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Endowing Human Pancreatic Ribonuclease with Toxicity for Cancer Cells*

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Onconase® is an amphibian protein that is now in Phase III clinical trials as a cancer chemotherapeutic. Human pancreatic ribonuclease (RNase 1) is homologous to Onconase® but is not cytotoxic. Here, ERDD RNase 1, which is the L86E/N88R/G89D/R91D variant of RNase 1, is shown to have conformational stability and ribonucleolytic activity similar to that of the wild-type enzyme but >10^3-fold less affinity for the endogenous cytosolic ribonuclease inhibitor protein. Most significantly, ERDD RNase 1 is toxic to human leukemia cells. The addition of a non-native disulfide bond to ERDD RNase 1 not only increases the conformational stability of the enzyme but also increases its cytotoxicity such that its IC_{50} value is only 8-fold greater than that of Onconase®. Thus, only a few amino acid substitutions are necessary to make a human protein toxic to human cancer cells. This finding has significant implications for human cancer chemotherapy.

Onconase® (ONC)[1] is a ribonuclease isolated from *Rana pipiens* (Northern leopard frog) which is selectively toxic to cancer cells both *in vitro* and *in vivo* (1–3). ONC has been tested in Phase I and Phase II human clinical trials for treatment of numerous solid tumors, including lung and pancreatic cancers (4, 5). In these trials, ONC appeared to have a favorable impact on the median survival time of the patients. Renal toxicity is dose-limiting but is reversible on discontinuation of treatment.

The mechanism of ONC cytotoxicity is not well understood. It is evident, however, that enzymatic activity is required for ONC cytotoxicity because alkylation of ONC active site residues not only abates enzymatic activity but also renders the enzyme nontoxic (6). Despite sharing a common enzymatic activity, mammalian homologs of ONC, such as bovine pancreatic ribonuclease A (RNase A) and human pancreatic ribonuclease (RNase 1), are not cytotoxic.

The creation of cytotoxic variants of RNase A and RNase 1 could help to reveal the mechanism of ribonuclease cytotoxicity. Moreover, endowing mammalian ribonucleases with cytotoxicity could yield cancer chemotherapeutics that lack the undesirable side effects of ONC. Toward this end, D'Alessio and co-workers (7, 8) have created artificial dimers of RNase A and RNase 1 which are toxic to cancerous cells. Others have conjugated monomers of RNase A and RNase 1 to peptides, proteins, and antibodies to enhance uptake by target cells (9–13). For example, Youle and co-workers (14) have attached transferrin to residue 89 of RNase 1. In the presence of 10 μM all-*trans*-retinoic acid, the transferrin-RNase 1 conjugate is toxic to cells that express the transferrin receptor. Still, data on unconjugated monomeric variants of RNase A and RNase 1 are likely to yield the most relevant information on the mechanism of ONC cytotoxicity. Moreover, artificial dimers and ribonuclease conjugates require *in vitro* processing following initial purification or can be readily isolated only as multiple isoforms (or both). These limitations could complicate their use as chemotherapeutic agents and prevent their use in gene therapy protocols.

The cytosol of most mammalian cells contains a potent inhibitor of RNase A and RNase 1. Ribonuclease inhibitor (RI) is a 50-kDa protein that binds to members of the pancreatic ribonuclease family with 1:1 stoichiometry and dissociation constants near or below 1 pM (14–16). Intriguingly, ONC is not inhibited by RI (6, 17–19). This lack of inhibition could allow ONC to catalyze degradation of cellular RNA and consequently cause cell death. According to this hypothesis, RNase A and RNase 1 are not cytotoxic because RI efficiently abates their enzymatic activity within the cytosol.

We suspected that by manipulating the interaction between RI and RNase 1, we could create a cytotoxic variant of RNase 1. Here, we describe amino acid substitutions in an RNase 1 surface loop which preserve conformational stability and ribonucleolytic activity but reduce inhibition by RI. In addition, we describe other amino acid substitutions in RNase 1 which affect conformational stability and ribonucleolytic activity. Finally, we report on the cytotoxicity of each RNase 1 variant. We find that monomeric RNase 1 can be transformed into a potent cytotoxin with only limited amino acid substitutions. This finding has significant implications for human cancer chemotherapy.

EXPERIMENTAL PROCEDURES

Materials—K-562 cells, which derive from a continuous human chronic myelogenous leukemia line, were from the American Type Culture Collection (Manassas, VA). *Escherichia coli* strain DH5α was from Life Technologies, Inc. *E. coli* strain BL21(DE3) and the pET22b(+) expression vector were from Novagen (Madison, WI). ONC was prepared as described (20). RI (human) was from Promega (Madison, WI).

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[1] The abbreviations used are: ONC, Onconase® (a registered trademark of Alfacell, Inc.); RNase A, bovine pancreatic ribonuclease A; RNase 1, human pancreatic ribonuclease; RI, ribonuclease inhibitor; 6-FAM, 6-carboxyfluorescein; 6-TAMRA, 6-carboxytetramethylrhodamine; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; MES, 2-(*N*-morpholino)ethanesulfonic acid.

Enzymes used for DNA manipulation were from Promega or New England Biolabs (Beverly, MA). The ribonuclease substrate 6-carboxyfluorescein~dArUdAdA~6-carboxytetramethylrhodamine (6-FAM~dArU(dA)$_2$~6-TAMRA (21)) was from Integrated DNA Technologies (Coralville, IA). Ribosomal RNA (rRNA; 16 S and 23 S) was from Roche Molecular Biochemicals (Mannheim, Germany). Poly(cytidylic acid) (poly(C)) was from Midland Certified Reagents (Midland, TX). [*methyl*-^{3}H]Thymidine was from PerkinElmer Life Sciences. All other chemicals were of commercial reagent grade or better and were used without further purification.

DNA oligonucleotides for PCR, site-specific mutagenesis, and DNA sequencing were from Integrated DNA Technologies. PCR reagents were from CLONTECH (Palo Alto, CA). DNA was sequenced at the University of Wisconsin Biotechnology Center with a BigDye cycle sequencing kit from PerkinElmer Life Sciences and an ABI 377XL automated DNA sequencer from PE Biosystems (Foster City, CA).

Terrific broth (TB) contained (in 1.00 liter) 12 g of Bacto-tryptone, 24 g of Bacto-yeast extract, 4 ml of glycerol, 2.31 g of KH_2PO_4, and 12.54 g of K_2HPO_4. Phosphate-buffered saline (PBS) contained (in 1.00 liter) 0.20 g of KCl, 0.20 g of KH_2PO_4, 8.0 g of NaCl, and 2.16 g of $Na_2HPO_4{\cdot}7H_2O$.

Instruments—Fluorescence-based assays of ribonucleolytic activity and its inhibition by RI were made with a QuantaMaster 1 photon counting fluorometer equipped with sample stirring from Photon Technology International (South Brunswick, NJ). Ultraviolet absorbance measurements were made with a Cary model 3 or a Cary 50 Biospectrophotometer from Varian (Palo Alto, CA).

Protein Production and Purification—A synthetic cDNA that codes for Met(−1) RNase 1 was a generous gift from R. J. Youle (22). This cDNA was excised from pET11d and inserted into pET22b(+) by using the *Bam*HI and *Xba*I sites. The resulting plasmid was termed pHP-RNase.

cDNAs coding the RNase 1 variants were made by site-directed mutagenesis and PCR-based mutagenesis of pHP-RNase. RNase 1 residues Lys^{41}, Leu^{86}, Asn^{88}, Gly^{89}, and Arg^{91} were replaced using site-directed mutagenesis. Oligonucleotide PL28 (5′-CGAAAGTGTTAACAGG**CCT**GCAACGACCA-3′) was used to replace the AAA codon of Lys^{41} with an AGG codon of arginine (reverse complement in bold) and to incorporate a translationally silent *Stu*I site (underlined). Oligonucleotide BMK15 (5′-GTAAGCGCAGTTCGGGTA**ATC**AGA**ATCACG**C GT**TTC**ACGGCAGTCAGTGATATG-3′) incorporates four codon changes. This oligonucleotide replaces 1) the CUG codon of Leu^{86} with a GAA codon of glutamate, 2) the AAC codon of Asn^{88} with a CGU codon of arginine, 3) the GGU codon of Gly^{89} with a GAU codon of aspartate, and 4) the CGU codon of Arg^{91} with a GAU codon of aspartate (reverse complements in bold). In addition, oligonucleotide BMK15 incorporates a translationally silent *Mlu*I site (underlined). RNase 1 residues Arg^4 and Val^{118} were replaced with cysteine residues using PCR-based mutagenesis. Oligonucleotide PL29 (5′-GGAGATATA*CATATG*AAAGAATCT**TGC**GCAAAAAAATCCCAGCG-3′) replaces the CGU codon of Arg^4 with a UGC codon of cysteine (bold) and incorporates translationally silent *Fsp*I (underlined) and *Nde*I (italics) sites. Oligonucleotide PL30a (5′-TC*GGATCCC*TACTAAGAGTCTTCAACGCTAGCGTCGAAATG**ACA**CGG-3′) replaces the GUU codon of Val^{118} with a UGU codon of cysteine (reverse complement in bold) and incorporates translationally silent *Nhe*I (underlined) and *Bam*HI (italics) sites. The PCR product was band purified, treated with restriction endonucleases *Nde*I and *Bam*HI, and inserted into a pET22b(+) fragment prepared with the same restriction endonucleases. The nucleotide sequence of all RNase 1 variants was confirmed with dye terminator cycle sequencing.

RNase 1, its variants, and ONC were produced and purified using methods described previously (20). Briefly, *E. coli* strain BL21(DE3) was transformed with the appropriate plasmid and grown in TB to an A_{600} of 2.0. Protein expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside to 0.5 mM. Cells were harvested by centrifugation and lysed with a French pressure cell. Inclusion bodies were recovered by centrifugation, then reduced and denatured. Proteins were folded by dropwise addition into 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 M L-Arg, 3.0 mM reduced glutathione, and 0.6 mM oxidized glutathione at 10 °C. After a 15-h incubation, the solution was adjusted to pH < 7.0 and concentrated by ultrafiltration. Concentrated samples were applied to a Superdex G-75 gel filtration fast protein liquid chromatography column (Amersham Pharmacia Biotech) equilibrated in 50 mM sodium acetate buffer (pH 5.0) containing 0.1 M NaCl and 0.02% w/v NaN_3. Protein from the major peak was collected and applied to a Mono S cation exchange fast protein liquid chromatography column (Amersham Pharmacia Biotech). RNase 1 and its variants were eluted with a 0.3–0.6 M linear gradient of NaCl in 50 mM sodium acetate buffer (pH 5.0). The concentration of RNase 1 and its variants was determined by UV spectroscopy using an extinction coefficient of ϵ_{280} = 0.53 ml·mg^{-1}·cm^{-1}, which was calculated with the method of Gill and von Hippel (23). The extinction coefficient for RNase 1 differed by less than 1% when calculated with the method of Pace and co-workers (24). Both methods predict that adding a disulfide bond to RNase 1 will change the extinction coefficient by less than 3%. Hence, the same extinction coefficient was used for all RNase 1 variants. The concentration of ONC was determined by UV spectroscopy using an extinction coefficient of ϵ_{280} = 0.87 ml·mg^{-1}·cm^{-1} (20).

Assays of Conformational Stability—The conformational stability of RNase 1 and its variants was measured by recording the change in absorbance at 287 nm with increasing temperature (25). The temperature of the ribonuclease solution (0.1–0.2 mg/ml) in PBS was increased continually from 20 to 80 °C at 0.2 °C/min. The A_{287} was recorded at 1 °C intervals and fit to a two-state model for denaturation, where the temperature at the midpoint of the transition is the melting temperature (T_m).

Assays of Ribonucleolytic Activity—Steady-state kinetic parameters for catalysis of RNA cleavage by RNase 1 and its variants were determined with a fluorogenic substrate, 6-FAM~dArU(dA)$_2$~6-TAMRA (21). This substrate consists of a single ribonucleotide embedded within three deoxyribonucleotides. When the substrate is intact, the 6-TAMRA group quenches the fluorescence of the 6-FAM group. Upon ribonucleolytic cleavage, fluorescence intensity increases by 180-fold. Assays were carried out in 2.00 ml of 0.1 M MES-NaOH buffer (pH 6.0) containing 0.1 M NaCl, 60 nM substrate, and 0.060–6.25 nM enzyme. Reaction progress was observed by recording fluorescence emission at 515 nm upon excitation at 490 nm. Values of k_{cat}/K_m were determined by a linear least squares regression analysis of initial velocity data using Equation 1,

$$k_{cat}/K_m = \left(\frac{\Delta F/\Delta t}{F_{max} - F_0}\right)\frac{1}{[E]} \qquad \text{(Eq. 1)}$$

where $\Delta F/\Delta t$ is the slope from the linear regression, F_{max} is the maximal fluorescence intensity, F_0 is initial fluorescence intensity, and [E] is the total enzyme concentration. F_{max} was determined by adding RNase A to the assay mixture (to ~0.1 μM) after the correlation coefficient for the least squares regression of the initial velocity data was $r^2 > 0.99$.

Assays of Inhibition by Ribonuclease Inhibitor—Two assays were used to assess the binding of the RNase 1 variants to RI. The first was a qualitative gel-based assay, and the second was a quantitative spectrophotometric assay. RNase 1 variants were screened for ribonucleolytic activity in the absence and the presence of RI with an agarose gel-based assay as described previously (20), with the following modifications. Each ribonuclease (10 ng) was added to PBS containing 10 mM dithiothreitol and 0, 20, or 40 units of RI. After a 10-min incubation on ice, 4 μg of 16 S and 23 S rRNA was added to the assay mixture. The assay mixture was incubated at 37 °C for 10 min and then prepared for electrophoresis by the addition of 10 mM Tris-HCl buffer (pH 7.5) containing 50 mM EDTA, 30% v/v glycerol, 0.25% w/v xylene cyanol FF, and 0.25% w/v bromphenol blue. Finally, the assay mixtures were subjected to electrophoresis through a 1% w/v agarose gel containing 0.4 μg/ml ethidium bromide. The intensity and position of the rRNA band correlate with the degree of ribonucleolytic activity. As ribonucleolytic activity in an assay mixture decreases, both the band intensity and its apparent M_r increase.

Values of K_i for the RNase 1 variants were determined by using fluorescence spectroscopy to measure the steady-state rate of 6-FAM~dArU(dA)$_2$~6-TAMRA cleavage in the presence of increasing concentrations of RI. RI was prepared and quantitated as described previously (26, 27). Assays were carried out in 2.00 ml of 0.10 M MES-NaOH buffer (pH 6.0) containing 0.1 M NaCl, 5 mM dithiothreitol, and 60 nM substrate. After an initial 2–5-min equilibration, ribonuclease was added to the assay mixture (to 25–750 pM), and the steady-state rate of substrate cleavage was recorded. RI was then added to the assay mixture in a stepwise manner, and the steady-state rate was recorded after each addition. RI was added until the steady-state rate was <2% of that in the absence of RI. Values of K_i were calculated by fitting the steady-state rates to an equation that describes inhibition by tight binding inhibitors (28). In all determinations, the ribonuclease concentration was less than the value determined for K_i.

Assays of Cytotoxicity—The effect of RNase 1 variants on proliferation of transformed cells was measured as described previously (20) with the following modification. RNase 1 variants were stored in PBS containing 0.1% w/v human serum albumin and were sterile filtered before being added to the cells. After a 44-h incubation with the ribonucleases, cells were treated with [*methyl*-^{3}H]thymidine for 4 h. Cellu-

lar DNA was recovered, and its radioactivity was counted in a liquid scintillation counter. Results are expressed as the percentage of [*methyl*-^{3}H]thymidine incorporated into the DNA of cells treated with PBS containing 0.1% w/v human serum albumin but no exogenous ribonuclease. Data are the average of triplicate determinations with each ribonuclease concentration. Cytotoxicity assays were repeated at least twice, with no significant variation in the results.

To determine whether the RNase 1 variants act as cytotoxic agents or, alternatively, as cytostatic agents, cells were stained with trypan blue after exposure to the ribonucleases. Briefly, 5.0×10^4 K-562 cells were incubated for 72 h with 5 μM ONC, 10 μM RNase 1, or 10 μM S-S/ERDD RNase 1. The cells were then stained with 0.02% w/v trypan blue in PBS. The total number of cells and the number of cells stained by trypan blue were determined by visual inspection using light microscopy. Representative cells from each ribonuclease treatment were photographed using a Carl Zeiss axioscope (Germany) equipped with a Princeton Instruments digital camera.

RESULTS

Design of RNase 1 Variants—Our goal was to create variants of RNase 1 which are cytotoxic. First, we replaced amino acid residues in RNase 1 to reduce the affinity for RI. Second, we replaced an active site residue to reduce further the affinity for RI but at the expense of ribonucleolytic activity. Third, we incorporated a non-native disulfide bond into RNase 1 to enhance conformational stability. Our design was intentionally restricted to monomeric RNase 1 variants containing only the 20 natural amino acids. This restriction facilitates the production of the variants in *E. coli*. Further, a cytotoxic RNase 1 variant so restricted may be incorporated directly into a gene therapy regimen. The three types of amino acid changes are described below.

RNase A residues 85–94 comprise a solvent-exposed surface loop flanked by half-cystine residues. In the structure of the crystalline RI·RNase A complex, this loop makes many contacts with RI (29, 30). Substitution of RNase A residue Gly^{88} with arginine (G88R RNase A) lessens inhibition by RI because of a steric and electrostatic strain introduced into the RI·RNase A complex (20). The structure of wild-type RNase 1 and its complex with RI is unknown. The structure of RNase 1 variants reveals that elements of secondary structure are conserved between RNase 1 and RNase A (31, 32). The conformations of RNase 1 surface loops, including residues 85–94, do, however, deviate significantly from their bovine counterparts. Moreover, the 85–94 loop of RNase 1 appears to be highly flexible. Vilanova and co-workers (31) have suggested that these features of RNase 1 may exclude a strategy analogous to that used to create the cytotoxic G88R RNase A variant. Nevertheless, we attempted to introduce steric and electrostatic strain into the RI·RNase 1 complex by altering the 85–94 loop. First, we created the N88R variant of RNase 1. We found that N88R RNase 1 binds to RI with high affinity (data not shown), consistent with results described by Batra and co-workers (16) and by Vilanova and co-workers (31) for the N88R/G89S variant of RNase 1. Consequently, we resorted to multiple substitutions (Table I). The L86E substitution was added to make the RNase 1 residues that flank Cys^{84} identical to those of RNase A (Fig. 1). Our hope was that this change would likewise equate the three-dimensional structures near Cys^{84}. The G89D and R91D substitutions were made to introduce additional steric and electrostatic strain into the RI·RNase 1 complex. Hereafter, the variant that contains the L86E, N88R, G89D, and R91D substitutions is referred to as ERDD RNase 1.

RNase A residue Lys^{41} is in the enzymic active site. The role of Lys^{41} in catalysis is to donate a hydrogen bond to the rate-limiting transition state (33). Arginine can replace Lys^{41} in RNase A catalysis, albeit with a 10^2-fold reduction in catalytic efficiency (33, 34). Lys^{41} makes van der Waals contacts with RI residues Tyr^{430} and Asp^{431} in the crystalline RI·RNase A complex (29, 30). Adding the K41R substitution to G88R RNase A reduces the affinity for RI by 20-fold (26). Moreover, K41R/G88R RNase A is 3-fold more toxic to K-562 cells than is G88R RNase A (26). Lys^{41} is conserved in RNase 1 (Fig. 1). Thus, we added the K41R substitution to ERDD RNase 1 to observe the impact of a decreased susceptibility to inactivation by RI but a loss of catalytic efficiency on the cytotoxic activity of ERDD RNase 1.

TABLE I
Amino acid substitutions in human pancreatic ribonuclease

Substitution	Secondary structure[a]	RI contact[b]
R4C	α-Helix	None
K41R	β-Strand	Tyr^{430}, Asp^{431}
L86E	β-Strand	Lys^{316}
N88R	Surface loop	Trp^{257}, Trp^{259}, Tyr^{433}
G89D	Surface loop	Glu^{202}, Trp^{257}, Trp^{259}
R91D	Surface loop	Trp^{257}, Glu^{283}, Trp^{314}
V118C	β-Strand	None

[a] From the structure of crystalline RNase A (62).
[b] From the structure of the crystalline RI·RNase A complex (29, 30).

The cytotoxic activity of a ribonuclease correlates with its conformational stability (27). Hence, we incorporated a non-native disulfide bond into ERDD RNase 1 in an attempt to increase its conformational stability and thereby its cytotoxicity. Previously, we and others described a particular variant of RNase A and RNase 1 in which Ala^4 and Val^{118} are each replaced with cysteine (27, 35). These cysteine residues form a disulfide bond that increases the conformational stability and decreases the proteolytic susceptibility of each protein. The RNase 1 variant that includes the Cys^4-Cys^{118} disulfide bond is designated with the prefix S-S.

Protein Production and Purification—RNase 1 and its variants were produced in *E. coli*. All proteins eluted as a single species during cation exchange chromatography and migrated as single bands of appropriate M_r during SDS-polyacrylamide gel electrophoresis (data not shown). RNase 1 and its variants also migrated as single bands on zymogram electrophoresis (36, 37), indicating that the preparations were free from contaminating ribonucleolytic activity (data not shown). Our expression and purification protocol yielded ~20 mg of wild-type RNase 1 and 5–20 mg of the RNase 1 variants/liter of *E. coli* growth medium.

Conformational Stability—The melting temperature (T_m) of RNase 1 was 56 °C (Table II). This value is slightly higher than that determined by Vilanova and co-workers (38). The discrepancy could reflect differences in solution conditions. The T_m of K41R RNase 1 was identical to that of the wild-type enzyme. The ERDD substitutions decreased the T_m by 4 °C compared with RNase 1. When the K41R and ERDD substitutions were combined in a single variant, the T_m was lower by 7 °C. As intended, adding the Cys^4-Cys^{118} disulfide bond increased the T_m of the ERDD variant, by 5 °C.

Ribonucleolytic Activity—The catalytic activity (k_{cat}/K_m) of RNase 1 and its variants was determined with a fluorogenic ribonuclease substrate (21). The value of k_{cat}/K_m for RNase 1 was 1.4×10^6 M^{-1} s^{-1} (Table II). Replacing Lys^{41} with arginine (K41R RNase 1) reduced the value of k_{cat}/K_m by 60-fold. A similar change was observed when the analogous substitution was made in RNase A (26, 34). The amino acid substitutions of ERDD RNase 1 did not have a deleterious effect on ribonucleolytic activity but instead caused a modest, 2-fold, increase in the value of k_{cat}/K_m compared with RNase 1. The ERDD substitutions likewise caused a 2-fold increase in the k_{cat}/K_m value in K41R RNase 1. Addition of the Cys^4-Cys^{118} disulfide bond reduced the k_{cat}/K_m value for ERDD RNase 1 by almost 3-fold to 1.0×10^6 M^{-1} s^{-1}.

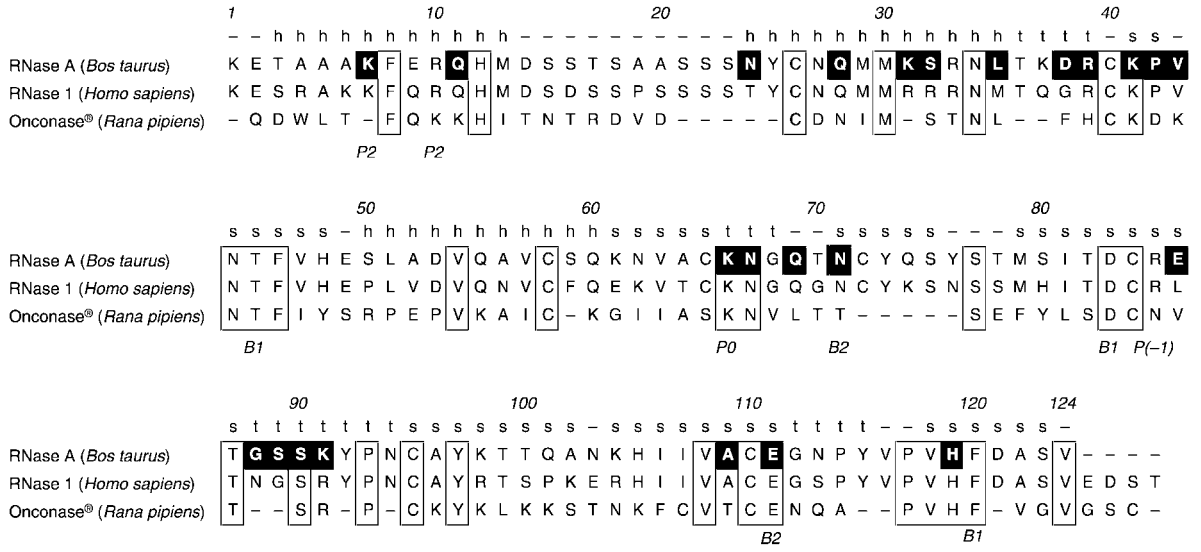

FIG. 1. **Amino acid sequences of RNase A, human pancreatic ribonuclease, and ONC.** Sequences were aligned using the PILEUP program, version 9, from the Genetics Computer Group (Madison, WI) with gap weight 1.00 and gap length weight 0.100. Residues are numbered according to RNase A. The RNase A secondary structure is identified with *h* (α-helix), *s* (β-strand), or *t* (turn). RNase A phosphoryl group binding subsites (*P*(−*1*), *P0*, *P1*, *P2*) and nucleobase binding subsites (*B1*, *B2*) are labeled. Residues conserved among all three ribonucleases are *boxed*. RNase A residues that contact RI in the RI·RNase A complex are *white* on *black* (29).

TABLE II
Attributes of human pancreatic ribonuclease, its variants, and ONC

Ribonuclease	T_m[a]	k_{cat}/K_m[b]	K_i[c]	IC_{50}[d]
	°C	(10^6 M^{-1} s^{-1})	nM	μM
RNase 1	56	1.4 ± 0.1	ND[e]	—[f]
K41R RNase 1	56	0.022 ± 0.06	ND[e]	—[f]
ERDD RNase 1	52	2.7 ± 0.3	0.21 ± 0.02	7
K41R/ERDD RNase 1	49	0.048 ± 0.001	0.7 ± 0.2	10
S-S/ERDD RNase 1	57	1.0 ± 0.1	2.6 ± 0.8	3
ONC	90	0.00022 ± 0.00001	≥10^3	0.4

[a] Values of T_m (± 2 °C) for RNase 1 and its variants were determined in PBS by UV spectroscopy. The T_m of ONC is from Ref. 20 and was determined by CD spectroscopy.
[b] Values of k_{cat}/K_m (± S.E.) are for catalysis of 6-FAM~dArUdAdA~6-TAMRA cleavage at pH 6.0 and 25 °C. The k_{cat}/K_m value for ONC is from Ref. 63.
[c] Values of K_i (± S.E.) for the RNase 1 variants are for inhibition of catalysis of 6-FAM~dArU$(dA)_2$~6-TAMRA cleavage at pH 6.0 and 25 °C by RI. The K_i value for ONC is an estimate from Ref. 15.
[d] Values of IC_{50} are for toxicity to K-562 cells (Fig. 3).
[e] ND, not determined.
[f] —, no toxicity was observed at a ribonuclease concentration of 10 μM.

Inhibition by Ribonuclease Inhibitor—An agarose gel-based assay was used as a qualitative measure of the affinity of RI for the RNase 1 variants. RNase 1 hydrolyzed rRNA in the absence of RI but was inhibited fully by a 2- or 4-fold excess of RI (Fig. 2). Like that of wild-type RNase 1, the ribonucleolytic activity of the K41R enzyme was inhibited by RI. In contrast, incorporation of the ERDD substitutions in wild-type RNase 1 or K41R RNase 1 yielded variants that retained ribonucleolytic activity in the presence of RI. Addition of the Cys^4-Cys^{118} disulfide bond to ERDD RNase 1 (S-S/ERDD RNase 1) further reduced susceptibility to RI.

The interaction between each RNase 1 variant and RI was quantitated by measuring cleavage of 6-FAM~dArU$(dA)_2$~6-TAMRA in the presence of increasing concentrations of RI (Table II). The results of this assay mirror those obtained with the agarose gel-based assay. Because of the extraordinarily tight binding of RI to wild-type RNase 1, it was not possible to determine a K_i value with this assay. Values of K_i for the interaction between RI and wild-type RNase 1 have been reported to be 2.0×10^{-11} M (16), 5.2×10^{-12} M (14) and 2.0×10^{-13} M (15). Because the concentration of ribonuclease used in the determination of these values was greater than the value of

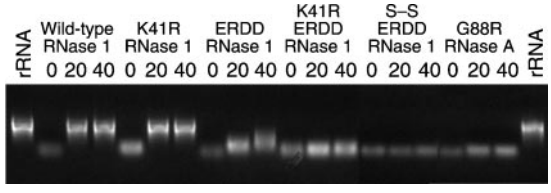

FIG. 2. **Agarose gel-based assay for inhibition of ribonucleolytic activity by RI.** Inhibition was assessed by visualizing the cleavage of 16 S and 23 S rRNA by a 10 ng of ribonuclease in the absence or presence of 20 or 40 units of RI (where 1 unit of RI is the amount required to inhibit the activity of 5 ng of RNase A by 50%).

K_i, these values provide only an upper limit for the actual K_i value (20, 28, 39). Alone, the K41R substitution does not disturb the interaction of RI and RNase 1 substantially, making it difficult to measure the K_i value with an assay based on ribonucleolytic activity. The value of K_i for ERDD RNase 1 was 2.1×10^{-10} M. Addition of the K41R variation to ERDD RNase 1 increased the K_i by ~3-fold. Addition of the Cys^4-Cys^{118} disulfide bond to ERDD RNase 1 yields a variant with K_i =

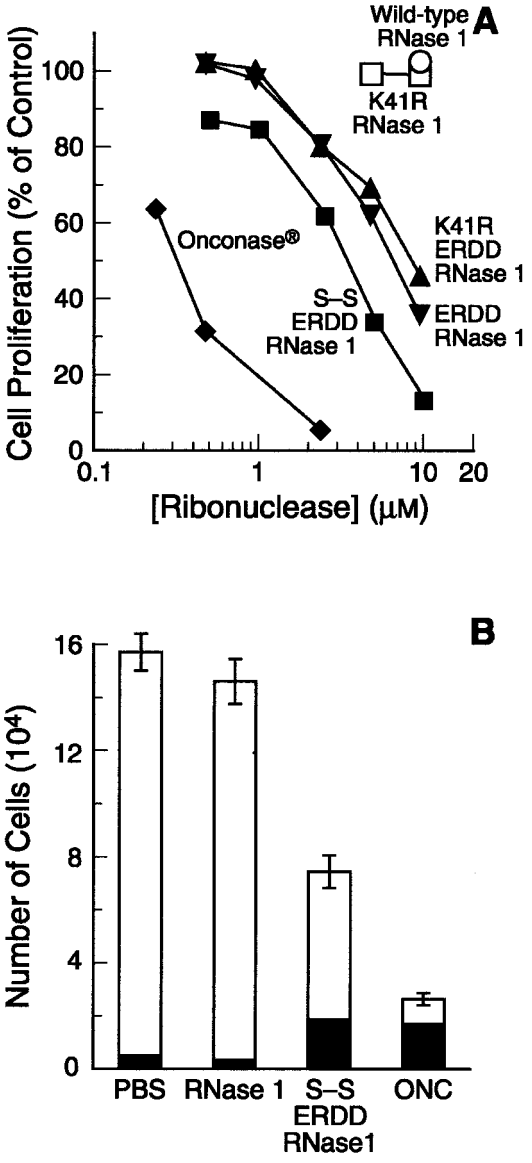

FIG. 3. **Proliferation of K-562 cells cultured in the presence of a ribonuclease.** *Panel A*, cell proliferation was measured by monitoring the incorporation of [*methyl*-^{3}H]thymidine into cellular DNA after a 44-h incubation with a ribonuclease. Values reported are the mean of three cultures and are expressed as the percentage of control cultures that lacked exogenous ribonucleases. *Panel B*, cells were counted by using a hemocytometer. K-562 cells (1×10^4) were cultured in the absence of exogenous ribonuclease or the presence of 5 μM ONC or 10 μM S-S/ERDD RNase 1 for 72 h. Viable cells were identified by their ability to exclude the vital dye trypan blue. *Bars* represent the total number of cells counted; *filled portions* represent the number of cells stained by trypan blue.

2.6×10^{-9} M. This value of K_i is 10-fold greater than that for ERDD RNase 1.

Cytotoxicity—We tested the effect of wild-type RNase 1 and its variants on cancer cell viability by measuring the ability of cells to incorporate a ^{3}H-labeled nucleotide (Fig. 3*A*). At the ribonuclease concentrations used in this assay ($\leq$10 μM), wild-type RNase 1 had no effect on cell viability. Likewise, K41R RNase 1 did not affect cell viability. In contrast, incorporation of the ERDD substitutions resulted in a RNase 1 variant that inhibited proliferation of K-562 cells with an IC_{50} of 7 μM. Incorporation of the K41R substitution into ERDD RNase 1 increased the IC_{50} value to 10 μM. Addition of the Cys^4-Cys^{118} disulfide bond to ERDD RNase 1 reduced the IC_{50} value to 3 μM. The IC_{50} value for ONC was 0.4 μM.

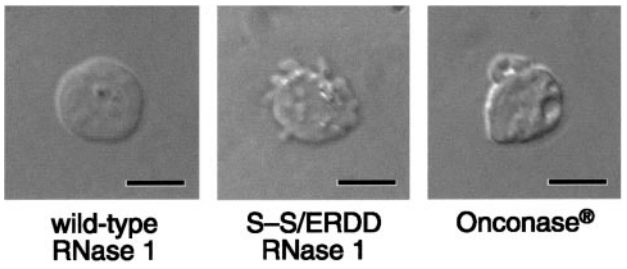

FIG. 4. **K-562 cell morphology after treatment with a ribonuclease.** K-562 cells were cultured in the presence of 10 μM RNase 1, 10 μM S-S/ERDD RNase 1, or 5 μM ONC for 72 h. Cells were then stained with trypan blue and visualized using light microscopy. The RNase 1 *panel i*s representative of a cell that was not stained by trypan blue. The S-S/ERDD RNase 1 and ONC *panels* are representative of cells that were stained by trypan blue. *Bar*, 12.5 μm.

As an alternative means to quantitate the effects of the RNase 1 variants on K-562 cell viability, we used trypan blue staining to count the number of viable cells following a 72-h exposure to the ribonucleases. ONC and S-S/ERDD RNase 1 reduced K-562 cell proliferation significantly compared with cells treated with PBS or RNase 1 (Fig. 3*B*). ONC and S-S/ERDD RNase 1 also rendered K-562 cells susceptible to staining by trypan blue; 63% of ONC-treated cells and 25% of S-S/ERDD RNase 1-treated cells were stained with trypan blue (Fig. 3*B*). When viewed at 40$\times$ magnification, K-562 cells treated with either ONC or S-S/ERDD RNase 1 and stained by trypan blue had the morphology expected from an apoptotic death (Fig. 4). Thus, it appears that like ONC, S-S/ERDD RNase 1 kills K-562 cells.

DISCUSSION

Mechanism of Ribonuclease Cytotoxicity—The mechanism of ribonuclease-mediated cytotoxicity is not known in detail (3). Still, the existing data are consistent with a mechanism that includes association with the cell surface, internalization, and translocation to the cytosol (Fig. 5; 12–17, 20, 29, 36). The first requisite step in ribonuclease cytotoxicity is an interaction between the ribonuclease and the plasma membrane of a target cell (Fig. 5). ONC binds to specific sites on the plasma membrane of cultured glioma cells with K_d values of 6.2×10^{-8} M and 2.5×10^{-7} M (6). The receptors have not been identified, and it is unclear whether these receptors are intrinsic to the glioma cells or if ONC binds to other cancer cells by using the same interactions. Similarly, cytotoxic ribonucleases from *Rana catesbeiana* (bullfrog) and *Rana japonica* (Japanese rice paddy frog) bind specifically to the plasma membrane of cancer cells (40–42).

After binding to a plasma membrane receptor, cytotoxic ribonucleases appear to be internalized by endocytosis (Fig. 5). Small molecules that inhibit ATP synthesis also prevent ONC cytotoxicity, consistent with the energy requirements of endocytosis (6). Postulating an endocytic mechanism, however, introduces a topological problem. Ribonucleases kill by hydrolyzing RNA and therefore must reach the cytosol to exert their lethal enzymatic activity. The site and mechanism of bilayer transversal are unknown. Because only a few molecules of a cytotoxic ribonuclease are required to kill a cell (43), delineating the transbilayer movement of ribonucleases in molecular terms remains a formidable challenge (44).

Once in the cytosol, ribonucleases encounter RI (Fig. 5). Mammalian homologs of RNase A, including RNase 1, are highly susceptible to inactivation by RI, provoking speculation that RI acts to preserve cellular RNA should a pancreatic-type ribonuclease inadvertently enter the cytosol (19). Consistent with this hypothesis, the catalytic activity of cytotoxic ribo-

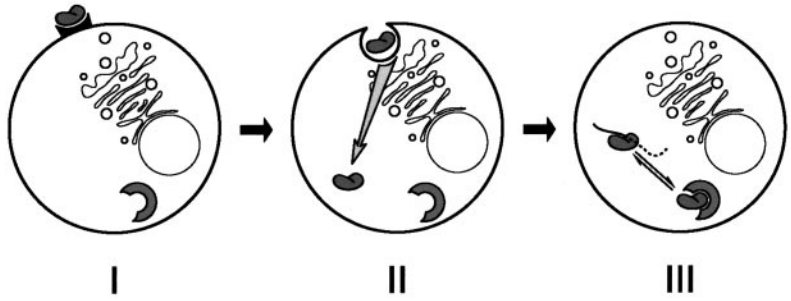

FIG. 5. **Putative cellular routing of a cytotoxic pancreatic-type ribonuclease.** *I*, the ribonuclease first interacts with the surface of a target cell. *II*, the ribonuclease is internalized and crosses a lipid bilayer to reach the cytosol. The site of bilayer transversal is unknown. *III*, in the cytosol, the ribonuclease encounters the RI protein. A ribonuclease that evades RI catalyzes cleavage of cellular RNA and thereby causes cell death.

nucleases, including ONC and the *R. catesbeiana* ribonuclease, is not inhibited by RI (15, 45). Thus, cytotoxic ribonucleases are able to destroy information carried by cellular RNA. Ultimately, this insult proves to be lethal (Figs. 3*B* and 4).

A Cytotoxic RNase 1 Variant—If the interaction between a ribonuclease and RI is indeed a determinant of cytotoxicity, then RNase 1 variants that retain ribonucleolytic activity in the presence of RI could destroy cellular RNA and cause cell death. To weaken the interaction between RI and RNase 1, we made four amino acid substitutions in a surface loop to yield ERDD RNase 1 (Table I). The ERDD substitutions reduce the T_m by 4 °C and cause an unexpected 2-fold increase in catalytic activity compared with RNase 1. Significantly, the ERDD substitutions yield a variant with reduced affinity for RI. Indeed, the K_i value for ERDD RNase 1 is 2.1×10^{-10} M, which is $\sim 10^3$-fold greater than that of RNase 1. Weakening the interaction with RI correlates with a pronounced cytotoxic activity because ERDD RNase 1 is toxic to K-562 cells with an IC_{50} value of 7 μM (Fig. 3). In contrast, a 10 μM dose of RNase 1 has no effect on K-562 cell proliferation, consistent with previous investigations on SVT2 mouse fibroblast cells (8), Bl6/BL6 mouse melanoma cells (12), U251 rat glioma cells (15), and 9L rat glioma cells (22). Surprisingly, Batra and co-workers (16) report that RNase 1 is indeed cytotoxic to K-562 cells (IC_{50} = 10 μM) and to J774A.1 mouse monocyte-macrophage cells (IC_{50} = 5 μM). Based on data presented in this paper (Figs. 3 and 4) and elsewhere (8, 12, 15, 22), we conclude that RNase 1 has insignificant cytotoxic activity. Thus, we and others digress from Batra and co-workers, and we urge caution in interpreting their data on cytotoxicity as well as their data on inhibition by RI (see above). We do believe, however, that RNase 1 has all of the necessary features of a cytotoxic ribonuclease except for resistance to RI. RNase 1 likely associates with the cell surface and then translocates to the cytosol. If resistant to RI, RNase 1 has sufficient stability and catalytic activity to cause cell death.

Residues 31–33 of RNase 1 comprise a putative nuclear localization signal (Fig. 1) (46). This sequence is not conserved in RNase A or ONC but is present in angiogenin, a homolog that causes neovascularization. Angiogenin is internalized by subconfluent endothelial cells and accumulates in the nucleus (47–49). Nuclear localization appears to be essential for the angiogenic activity of angiogenin; the R33A variant of angiogenin is not localized to the nucleus and is not angiogenic (47, 48). To test whether residues 31–33 play a role in the cytotoxicity of RNase 1, we created the R32A and the R33A variants of ERDD RNase 1. The IC_{50} values for both R32A/ERDD RNase 1 and R33A/ERDD RNase 1 were identical to that of ERDD RNase 1 (data not shown). Thus, the mechanism of ERDD RNase 1 cytotoxicity is unlikely to involve nuclear localization.

D'Alessio and co-workers have described a dimeric variant of RNase 1 with cytotoxic activity (8). Interestingly, the dimer was cytotoxic only after it was treated with an aminopeptidase to remove the N-terminal methionine residue [Met(-1)] incorporated during heterologous expression in *E. coli*. The cDNA used to produce RNase 1 and its variants described this paper also codes for an N-terminal methionine. We made no effort to remove Met(−1) from RNase 1 or its variants. Nonetheless, the ERDD, K41R/ERDD, and S-S/ERDD variants of RNase 1 are potent cytotoxins (Fig. 3). Thus, it appears that a native N terminus is not necessary for a monomeric variant of RNase 1 to be cytotoxic.

Residues 88–90 of RNase 1 comprise a consensus Asn-Xaa-Ser/Thr *N*-glycosylation sequence (Fig. 1) (50). We have shown that replacing residues 86, 88, 89, and 91 yields a variant of RNase 1 which evades RI and is cytotoxic (Table II). Likewise, glycosylation of Asn^{88} may enable RNase 1 to evade RI. Interestingly, Asn^{88} of RNase 1 is glycosylated in humans, though only to a minor extent (51).

Enhancing RNase 1 Cytotoxicity—Despite the amino acid substitutions, ERDD RNase 1 still binds to RI with much higher affinity than does ONC (Table II). This difference is likely manifested in cytotoxic activity; ONC is almost 20 times more cytotoxic than is ERDD RNase 1. We sought to increase the cytotoxicity of ERDD RNase 1 by incorporating an additional amino acid substitution that would increase further its ability to evade RI.

Previously, we showed that K41R/G88R RNase A is more cytotoxic than is G88R RNase A (26). Based on this result, we anticipated that replacing Lys^{41} with an arginine residue in ERDD RNase 1 would potentiate the cytotoxic activity of this variant. Surprisingly, K41R/ERDD RNase 1 is no more toxic to K-562 cells than is ERDD RNase 1 (Fig. 3). As intended, the K41R substitution in ERDD RNase 1 does weaken further binding to RI. In addition, the K41R substitution causes changes deleterious to cytotoxic activity. First, it reduces catalytic activity by 60-fold (Table II). Second, it reduces further the conformational stability of the variant (Table II). We conclude that the K41R substitution does not potentiate the cytotoxic activity of ERDD RNase 1 because the favorable change in the K_i value does not compensate for the unfavorable loss of catalytic activity and conformational stability.

As an alternative means to potentiate the cytotoxicity of RNase 1, we added a non-native disulfide bond to the ERDD variant. We had used this strategy previously to increase the cytotoxicity of G88R RNase A (27). At 3 μM, the IC_{50} value of S-S/ERDD RNase 1 is nearly 3-fold less than that of ERDD RNase 1 (Fig. 3) and only 8-fold greater than that of ONC (Table II). Again, the change in cytotoxicity is a consequence of changes to conformational stability and affinity for RI.

To create S-S/ERDD RNase 1, residues Arg^4 and Val^{118} of ERDD RNase 1 were each replaced with a cysteine residue. The melting temperature of S-S/ERDD RNase 1 is 5 °C higher than that of ERDD RNase 1 (Table II). Thus, S-S/ERDD RNase 1 is less prone to denaturation than is ERDD RNase 1. Because the proteolytic susceptibility of a protein correlates with its conformational stability (27, 52, 53), the non-native Cys^4-Cys^{118} disulfide bond likely enhances the cytotoxicity of ERDD RNase 1 by preserving its structural integrity and hence its ribonucleolytic activity in the cytosol.

The Cys^4-Cys^{118} disulfide bond makes a second, favorable contribution to the cytotoxic activity of the S-S/ERDD variant. The K_i value for S-S/ERDD RNase 1 inhibition by RI is 10-fold greater than that of ERDD RNase 1 (Fig. 2 and Table II). The addition of the Cys^4-Cys^{118} disulfide bond to ERDD RNase 1 likely causes a subtle reorientation of the N-terminal α-helix and C-terminal β-strand, which disrupts intermolecular contacts with RI and thereby lowers the affinity for RI.

Estimation of Cytosolic Ribonucleolytic Activity—Cytotoxic ribonucleases kill cells because they are able to degrade RNA in the presence of RI. The amount of ribonucleolytic activity manifested in the cytosol can be approximated with Equation 2 (26, 54).

$$(k_{cat}/K_m)_{cytosol} = (k_{cat}/K_m)\left(\frac{K_i}{K_i + [RI]}\right) \quad \text{(Eq. 2)}$$

To calculate $(k_{cat}/K_m)_{cytosol}$, we use the values of k_{cat}/K_m and K_i in Table II and estimate that [RI] = 1 μM (55, 56). Thus, the values of $(k_{cat}/K_m)_{cytosol}$ for K41R/ERDD RNase 1, ERDD RNase 1, and S-S/ERDD RNase 1 are 3×10^1, 6×10^2, and 2.5×10^3 M^{-1} s^{-1}, respectively. It is intriguing that as the value of $(k_{cat}/K_m)_{cytosol}$ increases, the value of IC_{50} decreases.

Prospectus—The favorable therapeutic index of ONC appears to be preserved in cytotoxic mammalian ribonucleases. For example, a dimeric variant of RNase 1 was many times more toxic to malignant cell lines than to their nonmalignant counterparts (8). Similarly, we have found that G88R RNase A is more toxic to malignant cell lines than to nonmalignant cell lines.[2]

The basis for the therapeutic index of the cytotoxic ribonucleases is unknown. Changes to the plasma membrane, perhaps up-regulation of a receptor, may increase the susceptibility of cancerous cells to cytotoxic ribonucleases. Alternatively, the cellular routing of ribonucleases may differ between cancerous cells and their normal counterparts. Finally, because of their accelerated growth rate, cancerous cells may be more sensitive to the integrity of their RNA than are noncancerous cells.

The RNase 1 variants described in this paper could have advantages as cancer chemotherapeutics compared with other cytotoxic ribonucleases, natural or non-natural. Most importantly, the RNase 1 variants are derived from a human protein. The amino acid sequences of ERDD RNase 1 and S-S/ERDD RNase 1 are 97 and 95% identical, respectively, to that of RNase 1. In contrast, the amino acid sequence of ONC is only 25% identical to that of RNase 1 (Fig. 1) (57). Hence, the chemotherapeutic efficacy of the RNase 1 variants is less likely to be curtailed by an immune response. In addition, when ONC or RNase 1 are injected into mice, 50% of ONC but only 1% of RNase 1 is found in the kidney after 3 h (57, 58). Thus, renal retention could limit the efficacy of ONC, as it limits the dosage of ONC (4, 5), but have no effect on the efficacy of an RNase 1 variant.

The RNase 1 variants are toxic to K-562 cells in the absence of added small molecule chemotherapeutics. The IC_{50} value of ONC decreases when administered in combination with tamoxifen, trifluoroperazine, cisplatin, lovostatin, or vincristine (59–61). Likewise, the chemotherapeutic efficacy of ERDD RNase 1 and S-S/ERDD RNase 1 would likely increase as a component of a combination therapy regime.

Finally, the RNase 1 variants are small, monomeric proteins. They contain only natural amino acid residues and require no special *in vitro* processing. Accordingly, the variants can be produced and isolated readily on a large scale as well as incorporated directly into a gene therapy protocol.

[2] M. C. Haigis and R. T. Raines, unpublished result.

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