervals, and the absorbance change was fitted to a two-state model of denaturation, wherein the temperature of the midpoint of the transition curve corresponds to the value of T_m. Data are the average of three experiments.

Ribonucleolytic Activity Assay. The ribonucleolytic activity of wild-type RNase A was determined by quantitating its ability to cleave 6-FAM–dArUdAdA–6-TAMRA, as described previously.[12] Briefly, assays were carried out at ambient temperature in 2.00 mL of 0.10 M MES–NaOH buffer (pH 6.0) containing NaCl (0.10 M). Fluorescence data were fitted to eq 1, 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. Activity was measured without dendrimer (control) or in the presence of 1 or 10 μM dendrimer. Data are the average of three experiments.

$$k_{cat}/K_M = \frac{\Delta I/\Delta t}{(I_f - I_0)[E]} \quad (1)$$

RI-Binding Assay. Affinities for RI were determined by using a competition assay described previously.[5] Briefly, a serial dilution (2×) of the dendrimer in DPBS was prepared in Eppendorf Protein LoBind Tubes (Fisher Scientific). A stock solution of A19C–DEF G88R RNase A (20 nM; 2×), RI (24 nM; 2×), DTT (1 mM; 2×), and BSA (0.1 mg/mL; 2×) was made, and 50-μL aliquots of this stock solution were added to the wells of a 96-well NBS plate. Aliquots of the serially diluted solution of dendrimer were then added to the wells. The negative control contained no ribonuclease (PBS), and the positive control contained excess RNase A (0.2 μM; 2×). The plate was incubated for 30 min at ambient temperature, after which the fluorescence intensity was measured. The observed fluorescence intensity (I) is described by eq 2:

$$I = f_F I_F + f_B I_B \quad (2)$$

where f_F and f_B are the fractions of the free and RI-bound form of the fluorescence conjugate, respectively, and I_F and I_B are the fluorescent intensities of the free and RI-bound states, respectively. The value of f_B was determined by linear regression analysis using the intensities of the positive and negative controls, which represent 0 and 75.9% bound, respectively, based on a K_d value of 1.4 nM for the fluorophore-labeled G88R variant of RNase A.[2] The fraction bound (f_B) was then calculated by using eq 3:

$$f_B = \frac{I - I_F}{I_B - I_F} \quad (3)$$

The value of K_d was calculated by plotting f_B against the concentration of competing dendrimer and fitting the data to the mathematical expression for complete competitive binding of two different ligands.[8,9] Data are the average of two measurements, and the entire experiment was repeated in triplicate.

Cell-Proliferation Assay. The effect of RNase A, its variants, dendrimer, and co-treatment of dendrimer with ribonucleases on the proliferation of K-562 cells was assayed as described previously.[7] Briefly, for assays with a single sample, 5 μL of a serially diluted solution of ribonuclease, dendrimer, or PBS was added to 95 μL of DPBS containing K-562 cells (5.0×10^4 cells/mL). For co-treatments, 10 μL of a serially diluted solution of ribonuclease (2×) was premixed with 10 μL of a solution of dendrimer (2×). Then, 5 μL of this solution was added to 95 μL of DPBS containing K-562 cells (5.0×10^4 cells/mL). After 44 h, the cells were treated with [*methyl*-^{3}H]-thymidine for 4 h, and the incorporation of radioactive thymidine into cellular DNA was quantified by liquid scintillation counting. Results were reported as the percentage of [*methyl*-^{3}H]thymidine incorporated relative to control cells that had been treated with PBS. Data were 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 with eq 4, in which y is the total DNA synthesis following the [*methyl*-^{3}H]thymidine pulse, and h is the slope of the curve.

$$y = \frac{100\%}{1+10^{(\log(IC_{50}) - \log[\text{ribonuclease}])h}} \quad (4)$$

Flow Cytometry Assay. The internalization of ribonuclease variants with latent fluorophore attached was followed by monitoring the unmasking of fluorescence by intracellular esterases, as described previously.[10] Briefly, K-562 cells were collected by centrifugation, washed with PBS, and resuspended at a density of 1.25×10^6 cells/mL in fresh medium. Labeled ribonuclease (to 10 μM) and dendrimer (to 0, 1, or 10 μM) was added to 200 μL of medium containing K-562 cells (1.25×10^6 cells/mL). Cells were allowed to incubate at 37 °C for 3 h. To quench internalization, cells were placed on ice, collected by centrifugation at 1000 rpm for 5 min at 4 °C, washed with 750 μL of PBS, and collected again by centrifugation at 1000 rpm for 5 min at 4 °C. The cell pellets were suspended gently in ice-cold PBS (250 μL). Samples remained on ice until analyzed by flow cytometry.

Fluorescence was detected through a 530/30-nm band-pass filter. Cell viability was determined by staining with propidium iodide, which was detected through a 660-nm long-pass filter. The mean channel fluorescence intensity of 20,000 viable cells was determined for each sample using CellQuest software and used for subsequent analysis. Data are the average of three measurements for each concentration, and the entire experiment was repeated in triplicate.

References and notes

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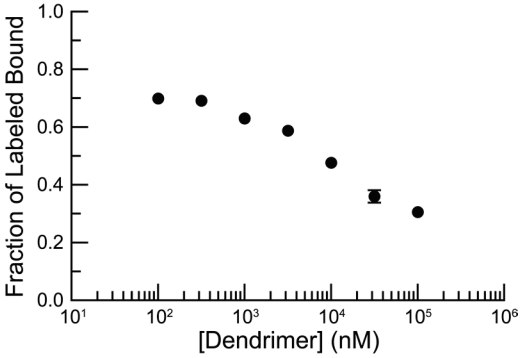

Figure S1. Binding of the ribonuclease inhibitor protein to the generation 2 poly(amidoamine) dendrimer. The value of K_d = (3.1 ± 0.5) μM for the RI·dendrimer complex was determined by disrupting the interaction of RI with DEF-labeled G88R RNase A in PBS. Data are the mean (± SE) from three separate experiments.