(54) CYTOTOXIC RIBONUCLEASE VARIANTS

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(57) ABSTRACT

This invention relates to cytotoxic variants of human ribonuclease 1 (RNase 1) identified through analysis of the interaction between RNase 1 and the human ribonuclease inhibitor (hRN) as defined by the three dimensional (3-D) atomic structure of the RNase1 hRN complex. Also disclosed is the 3-D structure of the hhRNase 1 complex and methods for designing the RNase 1 variants.

20 Claims, 6 Drawing Sheets
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FIG. 1a

(a)

RNase A (Bos taurus)  *
RNase 1 (human pancreas) Chain X  ■
RNase 1 (human pancreas) Chain Z  ▲

hydrogen bond(s) between RI and ribonuclease (r ≤ 3.30 Å)
van der Waals / hydrophobic contact(s) between RI and ribonuclease (r ≤ 3.90 Å)
cysteine
active-site residue

SEQ ID NO: 1
SEQ ID NO: 2
SEQ ID NO: 3
FIG. 3
CYTOTOXIC RIBONUCLEASE VARIANTS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. Nonprovisional application Ser. No. 11/454,418 filed Jun. 16, 2006 now U.S. Pat. No. 7,655,757, which claims priority to U.S. Provisional Application No. 60/091,311 filed Jun. 16, 2005. Each of these applications is incorporated by reference here in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

This invention was made with United States government support awarded by the following agency: NIH CA073808 and GM 064598. The United States government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Ribonucleases are enzymes that catalyze the degradation of RNA. A well studied ribonuclease is bovine pancreatic ribonuclease A (RNase A), the putative biological function of which is to break down the large amount of RNA that accumulates in the ruminal gut. The RNase A superfamily is a group of RNAse enzymes classified as similar to RNase A which possess a number of interesting biological properties including antiproliferative, cytotoxic, embryotoxic, spermatogenic, and antimetastatic activities. One member of this family is a homolog of RNase A, originally isolated from oocytes and early embryos of the Northern leopard frog Rana pipiens.

The frog (Rana pipiens) ribonuclease, when placed in a human cell, is not strongly-inhibited by RI and its RNAse activity destroys cellular RNA and kills the target cell. The anti-tumor properties, both in vitro and in vivo, of the frog ribonuclease are described and claimed in U.S. Pat. No. 5,559,212. This ribonuclease molecule is now known as Onconase® (ONC). The property of degrading RNA is essential to the cytotoxicity of ONC, ONC is currently being evaluated as a cancer therapeutic in clinical trials.

A significant limitation on the suitability of ONC as a chemotherapeutic is dose-limiting renal toxicity. ONC is retained in the kidney at concentrations much greater than mammalian members of the RNase superfamily. There may also be antigenic issues with ONC, since mice produce antibodies against ONC but not against RNase A, with which ONC shares about 30% of its amino acids. This suggests that other members of the RNase family may also be suitable candidates for evaluation as clinical therapeutics if they can be imbued with the cytotoxic properties similar to ONC.

In mammals, levels of RNase activity are controlled by a ribonuclease inhibitor (RI), which is a 50-kDa protein found in the cytosol of all mammalian cells. RI is a member of a leucine rich family of proteins and is composed of 15 alternating repeats arranged symmetrically in a horseshoe shaped molecule. RI has a large number of cysteine residues (32 in human RI) which means that it can only keep its shape and function in a reducing environment like the cytosol. RI acts to bind to members of the RNase superfamily, one RI to one molecule of RNase, and when so bound, RI completely inhibits the catalytic activity of the ribonuclease by sterically blocking the active site of the enzyme. The binding of RI to RNase is a very tight one, having a very high binding affinity.

Some RNase superfamily members, notably ONC and bovine seminal ribonuclease, possess the native ability to evade RI. The trait of evasion of RI is primarily responsible for the cytotoxicity of ONC and bovine seminal ribonuclease. It has also been found that RNase superfamily members, which are not natively cytotoxic, can be made cytotoxic by modifying their amino acid constituents, so as to inhibit binding to RI.

Using the three dimensional structure of the porcine RI (pRI)-RNase A complex, RI was engineered to be more toxic to human leukemic cells in vitro than ONC. Disruption of the RI-RNase A interface was accomplished by designing RNAase A variants with amino acid substitutions that disrupted complementarity regions at the pRI-RNase A interface. These amino acid substitutions targeted short range pRI-RNase A interactions by incorporating sterically disruptive amino acids or removing hydrogen bonds. This method is described in U.S. Pat. No. 5,840,296, incorporated by reference herein in its entirety. Analogous complementarity regions were applied to bovine seminal ribonuclease (BS-RNase, 87% sequence similarity) to produce a homologue of RNase A. However, a BS-RNase variant with mutations at the same complementarity regions was less cytotoxic than ONC or the most cytotoxic RNase A variant (D383/R393/D397/G388 RNase A). This strategy did not result in the level of cytotoxicity predicted for BS-RNase.

Furthermore, most of the work done so far in the creation of RNase A variants has been done with bovine RNase A. However, the sequence and structure of bovine RNase A (SEQ ID NO:1, GenBank Accession No. AA727577) differs from human pancreatic ribonuclease 1 (RNase 1) (SEQ ID NO: 2, GenBank Accession No. CAG29314, incorporated by reference herein in its entirety). RNase A and its homolog, RNase 1 share about 70% sequence identity of their amino acid sequences. While the bovine protein may prove out to be acceptable for use in human therapy, a conservative approach might be to utilize a variant of a human ribonuclease, on the theory that use of a human protein might minimize cross-species antigenic problems. Accordingly, it is desirable to design variants of human ribonucleases that may be more cytotoxic and effective for therapeutic, diagnostic or research use.

BRIEF SUMMARY OF THE INVENTION

The present invention is summarized as variants of human ribonuclease 1 (RNase 1) identified through analysis of the interaction between RNase 1 and the human ribonuclease inhibitor (hRI), as defined by the three dimensional (3-D) atomic structure of the hRI-RNase 1 complex.

In one aspect, the present invention defines an RNase 1 that has improved cytotoxic properties compared to all previously disclosed engineered ribonucleases.

In another aspect, the invention provides a variant RNase 1 having a modified amino acid sequence, wherein the variant RNase 1 retains its ribonuclease activity, and wherein the variant RNase 1 has a lower binding affinity for RI than that of the native RNase 1 and retains native ribonuclease activity.

In this aspect, the human RNase 1 variant includes at least two amino acid changes from its native sequence, the changes being chosen from a group consisting of hRI and RNase 1 through electrostatic repulsion, the first change being an amino acid substitution in the region of amino acid residues 85 to 94 of RNase 1, and the second change being an alteration, substitution or amino acid swap at a position selected from the group consisting of amino acid residues 4, 7, 11, 31, 32, 38, 39, 41, 42, 66, 67, 71, 111 and 118 of RNase 1, wherein the variant RNase 1 exhibits enhanced cytotoxic activity relative to the native RNase 1.
In a related aspect, the human RNase 1 variant includes at least two amino acid changes from its native sequence, the changes causing evasion of human hRI by RNase 1 through electrostatic repulsion, the first change being an amino acid substitution at amino acid residue 88 or 91 of RNase 1, and the second change being an alteration, substitution or amino acid swap at a location selected from the group consisting of amino acid residues 4, 7, 11, 31, 32, 38, 39, 41, 42, 66, 67, 71, 111 and 118 of RNase 1, wherein the variant RNase 1 exhibits enhanced cytotoxicity activity relative to the native RNase 1.

The present invention further provides variants of RNase 1 with amino acids modified from the native sequence. Exemplary variants are provided in Table 5 herein below. Additional variants that have the desired function are also within the scope of the invention.

In a preferred aspect, the RNase 1 variant is defined by R39D/N67D/N88A/G89D/R91D and has at least 10-fold lower affinity and 2700-fold lower association rate for hRI than wild-type (native) RNase 1.

In another aspect, the present invention provides a method for modifying the amino acid sequence of a native RNase 1 to produce a novel, cytotoxic RNase 1.

The present invention is a method for modifying the amino acid sequence of RNase 1 to produce a variant RNase 1, which retains its ribonucleolytic activity, and wherein the variant RNase 1 has a binding affinity for hRI that is lower than that of the native RNase 1 and retains native ribonucleolytic activity.

The present invention is also a method for inhibiting the proliferation of cancer cells, comprising delivering to the cells an effective amount of a modified RNase 1, wherein the variant RNase 1 has a binding affinity for hRI that is lower than that of the native RNase 1 and retains native ribonucleolytic activity.

In another aspect, the invention provides a method of engineering cytotoxic RNase 1 variants by identifying electrostatic anchor residues in the three-dimensional structure of the hRIP/RNase 1 complex and modifying the anchor residues identified in RNase 1 to inhibit binding to hRI through electrostatic repulsion, wherein the variants retain native ribonucleolytic activity, have a lower binding affinity for hRI than that of the native RNase 1, and exhibit enhanced cytotoxicity activity relative to the native RNase 1.

In another aspect, the invention provides a method of engineering RNase 1 variants that retain native ribonucleolytic activity, have a lower binding affinity for hRI than that of the native RNase 1, and exhibit enhanced cytotoxicity activity relative to the native RNase 1.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although suitable methods and materials for the practice or testing of the present invention are described below, other methods and materials similar or equivalent to those described herein, which are well known in the art, can also be used.

Other objects, advantages and features of the present invention will become apparent from the following specification taken in conjunction with the accompanying drawings.

**BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS**

FIGS. 1A-B show R1 contact residues of RNase 1 and RNase A. (A) Amino acid sequence alignment of RNase A and RNase 1. (B) Three-dimensional structure of RNase 1 chain Z from PDB identification No. 1Z7X.

FIGS. 2A-B show a color-coded comparison of the β4-β5 loop in RNase 1 and RNase A when bound to RI.

FIGS. 3A-C show electron density at 1σ of key shape complementarity residues between hRI and RNase 1.

FIGS. 4A-B show hRI affinity and cytotoxicity of RNase 1 and its variants. Legend for FIG. 4A is as follows: R39D/N67D/N88A/G89D/R91D (Ⅰ); R39D/N88A/G89D/R91D (Ⅱ); R39D/N67D/N88A/R91D (Ⅲ); R39D/N67D/G89D/R91D (Ⅳ); R39D/N67D/R88A/G89D/R91D (Ⅴ). Legend for FIG. 4B is as follows: D38R/R39D/N67R/G89R RNase A (A); R88R RNase A (Ⅰ); R39D/N67D/N88A/G89D/R91D RNase 1 (A); R39L/N67L/R88A/G89L/R91L RNase 1 (Ⅰ); R67D/N88A/G89D/R91D RNase 1 (Ⅱ); G39R/R39G/N67R/N88R RNase 1 (Ⅲ); and wild-type RNase 1 (Ⅳ).

**DETAILED DESCRIPTION OF THE INVENTION**

The present invention relates to novel human ribonucleases 1 variants engineered to exhibit an increased level of cytotoxic activity relative to the native RNase 1. This was made possible for the first time through the determination of the three-dimensional (3-D) atomic crystal structure of the human ribonuclease inhibitor (hRIP, SEQ ID NO: 4) molecule bound to the human ribonuclease 1 (RNase 1) molecule. The structure of the hRIP/RNase 1 complex has a 1.95 Å resolution and the atomic coordinates were deposited in the publicly available sequence database, Protein Data Bank (PDB), accession No. 1Z7X.

Using the 3-D structure of the hRIP/RNase 1 complex, the interaction between hRI and RNase 1 in complex was characterized and used to determine the energetic contribution of specific RNase 1 residues to RI binding. The interaction between long range electrostatics and the rate of association was analyzed to identify electrostatic contributions of anchor residues in the hRIP/RNase 1 complex. These residues were rationally modified to (i) evade hRI by inhibiting the binding of the anchor residues through electrostatic repulsion and (ii) increase cytotoxic activity relative to the native RNase 1. Using the logic described here, it is believed that we were able to overcome a major obstacle to the development of chemotherapeutics based on human ribonucleases.

In a broad embodiment, the invention provides an engineered ribonuclease variant of RNase 1 having at least two amino acid changes from its native sequence, the changes causing evasion of hRI by RNase 1 through electrostatic repulsion, the first change being an amino acid substitution in the region of amino acid residues 85 to 94 of RNase 1, and the second change being an alteration, substitution or amino acid swap at a location selected from the group consisting of amino acid residues 4, 7, 11, 31, 32, 38, 39, 41, 42, 66, 67, 71, 111 and 118 of RNase 1, the variant RNase 1 having cytotoxic activity relative to the native RNase 1. Such variants are designated herein by the notation XNNY, where Y is the substituted amino acid residue for the residue X normally found at location NN (e.g., R4C).

As used herein, the terms, “native”, “wild-type”, “unmodified” are synonymous with each other. They refer to a gene product that has the characteristics of that gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designed the “normal” or “wild-type” form of the gene.
In contrast, the terms “variant,” “modified”, or “mutant” refer to a gene product that displays modifications in sequence and/or functional properties (i.e., altered characteristics) when compared to the wild-type gene product. The invention provides for variants of RNase 1. Exemplary variants are described in Table 5.

In one embodiment, the invention provides a RNase 1 variant having an amino acid change at residues 38, 39, 67, 88, 89, 91 and an 118 causing evasion of human ribonuclease inhibitor (hRI) by RNase 1 through electrostatic repulsion relative to the native sequence. SEQ ID NO:2, wherein the variant RNase 1 retains its native ribonuclease activity, has a lower binding affinity for RI than that of the native ribonuclease and exhibits enhanced cytotoxic activity relative to the native RNase 1.

Based on that embodiment, we synthesized R4C/G38R/R39G/N67R/N88R/G89R/R91G/V118C RNase 1, which is an instructive variant of human pancreatic ribonuclease. This enzyme has changes to eight residues, inspired by the atomic structure of the hRIPRNase 1 complex as presented here. Residues Gly38, Arg39, Asn67, Asx88, Gly89, and Arg91 are all near the interface of the hRIPRNase 1 complex. The replacement of these residues in the variant is designed to interfere with the interface. Arg4 and Val118 are also near the hRIPRNase 1 interface. Their replacement with cysteine residues is designed to interfere with this interface as well as allowing for the formation of a new disulfide bond between Cys4 and Cys118, which is likely to confer extra conformational stability to the enzyme. This RNase 1 variant (1) retains nearly all of the enzymatic activity of the native RNase 1, (2) evades hRI, and (3) exhibits significant cytotoxic activity.

In a preferred embodiment, the invention provides RNase 1 variants having an amino acid change at residues 39, 67, 88, 89, and 91 relative to the native sequence. SEQ ID NO:2, wherein the variants retain native ribonuclease activity, have a lower binding affinity for RI than that of the native RNase 1, and exhibit enhanced cytotoxic activity relative to the native RNase 1.

These RNase 1 variants were designed by applying the relationship between long-range electrostatics and the rate of association. A preferred RNase 1 variant is defined by R39D/N67D/N88A/G89D/R91D and has 10-fold lower affinity and 2700-fold lower association rate for hRI than wild-type RNase 1. The 2700-fold lower association rate was 25-fold gain of electrostatic repulsion by aspartate substitution in RNase 1 and 110-fold loss of electrostatic attraction at positions 39 and 91 in RNase 1.

It is noted that the hRIPRNase 1 complex, electrostatic attraction of key charged residues like Arg39 and Arg91 helps hRI recognize RNase 1 more than sterically constrained residues like Asn67 do. RI uses its horseshoe shaped structure to allow long-range electrostatic interactions by key solvent-exposed charged residues (especially Arg39 and Arg91) to determine its tight interaction with RNase 1.

Other novel variants of RNase 1 are also described herein which retain native ribonuclease activity, have a lower binding affinity for RI than that of the native RNase 1, and exhibit enhanced cytotoxic activity relative to the native RNase 1. These include RNase 1 variants, which have an amino acid change relative to the native sequence, SEQ ID NO:2 at (1) residues 39, 67, 89 and 91, preferably R39D/N67D/R89A/R91D; (2) residues 39, 67, 88 and 91, preferably R39D/N67D/N88A/R91D; (3) residues 39, 67, 88 and 89, preferably R39D/N67D/88A/G89D; (4) residues 39, 88, 89 and 91, preferably R39D/N88A/G89D/R91D; (5) residues 38, 39, 67, and 88, preferably G38R/R39G/N67R/N88R; and residues 67, 88, 89 and 91, preferably 67D/N88A/G89D/R91D.

In another embodiment, the invention provides method of modifying RNase 1 to make cytotoxic RNase 1 variants by identifying electrostatic anchor residues in the three-dimensional structure of the hRIPRNase 1 complex; and modifying the anchor residues identified in RNase 1 to inhibit binding to hRI through electrostatic repulsion, wherein the variants retain native ribonuclease activity, have a lower binding affinity for hRI than that of the native RNase 1, and exhibit enhanced cytotoxic activity relative to the native RNase 1. Thus, through exploitation of the electrostatic attraction between hRI and RNase 1 we were able to develop variants of RNase 1 that are capable of eliciting the inhibitory binding of hRI to overcome a major hurdle in the development of human ribonuclease-based chemotherapeutics.

In another embodiment, the invention provides a method for inhibiting the proliferation of cancer or tumor cells, comprising delivering to the cells an effective amount of a variant RNase 1, wherein the variant RNase 1 exhibits enhanced cytotoxic activity relative to the native RNase 1.

By “enhanced cytotoxicity” it is meant that the modified RNase 1 exhibits greater cytotoxicity than the corresponding unmodified or native (wild-type) RNase 1. In the examples below, cytotoxicity was evaluated using the human erythroblast leukemia cell line K-562. It is anticipated that the modified ribonuclease of the present invention is cytotoxic against other tumor cells in addition to that which is described herein. Inhibition of cell proliferation is determined by calculating the percentage of viable K-562 cells treated with the modified or unmodified RNase 1, where 100% viability is considered to be the number of viable cells that were treated with a solution of phosphate-buffered saline (PBS).

By “effective amount” is meant that amount of ribonuclease needed to cause a significant reduction in the proliferation of the tumor cells.

Preferably, the modified ribonuclease reduces cell viability by at least about 10%. More preferably, the modified ribonuclease reduces cell viability by at least about 20%. Most preferably, the modified ribonuclease reduces cell viability by about 50%, or even as much as about 75%.

In another embodiment, the invention provides a hRIPRNase 1 complex as defined by the Protein Data Bank identification No. 1Z7X. The 3-D atomic crystal structure of the hRIPRNase 1 complex is described here for the first time. The atomic coordinates for the crystal structure of the hRIPRNase 1 complex are set forth here in Appendix 1. These atomic coordinates were also deposited at the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB) and assigned the accession number PDB ID No: 1Z7X. The 3-D structure of the hRIPRNase 1 complex can be used as a tool to rationally design RNase 1 variants that evade hRI and that exhibit enhanced cytotoxic activity relative to the native enzyme.

The research value of the 3-D structure of the hRIPRNase 1 complex is understood to those skilled in the art. It will also be appreciated that the structure obtained from X-ray crystallography is only a static snapshot of the protein-ligand complex. In reality, proteins like RNase 1 are highly flexible molecules, changing their conformation on various time scales. Access to potential binding sites may only be available in certain conformations. It is envisioned that techniques, namely Molecular Dynamics, normal Mode or Monte Carlo methods, may be used to capture one or more representative structures for designing other RNase 1 variants.
While this patent specification contains several examples of protein and amino acid sequences, it should be understood that all protein sequences are subject to minor changes and modifications without fundamentally changing the proteins or the concept of the present invention. Conservative changes of amino acids of similar size and polarity are always possible and rarely change the functioning of a protein. The whole RNase 1 is subject to further modifications of sequence, either by minor amino acid addition, deletion of substitution without adversely affecting the activity as a RNase 1. These kinds of changes in amino acid sequence are interpreted to be within the scope of the language used herein.

A conservative amino acid substitution includes one or more amino acid residues within the sequence that can be substituted by another amino acid of a similar polarity, which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. Amino acids containing aromatic ring structures are phenylalanine, tryptophan, and tyrosine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine, and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Such alterations will not be expected to affect apparent molecular weight as determined by polyacrylamide gel electrophoresis, or isoelectric point. Abbreviations of amino acids are known in the art.

The invention is further clarified by consideration of the following examples, which are intended to be purely exemplary of the method of the present invention.

EXAMPLES

1. Experimental Overview

Design of RNase 1 Variants

In general, it is understood by those skilled in the art that the equilibrium dissociation constant (Kd) of a protein complex is governed by the intermolecular factors influencing the rate of association (k_on) and dissociation (k_off). The rate of dissociation is influenced by factors that act over short distances, including van der Waals interactions, hydrogen bonds, hydrophobic interactions, and salt bridges. The rate of association, however, depends primarily on diffusion but can be increased through Coulombic electrostatic forces. The majority of the long-range electrostatic forces destabilize protein-protein interactions due to a large energetic penalty to desolvate the charged amino acids. However, when speed is a necessity, the rate of association and consequently the affinity of a complex can be increased by optimizing the electrostatic energy.

In designing proteins to have lower affinity for their interacting protein partner, either component of the kinetic rate (k_on or k_off) could be targeted. Previous inhibition studies of the R1+RNase interface have focused on short range intermolecular contacts between R1+RNase, effectively raising the dissociation rate. Detrimentally affecting the affinity of RNase 1, the human homologue of RNase A, using short range interactions has proven more difficult.

To overcome this hurdle, we determined the crystal structure of the hR1+RNase 1 complex at 1.95 Å resolution and employed the structural information to design variants of RNase 1 with micromolar affinity for hR1 (GenBank Accession No. P13489). We also investigated with RNase 1 the analogous complementarity residues identified in RNase A and revealed the energetic contribution to R1 binding from these RNase 1 residues. Based on the contribution of these charged residues (e.g., Arg59 and Arg91) to the rate of complex association, we define a role for "electrostatic anchor" residues in determining protein-protein interactions. Electrostatic anchor residues determine protein-protein recognition by (1) contributing substantial enhancement to the association rate, and (2) maintaining complex formation through tight hydrogen bonds. Overall, the evasion of R1 by RNase 1 requires both steric and electrostatic contributions, but is driven to micromolar affinity by a significant decrease in the association rate constant.

Accordingly, the cytotoxic RNase 1 variants of the present invention were developed by (1) analyzing the molecular recognition patterns of R1 in complex with RNase 1 and RNase A, two ribonucleases with high sequence identity and (2) dissecting the difference in the energetic (e.g., steric and electrostatic) distribution of specific residues involved in R1-binding to design rationally-based RNase 1 variants. One of the outcomes of this design strategy was engineering cytotoxic RNase 1 variants with at least 10-fold lower affinity and 2700-fold lower association rate for hR1 than wild-type RNase 1.

Differences in Pancreatic Ribonuclease Recognition by R1

The fast atomic density evaluator (FADE) algorithm revealed regions of high shape complementarity between pR1 and RNase A. By inserting disruptive mutations in regions identified to have high shape complementarity, D38R/R39D/N67R/G88R RNase A (K_f=510 nM for hR1) and C31A/C32A/G38R/K39D/G88R BS-RNase (K_f=110 nM for hR1) were developed that had significantly decreased affinities for R1. Using the same logic and shape complementarity regions, we designed G38R/R39G/N67R/N88R RNase 1 (Table 5). When applied to RNase 1, this strategy failed to reduce the affinity of hR1 for RNase 1. The binding affinity of hR1 for this quadruple variant of RNase 1 was near the affinity of hR1 for wild-type RNase 1. Consequently, we wanted to determine what separated the RI recognition of RNase 1 from RNase A.

Residue 39. Arg39 of RNase A had the highest shape complementarity score for pR1+RNase A and was proposed to be a secondary anchor residue. When Arg39 was mutated to aspartate in G88R RNase A to create R39D/G88R RNase A, the R39D mutation instilled 725-fold lower RI affinity in RNase 1, Arg39 has even lighter interactions with hR1 with the formation of 3 hydrogen bonds and consequently the energetic contribution of R39D is second highest among the residues studied at ΔΔG=-2.2 kcal/mol.

Similar to RNase A, a mutation of Arg39 to Asp decreases the affinity of hR1 for RNase 1, but the cytotoxicity of variants with R39D is proportionally lower than other variants with similar RI evasion. Part of the negative influence of an R39D substitution on the cytotoxicity of RNase 1 variants can be accounted for by the 3-fold decrease in the catalytic activity, but the increased activity does not completely account for the high cytotoxicity of N67D/N88A/G88D/R91D RNase 1. The disproportionately large decrease in cytotoxicity in variants of RNase 1 with R39D advocates a role for Arg39 in cell surface binding. Arg39 is positioned between two positively charged patches on RNase 1 (FIG. 5) and so a negative charge at position 39 may weaken the cell surface binding of both positive patches, producing a proportionally greater decrease in internalization and cytotoxicity.

Residue 67. Previously, the recognition of Asn67 by hR1 was exploited to develop RI variants that selectively bind to
angiogenin but not RNase 1 or RNase A. By incorporating a tryptophan at positions 408 and 410 in hRI, a highly selective variant of hRI was engineered that only bound angiogenin. A tryptophan substitution at Asn67 of RNase 1 to sterically hinder the binding of residues 408-410 in hRI did not produce a comparable binding change (data not shown). Yet, an aspartate at position 67 does destabilize the complex by 1.9 kcal/mol (Table 5).

Asn67 was proposed to be a primary anchor residue in the pR1-RNase A interface, due to its burial of surface area and its lack of molecular motion. In agreement with the assertion that Asn67 plays a role in complex formation, we find that the energetic destabilization caused by mutation at position 67 is substantial (ΔΔG = 1.9 kcal/mol). However, Arg39 and Arg91 provide more overall energy to stabilization of the hR1-RNase 1 complex.

β4-β5 loop. To determine what separated the R1 recognition of RNase 1 from RNase A in the β4-β5 loop region, a 3-D structural comparison was performed as shown in Fig. 2 between the β4-β5 loop in RNase 1 (purple) and RNase A (blue) when bound to R1 (green). This was accomplished by aligning the alpha-carbons of RNase 1 and RNase A with the program Sequoia, and images were created with the program PyMOL. (A) Structure of β4-β5 loops, with R1 concealed. Side chains of residues 88-91 are shown as sticks. Amino acids are labeled with the color corresponding to the color of the ribbon. The green portion of the loop is not shown in the complex structure. (B) Orientation of the β4-β5 loop bound to RI. RNase A (chain E) was aligned to RNase 1 (chain Z) and then modeled into hRI (chain Y) based on the alignment to RNase 1. Hydrogen bonds are shown as dotted lines. Hydrogen bonds between hRI and RNase A are hypothesized based on the alignment of hRI and pR1.

By performing this comparative alignment, we discovered that in contrast to prior results, where Gly 88 to arginine mutations decreased the affinity of pR1 for RNase A by 10^4 M and the affinity of R1 for RNase 1 by 250-fold, substituting Asn88 with arginine in RNase 1 did not generate a similar decrease in affinity. In the crystal structure of the hR1•RNase 1 complex, the β4-β5 loop adopts a similar conformation to RNase A with pR1 (Fig. 2). One major difference between RNase 1 and RNase A in the β4-β5 loop is with residue 88 where Asn88 of RNase 1 hydrogen bonds with Glu264 instead of folding into the pocket formed by Trp261 and Trp263 like Gly88 in RNase A. Asn88 in RNase 1 is located on the outer surface of the hR1•RNase 1 interface and could accommodate the structural bulk of an arginine or carbohydrate chain while still maintaining high affinity for R1.

Gly89 of RNase 1 has been proposed to constitute the structural analogue of Gly88 in RNase A, but mutational studies at Gly89 in RNase 1 have, also, failed to produce variants with lower affinity for RI. Gly89 in RNase 1 overlaps more closely with Ser89 in RNase A (Fig. 2), but Gly89 is unable to hydrogen bond with Glu206 as seen for Ser89 in RNase A.

Gly89 in RNase 1 still has van der Waals contact with Trp261 and Trp263 in hR1•RNase 1, but Gly89 appears to have greater flexibility than Gly88 in RNase A. Consequently, hRI can adjust to an aspartate or arginine substitution at Gly89 in RNase 1 while maintaining near wild-type affinity (Fig. 2).

Among the five residues investigated, Arg91 had the greatest energetic influence on the hR1•RNase 1 complex (ΔΔG = 2.8 kcal/mol). Arg91 contacts the negatively charged side of the hRI surface (Fig. 5), where Arg91 forms two hydrogen bonds with Glu287 of hRI. Lys91 in RNase A was proposed to play a secondary latching role in anchoring RNase A to hRI, but in RNase 1, Arg91 may serve as a primary anchor residue to recognition by hRI.

Substituting Arg91 with an aspartate severed the tight hydrogen bonds to Glu287 of hRI, and replaced the attractive force of Arg91 with an electrostatic repulsion. This loss of charge-charge attraction and gain of electrostatic repulsion at position 91 caused the largest change in the overall binding affinity.

Electrostatic Anchor Residues

In a survey of 14 enzyme-inhibitor complexes, all fourteen complexes had a positive ΔG_{binding}, meaning electrostatics were a negative force to complex formation. However, R1•RNase complexes are atypical protein-protein interfaces. Binding of angiogenin to hRI has an electrostatic energy of interaction of -12.3 kcal/mol and a calculated rate increase due to electrostatics of 10^{-1}^{-1}. In contrast to other complexes, in R1•RNase complexes electrostatics play a key role in binding.

Fig. 3 shows electron density at 1° of key shape complementarity residues between hRI (green) and RNase 1 (purple). Specific residues shown in detail are (A) Arg39, (B) Asn67, and (C) Arg91. Highlighted regions are shown in wall-eyed stereo and interproton hydrogen bonds are displayed by black dotted lines. Images were created with the program PyMOL. Fig. 3 demonstrates that instead of sterically-constrained residues making initial contact with hRI, the electrostatics of the key solvent exposed charged residues like Arg39 and Arg91 drive the association rate.

Specifically, Arg39 and Arg91 contribute at least 0.3 kcal/mol more to the binding energy than Asn67, Asn88, or Gly89 do. The charged surface of Arg39 and Arg91 determines the association rate as substitution of these charges to leucine in R39/L67/N88/A264/K91 decreases the association rate by 110-fold. Hence, Arg39 and Arg91 serve a special role in the hR1•RNase 1 complex that we define as electrostatic anchor residues. A residue that anchors the formation of a protein-protein complex should provide the major energetic force to complex formation and be the major marker for the recognition of its protein-binding partner.

Electrostatic residues like Arg39 and Arg91 fit these criteria, as they are initially recognized by the electrostatic surface of hRI (Fig. 5). Specifically, Fig. 5 illustrates an electrostatic representation of the hRI•RNase 1 (green) and RNase 1 (purple) interaction. Protein contact potential of RNase 1, residues 39 and 91 are labeled (A), hRI (B), and hR1•RNase 1 (C) as shown. The intensity of the blue (positive) and red (negative) coloration is indicative of the local electrostatic environment. Vacuum electrostatics were calculated and images were created with the program PyMOL.

Furthermore, electrostatic residues Arg39 and Arg91 strongly affect the association rate of the complex (Table 6). Thus, Arg39 and Arg91 keep RNase 1 bound to hRI through tight hydrogen bonds (Fig. 3), allowing other contacts in the complex to form. Arg39 and Arg91 steer the formation of the hR1•RNase 1 complex over longer distances than the steric of Asn67 and ultimately contribute more binding energy to the affinity of the hR1•RNase 1 complex. Although, Arg39 and Lys91 in RNase A were proposed to play a role in RI binding, the key function that the electrostatics of these residues supply to the hR1•RNase 1 complex was underestimated.

Energetics of Evasion

Charged amino acids constitute 19% of all exposed amino acids on a protein surface, but in the average protein-protein interface fewer charged residues are exposed. Charge-charge interactions in protein-protein interfaces are disfavored energetically by a large energetic penalty to desolvate the exposed
charge residue upon binding. The energetic penalty of desolvation can be circumvented by leaving key charge interactions partially solvent exposed upon complex formation. In Fig. 3, the electron density for multiple solvent molecules are visible surrounding important charged interactions between hRI and RNase 1. RI seems to use its usual horseshoe-shape to expose greater surface area to solvent and only partially desolvate key charged residues. This exposure to solvent diminishes the energetic desolvation penalty incurred by RI upon RNase 1 binding and allows electrostatics to remain a driving energetic force to complex formation.

The positive charge on the RNase 1 surface (Fig. 5) facilitates substrate binding and consequently is necessary to maintain the biological activity of RNase 1. RI takes advantage of the necessity for a charged surface on RNase 1 to tightly and rapidly inhibit RNase 1 using long range electrostatics. Fig. 5 highlights the positive and negative charge distribution on RNase 1 and hRI, respectively. In the crystal structure, both Arg39 and Arg91 are tightly enclosed in the negative inner surface of RI (Fig. 5) and anchor RNase 1 to the negative surface of hRI.

By incorporating negatively-charged aspartate residues at key electrostatic anchors, we have lowered the equilibrium dissociation constant of hRI for RNase 1 by nearly seven orders of magnitude. Comparison of variants of RNase 1 with mutations at Arg39 and Asn67 illustrate how electrostatics help in the evasion of RI binding. The electrostatic repulsion of an aspartate at positions 39 and 67 destabilizes the complex by 2.2 and 1.9 kcal/mol, respectively (Table 5). Instead of an aspartate residue, the wild-type residue is changed to glycine at residue 39 and arginine at residue 67 (G39R/R39G/N67R/N88R RNase 1), a similar destabilization of the complex is not observed (ΔΔG = 3.0 kcal/mol). Thus, the electrostatics of residues Arg39 and Asn67 play a large role in determining the affinity of an RNase 1 variant.

Overall, the repulsion of RI binding by aspartate substitutions in RNase 1 is superadditive as the binding energy lost by the reversion of single mutations in RNase 1 (R39D/N67D/N88A/G39R/R91D RNase 1 (8.2 kcal/mol) is less than the binding energy lost with RNase 1 (R39D/N67D/N88A/G39R/R91D RNase 1 (9.3 kcal/mol). Examples of superadditive mutations in protein-protein complexes are uncommon, but have been observed with hRIPangiogenin. The superadditive results for hRI-RNase 1, however, are surprising, because previous mutations in hRI-RNase A were superadditive. The superadditivity of the mutations to the hRI-RNase 1 complex can be explained partially by the methods and partially by the type of mutations. By combining multiple substitutions in RNase 1, the native RNase 1 structure may have been contorted such that additional mutations develop disruptive contacts with RI that are not seen for single mutants. Also, the electrostatic repulsion of an aspartate substitution instead of an alanine substitution can perturb a larger surface area and increase the energetic destabilization of the mutation. For example, the ΔΔG values for all four aspartate substitutions in RNase 1 are larger than the ΔΔG value for deleting a single hydrogen bond (Asn88 to Ala88) (Table 5). Overall, we exploited the tight electrostatic attraction between hRI and RNase 1 to develop variants of RNase 1 with comparable affinity to the most evasive RNase A variants.

Rates of Association and Dissociation

Electrostatics steer the formation of protein-protein complexes over long distances and increase the rate of association over diffusion limited processes. We measured the difference in the kinetic rate constants between two variants of RNase 1 from Table 5 to determine which kinetic constant led to the increased evasion of R39D/N67D/N88A/G39R/R91D RNase 1. Overall, changes in the dissociation rate (3100-fold) and association rate (2700-fold) constants of R39D/N67D/N88A/G39R/R91D RNase 1 each account for half the decreased affinity for hRI as compared to wild-type RNase 1 (Table 6). The important contribution of the association rate to the micromolar affinity of R39D/N67D/N88A/G39R/R91D RNase 1 is seen more clearly when its rates are compared to R39D/N67D/N88A/G39R/R91D RNase 1 (Table 6).

The 50-fold increased RI-evasion of R39D/N67D/N88A/G39R/R91D RNase 1 over R39D/N67D/N88A/G39R/R91L RNase 1 is almost completely driven by long-range electrostatic repulsion through its effect on the association rate. The total influence of the electrostatics of residues 39, 67, 88, 89, and 91 on hRI-RNase complex formation can be approximated by combining the 110-fold decrease in association rate due to the loss of attractive forces by leucine substitutions and the 25-fold decrease in association rate with the gain of repulsive forces by aspartate substitutions. Overall, electrostatics contributes 2700-fold to the decreased affinity of R39D/N67D/N88A/G39R/R91D RNase 1 for hRI and the experimental results here reinforce previous calculations on the importance of electrostatics in the binding of RI to ribonuclease.

Molecules. E. coli BL21 (DE3) and pET22b (+) were from Novagen (Madison, Wis.). The fluorogenic ribonuclease substrate, 6-FAM-DAUr(dAdA)-6-TAMRA, was from Integrated DNA Technologies (Coralville, Iowa). Enzymes were from Promega (Madison, Wis.). K-562 cells were from the American Type Culture Collection (Manassas, Va.). Cell culture medium and supplements were from Invitrogen (Carlsbad, Calif.). [methyl-3H]Thymidine (6.7 Ci/mmol) was from Perkin-Elmer (Boston, Mass.). HitTrap NHis-ester columns were from Amersham Biosciences (Piscataway, N.J.). RNase A Type III-A for attachment to HitTrap NHis-ester columns was from Sigma-Aldrich (St. Louis, Mo.). MES buffer (Sigma-Aldrich, St. Louis, Mo.) was purified by anion exchange chromatography to remove trace amounts of oligomeric vinylsulfonic acid. All other chemicals were of commercial grade or better, and were used without further purification. TerriBroth (T3) contained in (1.0 L) tryptone (12 g), yeast extract (24 g), glycerol (4 ml), KH2PO4 (2.31 g), and KH2PO4 (12.54 g). Phosphate-buffered saline (PBS) pH 7.4 contained in (1.0 L) NaCl (8.0 g), KCl (2.0 g), Na2HPO4.7H2O (1.15 g), KH2PO4 (2.0 g), and NaN3 (0.10 g).

Instrumentation. Fluorescence measurements were made with a QuantMaster1 photoncounting fluorimeter with sample stirring (Photon Technology International, South Brunswick, N.J.). Thermal denaturation data were collected using a Cary 5 double-beam spectrophotometer equipped with a Cary temperature-controller (Varian, Palo Alto, Calif.). [methyl-3H]Thymidine incorporation into genomic DNA was quantified by liquid scintillation counting using a Microbeta TriLux liquid scintillation and luminescence counter (Perkin-Elmer, Wellesley, Mass.). The mass of RNase 1 and its variants was confirmed by matrix-assisted laser desorp-
RiRNA purification: RNAse I was purified from inclusion bodies using the same oxidative refolding procedure described previously. Variants of RNAse I were created by Quikchange site-directed mutagenesis or Quikchange Multi site-directed mutagenesis (Stratagene, La Jolla, CA) following the manufacturer’s protocol. Variants were purified using the same procedure used for wild-type RNAse I. Variants of RNAse I with free cysteine residues at position 19 were protected with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) before fluorophore attachment. Then, immediately before use, TNB-protected variants were deprotected using a three-fold molar excess of dithiothreitol (DTT) and desalted by chromatography using a PD-10 desalting column (Amersham Biosciences, Piscataway, NJ). RNAse I conjugates with 5-iodoacetamido fluorescein (Sigma-Aldrich, St. Louis, Mo.) were prepared by reaction with a ten-fold molar excess of 5-iodoacetamido fluorescein for 4.6 h at 25 °C. Conjugates were purified by chromatography using a Hitrap SP FF column. The molecular masses of RNAse I and its variants were confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry using a Voyager-DEPRO Biospectrometry Workstation.

hRI purification: hRI was purified similarly to procedures described previously. Briefly, a pET-22b(+) plasmid that contained cDNA for hRI was transformed into E. coli BL21 (DE3) and a single colony was used to inoculate LB medium (25 ml) containing ampicillin (150 µg/ml). A starter culture was grown for 16 h at 37 °C and 250 rpm and was used to inoculate cultures of TB medium (1.0 l) containing ampicillin (200 µg/ml). The cultures were grown at 37 °C and 225 rpm until OD600=3.0. Expression of the hRI cDNA was induced by IPTG (0.5 mM) and growing in an 18°C at 225 rpm. Bacteria were collected by centrifugation (12,000 x g for 10 min) and resuspended in 30 ml of 50 mM Tris-HCl buffer, pH 7.5, containing EDTA (10 mM) and DTT (10 mM). Bacteria were lysed by two passes through a French pressure cell, and the cellular debris was removed by ultracentrifugation. RNAse A was attached covalently to the resin in two 5-ml Hitrap NHS-ester columns, following the manufacturer’s protocol. The supernatant was loaded onto these two columns connected in series. The peak eluted from the RNAse A affinity column was dialyzed for 16 h against 4 L of 20 mM Tris-HCl buffer, pH 7.5, containing DTT (10 mM) and EDTA (1 mM) and purified further by chromatography using a Hitrap Q column. The purity of the eluted hRI was shown to be > 99% by SDSPAGE (data not shown).

Complex purification: Purified RNAse I (50 mg/ml) and hRI (10 mg/ml) were mixed at a molar ratio of 1.2 to 1.0, respectively. This solution was incubated at 25 °C for 60 min to allow for complex formation. The complex was loaded onto a 5-ml Hitrap Q column that had been pre-equilibrated with 20 mM Hepes-NaOH buffer, pH 7.5, containing DTT (10 mM) and glycerol (2% v/v). The complex was eluted with a linear gradient of NaCl (0-0.4 M) over 30 column volumes. Free RNAse 1 eluted with the flowthrough, and the hRI RNAse I complex eluted at about 0.15 M NaCl. Purified complex was dialyzed for 16 h at 4 °C against 20 mM Hepes-NaOH buffer, pH 7.5, containing DTT (10 mM) and glycerol (2% v/v). Finally, the complex was concentrated in a Vivaspin 20 Centrifugal concentrator (Vivasience AG, Hannover, Germany) at 6,000 x g to a final concentration of 10 mg/ml. Aliquots were flash frozen and stored at −80°C.

Crystalization: Crystals of the hRI RNAse I complex were obtained by hanging-drop vapor diffusion in 20 mM sodium citrate buffer, pH 4.2, containing methyl ether PEG 2000 (10% w/v), ammonium sulfate (1 mM), and DTT (25 mM) with the hanging drop solution containing a mixture of purified hRI RNAse I (0.9 µl) and citrate buffer solution (5.1 µl). Diffraction-quality crystals grew within a week at 25 °C. Protein crystals were soaked in reservoir solutions containing increasing amounts of ethylene glycol up to 25% (v/v), and were flash-cooled in a stream of cryogenic N2 (g).

Diffraction data were collected at SCEL-CAT Sector 22 at Argonne National Laboratories. The crystal was maintained at 100 K during data collection, and X-rays were tuned to a wavelength of 0.99997 A. The diffraction images were integrated and scaled using HKL2000. The phases were determined through molecular replacement using MOLREP from the CCP4 suite with PDB entry 1DFJ as the starting model. Arp-Warp was used to build the initial model, which was then completed with alternate cycles of model building with Xtal, refinement and refinement using REFMAC. The structural coordinates for the complexes of the ribonuclease A inhibitor with the ribonuclease A inhibitor complexed with ribonuclease inhibitor have been deposited in the Protein Data Bank (PDB) having an accession or identification No. 27TX, incorporated by reference herein in its entirety.

Ribonucleolytic activity: The ribonucleolytic activity of RNAse A and its variants was quantitated using 6-FAM-dArU (dA5)-6-TAMRA. Cleavage of this substrate at the uridine ribonucleotide leads to a 180-fold increase in fluorescence. Assays were carried out at 23(±2) °C 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:

\[ L = \frac{\Delta F}{\Delta(1/E)} = k_{cat} \frac{[S]}{[I]} \]
scavenge free hRI. The increase in fluorescence was followed as the hRI•RNase 1 variant complex dissociated irreversibly. To calculate the dissociation rate constant, k_d, the data were fitted to eq 1, wherein F_0 is the fluorescence before the addition of wild-type RNase A and F_oo is the fluorescence after complete dissociation of the complex.

\[ F = F_0 e^{-k_d(t - t_0)} \] (1)

Cytotoxicity: The effect of RNase 1 and its variants on the proliferation of K-562 cells was assayed as described previously. Briefly, after a 48-h incubation with ribonuclease, K-562 cells were treated with [methyl-3H]thymidine for 4-h and the incorporation of radioactive thymidine into the cellular DNA was quantified by liquid scintillation counting. Results are shown as the percentage of [methyl-3H]thymidine incorporated into the DNA as compared to the incorporation into control K-562 cells where only PBS was added. Data are the average of three measurements for each concentration, and the entire experiment was repeated in triplicate. Values for IC50 were calculated by fitting the curves using nonlinear regression to eq 2, where y is the total DNA synthesis following the [methyl-3H]thymidine pulse, and h is the slope of the curve.

\[ y = \frac{100}{1 + 10^{[log(IC50) - \log(h)]}} \] (2)

RESULTS

Important Interactions Between hRI and RNase 1

RNase 1 and RNase A share 70% sequence identity, but previous mutagenesis studies have suggested a variation in how they are recognized by RI. To structurally elucidate these differences in RI binding, crystals of the hRI•RNase 1 complex were grown under low ionic conditions as described herein below. The structure was refined to an R-value of 0.175 (R-free 0.236) and at a resolution of 1.95 Å (Table 1).

### TABLE 1

<table>
<thead>
<tr>
<th>Crystallographic, data processing, and refinement statistics.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESU based on R_e (Å)</td>
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<tr>
<td>Average B factor (Å²)</td>
</tr>
<tr>
<td>Number of water molecules</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ramachandran plot</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Residues in most favorable region</td>
<td>86.8%</td>
</tr>
<tr>
<td>Residues in additional allowed region</td>
<td>12.8%</td>
</tr>
<tr>
<td>Residues in generously allowed region</td>
<td>0.4%</td>
</tr>
<tr>
<td>Residues in disallowed region</td>
<td>0.0%</td>
</tr>
</tbody>
</table>

Values in parentheses refer to the highest resolution shell.

\[ \rho_{max} = \frac{\rho_{max}}{\rho_{max}} - \frac{\rho_{obs}}{\rho_{obs}} \] where \( \rho_{max} \) is the intensity of an individual measurement of the reflection and \( \rho_{obs} \) is the mean intensity of the reflection.

\[ \rho_{calc} = \frac{\rho_{calc}}{\rho_{calc}} - \frac{\rho_{calc}}{\rho_{calc}} \] where \( \rho_{calc} \) and \( \rho_{calc} \) are the observed and calculated structure-factor amplitudes, respectively.

\[ \text{R}_{\text{free}} \] was calculated as \( \text{R}_{\text{free}} \) using 5% of the randomly selected unique reflections that were omitted from structure refinement.

Tables 2, 3, and 4 summarize some of the results of the analysis of the raw data, which was included in Appendix A of the corresponding U.S. priority application Ser. No. 60/601, 311. The atomic coordinates were also submitted to the protein Data Bank (Accession No. 1Z7X). Table 3 lists data from the analysis of the interaction between hRI and RNase 1, and identifies those amino acid residues in the human RNase 1 structure which are less than 3.20 Angstroms from amino acid residues in hRI when RNase 1 is bound to hRI. The distance of 3.20 Angstroms is a maximal distance for the existence of a meaningful interaction between the two molecules and thus indicates residues in RNase 1 that can be substituted to alter the interaction between the two molecules. This list includes several of the residues, the variations in which have demonstrated conversion of RNase A into a cytotoxic molecule, notably residue 88.

### TABLE 2

<table>
<thead>
<tr>
<th>RNase 1 Residues ≤3.20 Å from hRI Residues in hRI•RNase 1 Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg4, Leu7, Gly11, Arg31, Arg32, Arg39, Lys41</td>
</tr>
</tbody>
</table>

### TABLE 3

<table>
<thead>
<tr>
<th>w•X Complex</th>
<th>R•N Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg4 NH1</td>
<td>Ala441 CB</td>
</tr>
<tr>
<td>Lys7 CE</td>
<td>Ser461 GXT</td>
</tr>
<tr>
<td>Gly11 NE2</td>
<td>Ser460 GXT</td>
</tr>
<tr>
<td>Arg31 NH1</td>
<td>Gly11 OE1</td>
</tr>
<tr>
<td>Arg31 NH2</td>
<td>Arg34 NH1</td>
</tr>
<tr>
<td>Arg32 NE</td>
<td>Arg37 OD2</td>
</tr>
<tr>
<td>Arg39 NE</td>
<td>Thr376 CH2</td>
</tr>
<tr>
<td>Lys41 CE</td>
<td>Asp436 OD1</td>
</tr>
<tr>
<td>Lys66 NZ</td>
<td>Arg407 OD1</td>
</tr>
<tr>
<td>An721 OD2</td>
<td>Trp238 GH</td>
</tr>
</tbody>
</table>

Tables 3 and 4 list the locations of closest interaction between hRI and human RNase 1, as revealed by analysis of the atomic locations of the molecules in the two distinct molecular complexes (w•X and Y•Z) that formed the crystal used in the structural analysis below. The 3-D structures of these two molecular complexes were similar, but not identical, as can be seen from Table 3 and Table 4.
TABLE 3-continued

<table>
<thead>
<tr>
<th>RNase 1 (Z) atom</th>
<th>hRI (Y) atom</th>
<th>distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys7 NZ</td>
<td>Gly444 OE2</td>
<td>3.18</td>
</tr>
<tr>
<td>Arg32 NE</td>
<td>Asp37 OD1</td>
<td>2.83</td>
</tr>
<tr>
<td>Arg39 NE</td>
<td>Gly402 OE2</td>
<td>2.79</td>
</tr>
<tr>
<td>Lys401 NZ</td>
<td>Asp436 OD1</td>
<td>2.08</td>
</tr>
<tr>
<td>Pro42 CG</td>
<td>Asp407 ND2</td>
<td>3.17</td>
</tr>
<tr>
<td>Lys666 CE</td>
<td>Cys409 SG</td>
<td>3.20</td>
</tr>
<tr>
<td>Asn71 ND2</td>
<td>Tyr433 OH</td>
<td>2.86</td>
</tr>
<tr>
<td>Asn86 OD1</td>
<td>Gly265 OE2</td>
<td>2.70</td>
</tr>
<tr>
<td>Arg91 OD1</td>
<td>Gly286 OE2</td>
<td>2.60</td>
</tr>
<tr>
<td>Gly111 OE2</td>
<td>Tyr433 OH</td>
<td>2.58</td>
</tr>
</tbody>
</table>

From this summary of the raw data, it can be understood that amino acid residues Arg39, Asn86, and Arg91 represent prime locations for modifying RNase 1 to interfere with the binding of hRI. As a result, RNase 1 would be able to evade the action of the inhibitor in vivo and increase cytotoxicity of RNase 1 for chemotherapeutic purposes.

The contacts from both chains of RNase 1 in the unit cell are provided in FIG. 1A. The secondary structure of RNase A is identified with (α-helix), β (β-strand), or t (turn). Residues in van der Waals or hydrophobic contact with RI are in blue. Residues with hydrogen bonds to RI are in red. Conserved cysteine residues are in yellow. Key catalytic residues are in black boxes. In chain X of RNase 1 (FIG. 1A), a bound citrate molecule forms tight hydrogen bonds to all three catalytic residues, His12, His118, and Lys41. The bound citrate perturbs the substrate binding cleft of RNase 1 causing Arg10 and Lys66 to undergo significant conformational changes. Lys66 in chain X forms a hydrogen bond to Asn406 of hRI. However, in chain Z (FIG. 1B), 3-D structure of RNase 1 chain Z from 1ZTX, only van der Waals interactions were observed with hRI. Residues are colored using the same scheme as in the sequence alignment in FIG. 1A, except that active-site residues are not highlighted. The image was created with the program PyMOL (DeLano Scientific, South San Francisco, Calif.). To facilitate comparison between the structures of hRI•RNase 1 and pRI•RNase A, chains Y (hRI) and Z (RNase 1) (without citrate bound) serve as the hRI•RNase 1 complex for comparison.

The root mean square deviation (rmsd) between the alpha carbons of hRI•RNase 1 and pRI•RNase A is 2.8 Å. Much of the deviation between the complexes originates from the alignment of pRI with hRI (rmsd=1.6 Å), because unlike hRI, pRI is observed to undergo a conformational change upon RNase A binding. The alpha carbons of RNase 1 and RNase A have less deviation (rmsd=0.6 Å). Angiotensin and EDN, the other human ribonuclease crystalized with hRI, gave rmsd values of 7.4 and 6.3 Å from RNase 1, respectively. The considerably higher rmsd for angiotensin and EDN reveals the structural variation among human ribonucleases and underscores the similarity between RNase 1 and RNase A.

The conservation of contact residues between the complexes of hRI•RNase 1 and pRI•RNase A is shown in FIG. 1A. The localization of RI-contact residues on the active-site face of RNase 1 is shown in FIG. 1B. The total number of contact residues (23) with RI is conserved between RNase 1 and RNase A. A divergence in the recognition of RNase 1 and RNase A by RI is in the number of ribonucleic acid residues observed to form a hydrogen bond to RI. In RNase 1, 13 residues form at least one hydrogen bond to RI, compared to 8 in RNase A.

Previous studies on RNase A and BS-RNase binding to RI focused on three structural regions, residues 38/39, residue 67, and residues in the (β4-β5 loop. FIGS. 2 and 3 emphasize the hydrogen bonding network and electron density of these regions for the hRI•RNase 1 complex. Arg39 of RNase 1 forms three hydrogen bonds with hRI that are absent with Arg39 in RNase A. Arg39 of RNase 1 makes a bidentate hydrogen bond with Gly401 and a main-chain hydrogen bond to Tyr434 of hRI. The hydrogen bond formed from Asn67 to RI shifts from Val405 (Leu409 in hRI) in pRI•RNase A to Tyr437 in hRI•RNase 1. The β4-β5 loop forms hydrogen bonds involving Asn86, Gly89, and Arg91 (FIG. 2). Arg91 of RNase 1 forms two tight hydrogen bonds (<3.0 Å) from the nitrogen of its guanidino group to Gly287 in hRI (FIG. 3).

In contrast, Lys91 in RNase A is directed away from Gly287 in pRI and no hydrogen bonds were observed (FIG. 2). Based on the structural environment of the complementarity regions, two variants of RNase 1 were designed. One variant of RNase 1 (G38R/R39G/N67R/N88R RNase 1) mimics the most cytotoxic variant of RNase A (D38R/R39G/ N67R/G89R RNase A), by swapping the amino acids in positions 38, 39, and 40. The other RNase 1 variant (R39D/N67D/N88A/ G90D/R91D RNase 1) utilizes the same regions, but instead of steric bulk utilizes electrostatic repulsion to inhibit the binding of RI.

Ribonucleolytic Activity

The ability of a ribonuclease to cleave RNA in the presence of RI is closely correlated to its cytotoxicity in vitro. For a ribonuclease variant to achieve its full cytotoxic potential, a mutation that decreases RI binding must not detrimentally affect the native catalytic activity. Consequently, variants of RNase 1 were assayed for their catalytic activity toward a tetranucleotide substrate. Values of kcat/Km for RNase A, RNase 1, and their variants are given in Table 5. The kcat/Km value for wild-type RNase 1 is 10-fold higher than the value previously reported. A similar increase in the catalytic activity was measured for RNase A when oligoglycin sulfonate, a potent inhibitor of ribonuclease, was removed from the reaction buffer.

<table>
<thead>
<tr>
<th>Biochemical parameters of RNase 1, RNase A, and their variants.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribonuclease</td>
</tr>
<tr>
<td>Wild-type RNase A</td>
</tr>
<tr>
<td>D38R/R39G/N67R/R</td>
</tr>
<tr>
<td>G89R RNase A</td>
</tr>
<tr>
<td>Ribonuclease</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Wild-type RNase 1</td>
</tr>
<tr>
<td>G38R/R39G/N67R/</td>
</tr>
<tr>
<td>N88R/RNase 1</td>
</tr>
<tr>
<td>R39D/N67D/N88A/</td>
</tr>
<tr>
<td>89GD/R91D RNase 1</td>
</tr>
<tr>
<td>R91D RNase 1</td>
</tr>
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<td>R39D/N67D/G89D/</td>
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<td>89GD/R91D RNase 1</td>
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1. Values of $k_{cat}/K_{M}$ (μE) were determined for catalysis of 6-fAM-dAdC(6-fAM)-dARNA cleavage at 25°C in 0.1 M MES-NaOH buffer (pH 6.5, 50 mM), containing 0.10 M NaCl (0.1 M NaCl).
2. Values of Tr (°C) for Rnase 1 and its variants were determined in PBS by UV spectroscopy.
3. Values of $K_{M}$ (μM) were determined for the complex with HIL at 25°C (10)
4. Values of $k_{cat}$ were calculated with the equation $k_{cat} = \frac{v}{[S]}$,
5. Values of $ΔΔG = ΔΔG_{Rnase1-Rnase1}$ were determined.
6. Values of $ΔΔG'$ for Rnase 1 are for incorporation of (methyl-3H) thymidine into the DNA of K-562 cells treated with the ribonuclease and were calculated with eq. 1.
7. From Itzhaki et al.
8. From Lee et al.
9. From Szewczu et al.

The $k_{cat}/K_{M}$ values for all variants of RNase 1 are within 6-fold of the wild-type enzyme. Unlike RNase A, substitutions at residues 38/39, residue 67, and the residues in the β4-β5 loop of RNase 1 can detrimentally affect the catalytic activity. The influence of these residues is observed in the 5-fold and 3-fold decrease in activity of G38R/R39G/N67R/N88R RNase 1 and R39D/N67D/N88A/G89D/R91D RNase 1, respectively. An anomaly to this trend is R39D/N67D/N88A/G89D/R91D RNase 1 where catalytic activity is unaffected by substitutions at these residues. The discrepancy could result from a compensating favorable hydrophobic interaction between the substituted leucines and the substrate nucleotide bases, although none of the positions mutated were previously proposed to be involved in substrate binding.

By reverting only one substitution in R39D/N67D/N88A/G89D/R91D RNase 1 to the wild-type amino acid (Table 5), the contribution of individual mutations to the $k_{cat}/K_{M}$ value can be deduced. For example, in R39D/N67D/N88A/G89D/R91D RNase 1, an aspartate residue at position 39 decreases the activity 2.5-fold with respect to R39D/N67D/N88A/G89D/R91D RNase 1. Substitutions of N67D or G89D are responsible for a 1.6-fold decrease in the $k_{cat}/K_{M}$ value, where as mutations of R91D and N88A lead to a 1.3-fold and 1.9-fold increase in the $k_{cat}/K_{M}$ value, respectively. The contribution to the catalytic activity of each substitution in R39D/N67D/N88A/G89D/R91D RNase 1 seems to be additive as the total change in the $k_{cat}/K_{M}$ value for all five single substitutions (2.6-fold) approaches the 3.5-fold reduction in the $k_{cat}/K_{M}$ value for R39D/N67D/N88A/G89D/R91D RNase 1.

In regard to R4C/G38R/R39G/N67R/N88L/G89R/R91G/ V118C RNase 1, it was found to retain nearly all of the enzymatic activity of the wild-type enzyme, having a $k_{cat}/K_{M}$ value of (1.4 x 10^5) M^{-1} s^{-1} under similar assay conditions.

**Thermal Stability**

The thermal stability of a ribonuclease is linked to its susceptibility to proteolysis and consequently its cytotoxicity. The $T_m$ values for all RNase 1 variants are shown in Table 5. The $T_m$ value of wild-type RNase 1 is close to the previously reported value. In agreement with previous studies, incorporation of charged patches on the surface of RNase 1 does not reduce the $T_m$ value by more than 6°C. Neither arginine nor asparagine substitutions at residues 38/39, residue 67, or residues in the β4-β5 loop significantly disturb the conformational stability, as G38R/R39G/N67R/N88R RNase 1 and R39D/N67D/N88A/G89D/R91D RNase 1 have $T_m$ values comparable to wild-type RNase 1 (61 and 58°C, respectively.) The largest change in the conformational stability is observed with certain combinations of asparagine substitutions. For instance, N67D/N88A/G89D/R91D and R39D/N67D/N88A/G89D/R91D decrease the $T_m$ value by 6°C and R39D/N67D/G89D/R91D by 3°C. Each of these RNase 1 variants has substitutions of both N67D and R91D, where as variants with only an N67D or R91D substitution have wild-type stability. Positions 67 and 91 are located on opposite sides of the RNase 1 active site, so an explanation for their synergistic contribution to thermal stability will require further study. Overall, all variants of RNase 1 are stable well above physiological temperature.

**Evasion of Ribonuclease Inhibitor**

RI binds multiple members of the RNase A superfamily with equilibrium dissociation constant values in the femtomolar range, forming one of the tightest noncovalent biological interactions. By mutating residues 38/39, 67, and 88 in RNase A (D38R/R39G/N67R/G89R RNase A), the equilibrium dissociation constant of the hIRNase A complex was increased by seven orders of magnitude (Table 5). The analogously resistant variant in RNase 1 (G38R/R39G/N67R/N88R RNase 1) maintained near wild-type affinity (Table 5). However, substituting the arginine residues in G38R/R39G/N67R/N88R RNase 1 with multiple asparagine residues and one alanine residue reduces the affinity of RI for RNase 1 by nearly 10^7-fold. The $K_{f}$ value for R39D/N67D/N88A/G89D/R91D RNase 1 (1.7 μM) is close to the highest measured for any
RNase A variant (2.9 μM). When the aspartate substitutions in R39D/N67D/N88A/G89D/R91D RNase 1 are replaced with the isoelectric amino acid, leucine (R39L/N67L/N88A/G89L/R91L RNase 1), the equilibrium dissociation constant increases 50-fold. Lecine substitution causes the disruption of 7 kcal/mol of RI-binding energy by the loss of electrostatic attraction and steric hindrance, but an additional 2.4 kcal/mol of binding energy is disturbed by the electrostatic repulsion of an aspartate residue at the same position.

The influence of electrostatics on RI evasion is further expanded in Table 6, where the individual kinetic rate constants for the complex of hRI and two fluorescent-labeled RNase 1 variants are shown. The dissociation rate increases 1400-fold over wild-type RNase 1 upon substitutions of R39L/N67L/N88A/G89L/R91L in R1, but remains nearly constant (2-fold increase) upon aspartate substitution (R39D/N67D/N88A/G89D/R91D RNase 1). The association rate is affected more proportionately by both leucine substitution (110-fold decrease) and by aspartate substitution (25-fold decrease). The substantial change in the association rate with both leucine and aspartate demonstrates the two electrostatic forces that can lower the km value, loss of attractive forces (leucine substitution) and gain of repulsive forces (aspartate substitution). Overall, the nearly 10-fold decrease in binding affinity of hRI for R39D/N67D/N88A/G89D/R91D RNase 1 contains equal contributions by the dissociation rate (3100-fold) and association rate (2700-fold). Yet, the additional 50-fold decrease in the Kd value of R39D/N67D/N88A/G89D/R91D RNase 1 over R39L/N67L/N88A/G89L/R91L RNase 1 is driven by a decreased association rate.

### Table 6

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<th>Ribonuclease</th>
<th>Kd (μM)</th>
<th>Ka (M⁻¹ s⁻¹)</th>
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<td>wild-type</td>
<td>6.8 x 10⁻⁶</td>
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<td>RNase 1</td>
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<tr>
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<tr>
<td>R39L/N67L/N88A/G89L/R91L</td>
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<tr>
<td>R39D/N67D/N88A/G89D/R91D RNase 1</td>
<td>3.1 x 10⁻⁶</td>
<td>1.1 x 10⁵</td>
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*Values for Kd (μM) were determined by following the release of a fluorescent-labeled RNase 1 variant from hRI over time and fitting the curves to eq 1. Values in parentheses represent the dissociation rate constant (Kd) for each wild-type RNase 1 variant. Values of Ka were calculated using the equation, Kd = Ks / Ka. Numbers in parentheses represent the dissociation rate constant (Kd) for each wild-type RNase 1 variant. Values of Ka were calculated using the equation, Kd = Ks / Ka. Where the Kd value was from Joo et al. and the Km was for hRI/RNase A from Lee et al.*

### The Impact of Individual Mutations in R39D/N67D/N88A/G89D/R91D RNase 1 to its overall binding constant is elucidated by the reversion of each substitution in R39D/N67D/N88A/G89D/R91D RNase 1 to the wild-type amino acid. Residues with little impact on RI affinity will have small ΔΔG values in Table 5. Small ΔΔG values reflect a small change in the ΔΔG value when that one residue was reverted to the wild-type amino acid in R39D/N67D/N88A/G89D/R91D RNase 1. The ΔΔG values rank the energetic contributions of the mutations as N88A, G89D, N67D, and R91D, respectively.

**Fig. 4A** plots the binding isotherm of each RNase 1 variant and illustrates the affinity of RNase 1 variants for hRI. Binding to hRI was determined by using a competition assay with fluorescently-labeled G89R RNase A (50 nM). The concentration of bound fluorescence was determined by following the decrease in fluorescent emission upon hRI binding. Data points are the mean (±S.E.) of at least three separate measurements. Variants in order of decreasing hRI-affinity: R39D/N67D/N88A/G89D/R91D ( ); R39D/N67D/N88A/G89D/R91D ( ); R39D/N67D/N88A/G89D/R91D ( ); R39D/N67D/N88A/G89D/R91D ( ). The results of this study illustrate this trend; as indicated above for the individual mutations. An aspartate at position 91 contributed 2.8 kcal/mol of energy to evasion, 0.6 kcal/mol more than any other residue. Conversely, aspartates at both positions 88 and 89 contributed only 0.5 kcal/mol, which is lower than the energetic contribution of any other single substitution, showing that different residues play a more important role in hRI/RNase 1 complex formation as compared to R1/RNase A.

In regard to R4C/G38R/R39G/N67R/N88L/G89R/R91G/V118C RNase 1, it was found that the value of Kd for the complex of hRI and R4C/G38R/R39G/N67R/N88L/G89R/R91G/V118C RNase 1 is (5.5 ± 1.6) x 10⁻⁴ M. The value of the equilibrium dissociation constant, Kd, for the complex of hRI with wild-type RNase 1 is not known, but likely to be near the value of 10⁻⁴ M for the complex of pore RNase 1 with bovine pancreatic ribonuclease (RNase A).

### Charge and Cytotoxicity

The cytotoxicity of a ribonuclease is governed by all of the preceding ribonuclease attributes and the molecular charge of a ribonuclease. The interplay between the molecular charge and the cytotoxicity of a ribonuclease is seen with the results in **Fig. 4B and Table 5**.

In particular, **Fig. 4B** shows the effect of ribonucleases on the proliferation of K-562 cells. The incorporation of [methyl-3H]thymidine into cellular DNA was used to monitor the proliferation of K-562 cells in the presence of ribonucleases. Data points are the mean (±SD) of at least three separate experiments carried out in triplicate. Variants in order of increasing cytotoxicity: D38R/R39D/N67R/G89R RNase A (A); G89R RNase A (C); R39D/N67D/N88A/G89D/R91D RNase 1 (A); R39L/N67L/N88A/G89L/R91L RNase 1 (B); N67D/N88A/G89D/R91D RNase 1 (C); G38R/R39G/N67R/N88R/R91R RNase 1 (D); and wild-type RNase 1 (...). All other variants of RNase 1 from Table 5 had curves comparable to that of G38R/R39G/N67R/N88R RNase 1 and, for clarity, are not shown.

**Fig. 4B** illustrates that D38R/R39D/N67R/G89R RNase A has the same conformational stability, a 6-fold higher Kd value, and a 3-fold lower Kd value than R39D/N67D/N88A/G89D/R91D RNase 1, but their IC₅₀ values differ by a disproportionately large 87-fold. R39D/N67D/N88A/G89D/R91D RNase 1 has an IC₅₀ of only 13.3 μM, making it more toxic than wild-type RNase 1 (**Fig. 4**), but 2-fold less toxic than G89R RNase A. The only biochemical characteristic that differs significantly between D38R/R39D/N67R/G89R RNase A (C) and R39D/N67D/N88A/G89D/R91D RNase 1 (B) is the net charge.

The IC₅₀ values of all the other variants of RNase 1 from Table 5 fall outside the measurable range of the assay. R39L/N67L/N88A/G89L/R91L RNase 1 and N67D/N88A/G89D/R91D RNase 1, however, have killed approximately 60% of the K-562 cells at 25 μM (**Fig. 4**), putting their IC₅₀ values just slightly above 25 μM. The lack of cytotoxicity for most RNase 1 variants other than R39D/N67D/N88A/G89D/R91D RNase 1 can be explained by an increased affinity for RI or less in thermal stability when compared to R39D/N67D/N88A/G89D/R91D RNase 1. The outlier to this trend is N67D/N88A/G89D/R91D RNase 1, whose Kd value is 5-fold lower than R39D/N67D/N88A/G89D/R91D but whose IC₅₀ value is estimated to be only 2-fold higher. Part of the anomalous cytotoxicity of N67D/N88A/G89D/R91D RNase 1 can be attributed to a 3-fold increase in the catalytic activity over R39D/N67D/N88A/G89D/R91D RNase 1, but the increased activity does not completely explain the disproportionately high cytotoxicity of N67D/N88A/G89D/R91D RNase 1.
Furthermore, in assays we conducted with human chronic myelogenous leukemia cell line K-562, wild-type RNase 1 has an IC\textsubscript{50} value of >50 μM. In contrast, R4G/C88R/R39G/N67R/N88L/G98R/R91G/V118C RNase 1 exhibits significant cytotoxic activity, having an IC\textsubscript{50} value of 15 μM.

Taken together, the preceding examples demonstrate the creation of cytotoxic ribonuclease variants by exploiting the electrostatic interaction between hR1 and RNase 1, such that the variants evade R1 binding, with little compromise to catalytic efficacy. It is to be understood that the present invention is not limited to the particular embodiments disclosed in this application, but embraces all such modified forms thereof that come within the scope of the following claims.

Human Ribonuclease as a Chemotherapeutic

Ribonucleases show great promise as cancer chemotherapeutics. ONC, a homologue of RNase 1 from the northern leopard frog, is currently in phase III clinical trials for the treatment of malignant mesothelioma. However, therapeutics of RNase 1 have multiple advantages over ONC, including enhanced catalytic activity, decreased renal toxicity, and decreased immunogenicity. To develop therapeutics of ribonucleases that are not naturally cytotoxic, requires the careful consideration of multiple biochemical attributes including thermal stability, catalytic activity, charge, and especially R1 evasion.

Variants of RNase 1 with lower affinity for hR1 have been difficult to engineer using natural amino acid substitutions. However, using the structural and electrostatic information obtained from the crystal structure of the hR1/RNase 1 complex, we have removed this hindrance to cytotoxicity by designing a variant of RNase 1 with an affinity for R1 in the micromolar range. R39D/N67D/N88A/G98R/R91D RNase 1 has near native catalytic activity and conformational stability, but its cytotoxicity is hindered by a lowered positive charge (Table 5 and Fig. 4). Ribonuclease variants with lower net charge have increased IC\textsubscript{50} values when compared to ribonucleases with similar activity, stability, and R1 affinity. However, the charge on a ribonuclease can be increased by adding additional positive charge to the termini, which increased the toxicity of RNase A. By greatly decreasing the inhibition of RNase 1 by hR1, we have eliminated a barrier to ribonuclease cytotoxicity and opened the door to human ribonuclease-based therapies.

The tight inhibitory complexes between ribonucleases and R1 provide a good model system for studying the influence of electostatics and the association of protein-protein complexes. To this end, we have looked at RNase 1, the structural and sequence homologue of RNase A, whose interaction with hR1 has been difficult to predict. By determining the X-ray crystal structure of RNase 1 in complex with hR1 and studying key electrostatic hotspots, we were able to create variants of RNase 1 with micromolar affinity for hR1. Substitution of key charged residues created the greatest change in binding affinity and suggests a new class of anchor residues for protein-protein interactions, electrostatic anchor residues. Mutation of electrostatic anchors like Arg39 or Arg91 changes the affinity by influencing the association rate of the complex. We reduced the affinity of hR1 for RNase 1 by 10\textsuperscript{-4}-fold by exploiting the electrostatic anchors on RNase 1. Accordingly, the variants of RNase 1 that evade R1 binding represent a large step in the development of chemotherapeutics using human ribonucleases.

All publications and patents mentioned in the above specification are herein incorporated by reference as if expressly set forth herein. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is understood that certain adaptations of the invention are a matter of routine optimization for those skilled in the art, and can be implemented without departing from the spirit of the invention, or the scope of the appended claims.

RELATED PUBLICATIONS

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We claim:

1. A method for inhibiting the proliferation of cancer cells, comprising delivering to the cells an effective amount of an engineered human Ribonuclease (RNase 1) polypeptide, said polypeptide comprising SEQ ID NO:2 except for one or more amino acid substitutions in the region of amino acid residues 85 to 94 and one or more amino acid substitutions selected from the group consisting of substitutions at position 4, 38, 39, 67, and 118 of SEQ ID NO:2.

2. The method of claim 1, wherein said amino acid substitutions at positions 4, 38, 39, 67 and 118 are R4C, G38R, R39G, N67R, and V118C.

3. The method of claim 1, wherein said amino acid substitution in the region of amino acid residues 85 to 94 is N88L.

4. The method of claim 1, wherein said amino acid substitution in the region of amino acid residues 85 to 94 is G89R.

5. The method of claim 1, wherein said amino acid substitution in the region of amino acid residues 85 to 94 is R91G.

6. A method for inhibiting the proliferation of cancer cells, comprising delivering to the cells an effective amount of an engineered human Ribonuclease (RNase 1) polypeptide, said polypeptide comprising SEQ ID NO:2 except for one or more amino acid substitutions in the region of amino acid residues 85 to 94 and one or more of the following amino acid substitutions: R4C, G38R, R39G, N67R, or V118C relative to SEQ ID NO:2.

7. A method for inhibiting the proliferation of cancer cells, comprising delivering to the cells an effective amount of an engineered human Ribonuclease (RNase 1) polypeptide, said polypeptide comprising SEQ ID NO:2 except for one or more amino acid substitutions in the region of amino acid residues 85 to 94 and one or more amino acid substitutions at position 4, 38, 39, 67 or 118 of SEQ ID NO:2.

8. The method of claim 7, wherein said amino acid substitutions at position 4, 38, 39, 67 or 118 are R4C, G38R, R39G, N67R, or V118C.

9. The method of claim 7, wherein said amino acid substitution in the region amino acid residues 85 to 94 is N88L.

10. The method of claim 7, wherein said amino acid substitution in the region of amino acid residues 85 to 94 is G89R.

11. The method of claim 7, wherein said amino acid substitution in the region of amino acid residues 85 to 94 is R91G.

12. The method of claim 1, wherein the amino acid substitutions are R4C, G38R, G39G, N67R, N88L, G89R, R91G, and V118C.


15. A method for inhibiting the proliferation of cancer cells, comprising delivering to the cells an effective amount of an engineered human Ribonuclease (RNase 1) polypeptide, said polypeptide comprising SEQ ID NO:2 except for the following amino acid substitutions: R4C, G38R, R39G, N67R, N88L, G89R, R91G, and V118C relative to SEQ ID NO:2.

16. The method of claim 1, 6 or 7, wherein the engineered RNase 1 retains ribonucleolytic activity relative to SEQ ID NO:2.

17. The method of claim 1, 6 or 7, wherein the engineered RNase 1 exhibits enhanced cytotoxic activity relative to SEQ ID NO:2.

18. The method of claim 1, 6 or 7, wherein the engineered RNase 1 exhibits a lower binding affinity for hRN than SEQ ID NO:2.

19. The method of claim 1, 6 or 7, wherein the engineered RNase 1 retains ribonucleolytic activity, exhibits enhanced cytotoxic activity relative to SEQ ID NO:2, and has a lower binding affinity for hRN than SEQ ID NO:2.

20. The method of claim 1, 6 or 7, wherein the one or more amino acid substitutions in SEQ ID NO:2 electrostatically repel and/or sterically hinder binding of human Ribonuclease Inhibitor (hRN) to the engineered RNase 1.