Materials. mPEG–maleimide\(^1\) (2 kDa; \(1\); Figure 2) was from SunBio (Anyang City, South Korea). mPEG–maleimide (20 kDa; \(2\); Figure 2), mPEG\(_2\)–maleimide (20 kDa; \(3\); Figure 2), and mPEG\(_2\)–\(N\)-hydroxysuccinimide (20 kDa; \(4\); Scheme S1) were from Nektar Therapeutics (Huntsville, AL).

Ribonuclease substrates 6-FAM–dArUdAdA–6-TAMRA and 6-FAM–dArUdGdA–6-TAMRA were from Integrated DNA Technologies (Coralville, IA); poly(C) was from Midland Certified Reagents (Midland, TX). \([\text{methyl}^3\text{H}]\)Thymidine (6.7 Ci/mmol) was from PerkinElmer (Boston, MA).

*Escherichia coli* BL21(DE3) cells, pET22b(+) and pET27b(+) plasmids were from Novagen (Madison, WI). K-562 (derived from a continuous human chronic myelogenous leukemia line) and DU 145 (human prostate carcinoma) cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cell culture medium and supplements (including DPBS) were from Invitrogen (Carlsbad, CA).

Homozygous \((nu/nu)\) nude mice (male) were from Harlan (Indianapolis, IN).

FPLC HiLoad 26/60 Superdex G75 and G200 gel-filtration columns, HiTrap desalting, SP HP, and Q columns (5 mL) were from GE Healthcare (Uppsala, Sweden). A BCA protein assay kit was from Pierce (Rockford, IL). Gel-filtration standards, SDS–PAGE molecular-mass standards, and pre-cast gels for poly(acrylamide) electrophoresis were from BioRad (Hercules, CA). Flat-bottom, black polystyrene, Costar assay plates (96-well) with non-binding surface were from Corning Life Sciences (Acton, MA). Acetylated BSA for pharmacokinetic assays was from Sigma Chemical (St. Louis, MO). Black non-treated 96-well plates for pharmacokinetic assays were from NUNC (Rochester, NY).

All other chemicals used were of commercial reagent grade or better, and were used without further purification.

Analytical instruments. \([\text{methyl}^3\text{H}]\)Thymidine incorporation into K-562 and DU 145 genomic DNA was quantitated by scintillation counting using a Microbeta TriLux liquid scintillation and luminescence counter (PerkinElmer, Wellesley, MA). Molecular mass was determined by MALDI–TOF mass spectrometry using a Voyager-DE-PRO Biospectrometry Workstation (Applied Biosystems, Foster City, CA) in the campus Biophysics Instrumentation Facility. Cuvette-scale fluorescence measurements were made with a QuantaMaster1 photon-counting fluorometer equipped with sample stirring (Photon Technology International, South Brunswick, NJ). Fluorescence-based competition assays performed in 96-well plate format were read with a Perkin–Elmer EnVison 2100 Plate Reader in the campus Keck Center for Chemical Genomics. Thermal denaturation data were acquired with a Cary 400 Bio double-beam spectrophotometer equipped with a Cary temperature controller (Varian, Palo Alto, CA) in the campus Biophysics Instrumentation Facility.

Production of ribonucleases and ribonuclease inhibitor. cDNA encoding RNase A variants was created by oligonucleotide-mediated site-directed mutagenesis of a pET22b(+) or

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\(^1\)Abbreviations: DMF, dimethyl formamide; DTT, dithiothreitol; 6-FAM, 6-carboxyfluorescein; MES, 2-(\(N\)-morpholino)ethanesulfonic acid; MALDI–TOF, matrix-assisted laser desorption ionization–time-of-flight; mPEG, monomethoxypoly(ethylene glycol); PBS, phosphate-buffered saline; poly(C), poly(cytidylic acid); RI, ribonuclease inhibitor; RNase A, bovine pancreatic ribonuclease; PAGE, poly(acrylamide) gel electrophoresis; SDS, sodium dodecyl sulfate; 6-TAMRA, 6-carboxytetramethylrhodamine; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet.
pET27b(+) plasmid that contained cDNA encoding wild-type RNase A or D38R/R39D/N67R/G88R variants, respectively.\textsuperscript{1,2} Ranpirnase, wild-type RNase A, G88R and D38R/R39D/N67R/G88R RNase A variants were produced as described previously.\textsuperscript{3,1,2} Free cysteine-containing variants of RNase A (A19C, G88C, and D38R/R39D/N67R/G88C) were prepared in a similar manner, except that the solution containing dissolved inclusion bodies was diluted by 10-fold with a degassed acetic acid solution (20 mM), centrifuged to remove precipitant, and dialyzed overnight against 20 mM acetic acid that had been purged with N\textsubscript{2}(g) or Ar(g). Ribonucleases were refolded for \textgeq 3 days at 4 °C following slow dilution into 0.10 M Tris–HCl buffer (pH 8.0) containing NaCl (0.10 M), L-arginine (0.5 M), EDTA (10 mM), reduced glutathione (1.0 mM), and oxidized glutathione (0.2 mM). The refolding solution was purged with N\textsubscript{2}(g) or Ar(g) prior to the addition of denatured protein to prevent oxidation of the nascent cysteine residue. Following purification by gel-filtration chromatography, the thiol groups of the engineered, unpaired cysteine residues introduced at positions 19 or 88 were protected from inadvertent air oxidation by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) as described previously.\textsuperscript{4} Finally, the 5-thio(2-nitrobenzoic acid)-protected ribonuclease was applied to a HiTrap SP cation-exchange column, eluted with a linear gradient of NaCl (0.15–0.40 M) in 50 mM NaOAc buffer (pH 5.0), and stored at 4 °C until subsequent modification with maleimide-derivatized monomethoxypoly(ethylene glycol) (mPEG or mPEG\textsubscript{2}). Protein concentration (excluding 5-thio(2-nitrobenzoic acid)-protected ribonuclease) was determined either by the bicinchoninic acid method\textsuperscript{5} or UV spectroscopy using \(\varepsilon_{278} = 0.72\) (mg/mL)\textsuperscript{−1}·cm\textsuperscript{−1} for RNase A and its variants\textsuperscript{6} and \(\varepsilon_{280} = 0.87\) (mg/mL)\textsuperscript{−1}·cm\textsuperscript{−1} for ranpirnase.\textsuperscript{1}

Human RI was prepared as described previously,\textsuperscript{7,2} and its integrity was assessed by its ability to titrate the ribonucleolytic activity of wild-type RNase A.

Following purification, ribonucleases and ribonuclease inhibitor proteins migrated as single bands during SDS–PAGE, confirming their purity and apparent molecular mass. In addition, the identity of purified ribonuclease variants was confirmed by MADLI–TOF mass spectrometry (Table S1).

**Thiol-specific PEGylation of RNase A variants.** The pH of the protein solution containing 5-thio(2-nitrobenzoic acid)-protected ribonucleases in HiTrap SP elution buffer was adjusted from 5 to 7.4–8.0 by addition of either 10% (v/v) 10× PBS and/or 1.0 M Tris–HCl buffer (pH 8.0). The protecting group was removed by adding DTT (5-fold molar excess) and allowing the reaction to proceed at for \textgeq 5 min, resulting in the immediate generation of the yellow 5-thio(2-nitrobenzoic acid).\textsuperscript{8} DTT and salt were removed from the ribonucleases using a HiTrap desalting column that had been equilibrated with 0.10 M sodium phosphate buffer (pH 6.0) containing EDTA (2 mM). A 10-fold molar excess of maleimide-derivatized mPEG (1 and 2) or mPEG\textsubscript{2} (3) was dissolved in a small volume of 0.10 M sodium phosphate buffer (pH 6.0) containing EDTA (2 mM) and added to the solution containing a deprotected ribonuclease (50–250 µM). PEGylation reactions were protected from light and allowed to proceed for 2 h at room temperature, or overnight at 4 °C. Reactions were terminated by ~6-fold dilution with 50 mM NaOAc buffer (pH 5.0) and application to a column of HiTrap SP HP cation-exchange resin that had been equilibrated with the same buffer. PEGylated and unmodified RNase A variants were eluted differentially from the resin with a linear gradient of NaCl (0–0.4 M) in 50 mM NaOAc buffer (pH 5.0) (Figure S1). For 2-kDa mPEG-modified A19C RNase A, the smaller PEG moiety did not sufficiently reduce the interaction with the ion exchange resin to achieve baseline separation from unmodified ribonuclease. Hence, this conjugate was instead purified by gel-filtration HiLoad 26/60 G75 Superdex gel-filtration column. All PEGylated variants of RNase A
were assayed by SDS–PAGE and found to be ≥98% pure, containing only trace amounts of unmodified ribonuclease and no free PEG (Figure S2). Proteins were concentrated and extensively dialyzed against 1× PBS. Protein concentrations of PEGylated RNase A variants were determined in the same manner as described above for the unmodified ribonucleases. The presence of the PEG moieties was found not to interfere with either method of quantitation.

A second batch of 20-kDa mPEG2−G88C RNase A was prepared in a manner (Scheme S1) slightly different than that above. Briefly, ~66 mg 20-kDa mPEG2−N-hydroxysuccinimide 4 was dissolved in 2 mL of 0.20 M NaHCO3 buffer (pH 8.1) containing NaCl (0.10 M) and reacted with a 20-fold molar excess of the trifluoroacetic acid salt of N-(2-aminoethyl)maleimide (5, which was synthesized as described previously9) for 30 min, protected from light. Maleimide-derivatized mPEG2 6 was then separated from unreacted compound 5 by applying the crude reaction mixture to a column of HiTrap desalting resin that had been equilibrated with DPBS (Invitrogen), and collecting the early, salt-free, PEG-containing fractions. This 20-kDa mPEG2−maleimide was reacted with G88C RNase A in the same manner as described above in lieu of maleimide 3. 20-kDa mPEG2−G88C RNase A prepared in this manner behaved in an indistinguishable manner to the conjugate prepared with commercial 20-kDa mPEG2−maleimide 3 during SDS–PAGE and assays of enzymatic activity.

**Scheme S1. Route for the synthesis of thiol-reactive branched mPEG2 6**

[Chemical diagram]

**Thiol-specificity of maleimide-mediated PEGylation.** Near pH 7, maleimides are ≥103-fold more reactive toward thiols than amines.10 To confirm that we were achieving this functional group specificity and to demonstrate that the variants of RNase A were not being modified at any other residues, 10 mg of wild type RNase A (which lacks thiol groups) was subjected to sham PEGylation with a 10-fold excess of 20-kDa mPEG-maleimide. The sham procedure reproduced exactly the conditions used to prepare the PEGylated conjugates (vide supra), including exposure to 5-fold molar excess of DTT and desalting in 0.10 M sodium phosphate buffer (pH 6.0) containing EDTA (2 mM). All detectable proteinaceous material from the sham PEGylation reaction eluted from a column of HiTrap cation-exchange resin as a single peak at a salt concentration (0.32 M) that is consistent with its being unmodified RNase A. This assignment was confirmed by both MALDI–TOF mass spectrometry (m/z 13,683; expected: 13,682) and SDS–PAGE in which its retention was identical to that of unmodified RNase A. Hence, we concluded that mPEG-maleimide modifies RNase A only on the additional cysteine residue.

**Analysis and characterization of PEGylated RNase A variants.** **Analytical size-exclusion chromatography.** Protein (1.0 mg/mL) in gel-filtration buffer was applied to a column of HiLoad 26/60 Superdex G200 gel-filtration resin and eluted with 50 mM NaOAc buffer (pH 5.0) containing NaCl (0.10 M) and NaN3 (0.05% w/v) at a flow rate of 4 mL min⁻¹. Gel-filtration standards were analyzed with the same column according to the guidelines of the manufacturer.
Measurements of Conformational Stability. Protein was dialyzed exhaustively against 1×PBS and diluted to a concentration of ~25 µM in PBS. Assays were performed by incremental heating (0.15 °C/min from 25–80 °C) and measurement of the absorbance at 287 nm which decreases as RNase A is denatured and its six tyrosine residues become more solvent exposed.11,12 Data were collected and analyzed by fitting the data to a two-state process using the program THERMAL from Varian Analytical Instruments (Walnut Creek, CA) and determining the value of $T_m$—the temperature at the midpoint of the transition between the folded and unfolded states.13

References

Table S1. Molecular mass of RNase A, its PEGylated variants, and ranpirnase

<table>
<thead>
<tr>
<th>Ribonuclease</th>
<th>Mass (m/z)</th>
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<tr>
<td></td>
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<td>Wild-type RNase A</td>
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</tr>
<tr>
<td>G88R RNase A</td>
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*Values of m/z were determined by MALDI–TOF mass spectrometry.*
Figure S1. Chromatograms for the purification of PEGylated variants of RNase A by cation-exchange chromatography using a HiTrap SP HP resin. Unmodified monomeric and dimeric protein eluted near 0.29 and 0.37 M NaCl, respectively; PEG conjugates eluted at lower concentrations of salt. Chromatograms from multiple purifications are superimposed to simplify comparisons. 20-kDa mPEG–G88C RNase A (red); 20-kDa mPEG–A19C RNase A, (blue); 20-kDa mPEG–A19C RNase A (green); and 2-kDa mPEG–G88C RNase A (black).

Figure S2. SDS–PAGE analysis of purified G88R RNase A and PEGylated variants of RNase A (1 μg each). Lane 1 and 6: Pre-stained molecular mass standards (myosin, β-galactosidase, bovine serum albumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme, and aprotinin). Lane 2: G88R RNase A. Lanes 3–5: G88C RNase A modified with 2-kDa mPEG maleimide 1, 20-kDa mPEG maleimide 2, and 20-kDa mPEG2 maleimide 3, respectively. Lanes 7–9: A19C RNase A modified with the same PEGs. Lane 10: D38R/R39D/N67R/G88C RNase A modified with 20-kDa mPEG. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250, destained, and then stained for PEG using barium iodide.14,15
Figure S3. Chromatograms of purified wild-type RNase A and PEGylated variants after gel-filtration chromatography. Chromatograms from multiple purifications are superimposed to simplify comparisons. Calibration curve (inset) was generated from gel filtration standards which (bovine thyroglobulin, 670 kDa; bovine γ-globulin, 158 kDa; chicken ovalbumin, 44 kDa; horse myoglobin, 17 kDa; vitamin B-12, 1.35 kDa).

Figure S4. Thermal denaturation curves for unmodified G88R RNase A and seven PEGylated variants of RNase A. 2-kDa mPEG maleimide 1 (⋯); 20-kDa mPEG maleimide 2 (—); and 20-kDa mPEG₂ maleimide 3 (—). Data were fitted to a two-state model.