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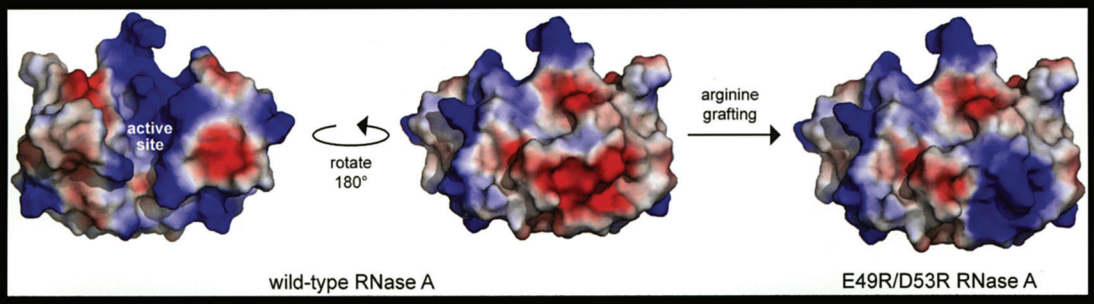


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# Increasing the potency of a cytotoxin with an arginine graft

**Stephen M. Fuchs[1,3], Thomas J. Rutkoski[1], Vanessa M. Kung[2], Ryan T. Groeschl[1] and Ronald T. Raines[1,2,4]**

[1]Department of Biochemistry, University of Wisconsin–Madison, 433 Babcock Drive, Madison, WI 53706-1544, USA, [2]Department of Chemistry, University of Wisconsin–Madison, 1101 University Avenue, Madison, WI 53706-1322, USA and [3]Present address: Lineberger Comprehensive Cancer Center, The University of North Carolina–Chapel Hill, 405 MEJ Building, Chapel Hill, NC 27599, USA.

[4]To whom correspondence should be addressed.
E-mail: raines@biochem.wisc.edu

**Variants and homologs of bovine pancreatic ribonuclease (RNase A) can exhibit cytotoxic activity. This toxicity relies on cellular internalization of the enzyme. Residues Glu49 and Asp53 form an anionic patch on the surface of RNase A. We find that replacing these two residues with arginine does not affect catalytic activity or affinity for the cytosolic ribonuclease inhibitor (RI) protein. This 'arginine graft' does, however, increase toxicity towards human cancer cells. Appending a nonaarginine domain to this cationic variant results in an additional increase in cytotoxicity, providing one of the most cytotoxic known variants of RNase A. These findings correlate the potency of a ribonuclease with its deliverance of ribonucleolytic activity to the cytosol, and indicate a rational means to enhance the efficacy of ribonucleases and other cytotoxic proteins.**

*Keywords*: cancer/Coulombic interaction/protein transduction domain/ribonuclease/ribonuclease inhibitor

## Introduction

Ribonucleases can be cytotoxic and thus have notable potential as chemotherapeutic agents (Youle and D'Alessio, 1997; Leland and Raines, 2001; Matoušek, 2001; Makarov and Ilinskaya, 2003; Benito *et al*., 2005; Arnold and Ulbrich-Hofmann, 2006). Bovine pancreatic ribonuclease (RNase A; EC 3.1.27.5) is the most-studied ribonuclease (Cuchillo *et al*., 1997; Raines, 1998). Although wild-type RNase A is not toxic to mammalian cells, properly engineered variants are cytotoxic (Rutkoski *et al*., 2005).

The cytotoxicity of a mammalian ribonuclease relies on several attributes (Bretscher *et al*., 2000; Dickson *et al*., 2003). First and foremost, ribonucleolytic activity is required for cytotoxicity (Ardelt *et al*., 1991; Kim *et al*., 1995). The cytotoxicity of a ribonuclease also correlates with its ability to evade the cytosolic ribonuclease inhibitor (RI) protein (Haigis *et al*., 2003; Dickson *et al*., 2005; Rutkoski *et al*., 2005; Johnson *et al*., 2007). Even single amino acid substitutions that weaken the affinity for RI make RNase A cytotoxic (Leland *et al*., 1998). The potency of cytotoxic RNase A variants is increased by enhancing their conformational stability (Klink and Raines, 2000). Lastly, a ribonuclease must reach the cytoplasm to engage and then degrade RNA. Mammalian ribonucleases bind to the cell surface through Coulombic interactions between cationic residues and anionic cell-surface molecules (Notomista *et al*., 2006), and are then endocytosed via a dynamin-independent pathway without the need for a specific receptor (Haigis and Raines, 2003). The efficiency of internalization is thus a key determinant of cytotoxicity (Wu *et al*., 1995), and can be increased by fusion to transferrin (Suzuki *et al*., 1999) or by making non-covalent or covalent oligomers that serve to increase molecular charge (Matoušek *et al*., 2003a; Libonati, 2004; Leich *et al*., 2006).

Cationic molecules are taken up readily by mammalian cells (Ryser and Hancock, 1965; Fuchs and Raines, 2006). By condensing the carboxyl groups of RNase A with ethylenediamine, Yamada and coworkers endowed the enzyme with cytotoxic activity but also diminished by 20-fold its enzymatic activity (Futami *et al*., 2002). This dichotomous result highlights a severe limitation of chemical cationization—the reaction is non-specific, producing a complex mixture of products and compromising function by allowing the modification of residues important for substrate binding or catalysis. In addition, chemical cationization attenuated the affinity of RNase A for RI, making it difficult to tease apart the individual contributions of increased positive charge and decreased affinity for RI in enhancing cytotoxic activity.

Here, we employ a new approach, 'arginine grafting' (Fuchs and Raines, 2007), to create a more cell-permeable variant of RNase A. The premise is that installing a patch of arginine residues improves affinity for the cell surface and hence cellular internalization, without affecting other properties of the enzyme. The resulting variant is a single, well-defined protein that can be produced by recombinant DNA technology alone. Previously, replacing five dispersed acidic residues with lysine was shown to endow a microbial ribonuclease with cytotoxic activity (Ilinskaya *et al*., 2002). By replacing only two proximal acidic residues with arginines, we create an arginine patch that increases the cytotoxicity of an RNase A variant. By also adding a protein transduction domain, we increase cytotoxicity even further.

## Materials and methods

### Cells and chemicals

*Escherichia coli* strains BL21(DE3) pLysS and BL21(DE3) were from Novagen (Madison, WI, USA). Human erythroleukemia cells (line K-562) were from the American Type Culture Collection (Manassas, VA, USA). [methyl-$^3$H] Thymidine (6.7 Ci/mmol) was from NEN Life Science Products (Boston, MA, USA). All other chemicals and

*Abbreviations*: DTT, dithiothreitol; 6-FAM, 6-carboxyfluorescein; hRI, human ribonuclease inhibitor; MALDI–TOF, matrix-assisted laser desorption ionization–time-of-flight; PBS, phosphate-buffered saline; $R_9$, nonaarginine; RI, ribonuclease inhibitor; RNase A, bovine pancreatic ribonuclease; 6-TAMRA, 6-carboxytetramethylrhodamine; Tris, tris(hydroxymethyl) aminomethane; *Z*, net molecular charge: Arg + Lys − Asp − Glu.

reagents were of commercial reagent grade or better, and were used without further purification.

### *Instruments*

The mass of each RNase A variant was ascertained by MALDI–TOF mass spectrometry using a Voyager-DE-PRO Biospectrometry Workstation (Applied Biosystems, Foster City, CA, USA) with 3,5-dimethoxy-4-hydroxycinnamic acid as a matrix in the campus Biophysics Instrumentation Facility. Fluorescence measurements were performed with a QuantaMaster 1 photon counting fluorometer equipped with sample stirring (Photon Technology International, South Brunswick, NJ, USA). Radioactivity was quantified with a Microbeta TriLux liquid scintillation counter (Perkin Elmer, Wellesley, MA, USA). Electrostatic potential maps were created with the program PyMol (DeLano Scientific, South San Francisco, CA, USA).

### *Site-directed mutagenesis*

Oligonucleotides were obtained from Integrated DNA Technology (Coralville, IA, USA). cDNA encoding variants of RNase A were created in plasmid pBXR, which directs the production of RNase A in *E.coli* (delCardayré *et al*., 1995), by using the QuikChange mutagenesis kit from Stratagene (La Jolla, CA, USA). All variants of RNase A possessed either the native Lys1 residue (wild-type RNase A, G88R RNase A, E49/D53R RNase A and E49/D53R/G88R RNase A) or an N-terminal methionine residue ($R_9$-tagged variants of RNase A), which has no effect on ribonucleolytic activity (Arnold *et al*., 2002). The C-terminal $R_9$ tag was distanced from the remainder of a protein by a triglycine linker.

### *Production and purification of protein variants*

Untagged variants of RNase A and Onconase [which is the most cytotoxic known homolog of RNase A (Matoušek *et al*., 2003b)] were produced in *E.coli* BL21(DE3) and purified as described previously (Leland *et al*., 1998). Variants of RNase A containing a C-terminal $R_9$ tag were produced in BL21(DE3)pLysS cells and purified as described previously (Fuchs and Raines, 2005).

### *Assay of enzymatic activity*

Ribonucleolytic activity was measured by monitoring the increase in the fluorescence of 6-FAM–dArU$(dA)_2$–6-TAMRA (Integrated DNA Technologies, Coralville, IA, USA) upon enzyme-catalyzed cleavage, as described previously (Kelemen *et al*., 1999) with minor modifications. Polyarginine-containing peptides are known to bind to glass surfaces (Chico *et al*., 2003). We observed this phenomenon with RNase A (Fuchs and Raines, 2005), and so performed all enzymatic activity assays in 10 mM Bis-Tris–HCl buffer, pH 6.0, containing NaCl (0.50 M). In this high-salt buffer, the binding of protein to a quartz cuvette was found to be insignificant.

### *Assay of conformational stability*

As RNase A is denatured, its six tyrosine residues become exposed to solvent and its molar absorptivity at 287 nm decreases significantly (Hermans and Scheraga, 1961). Unfolding was monitored in PBS by the change in absorbance at 287 nm as the temperature was raised at a rate of 0.15°C/min. Data were fitted to a two-state model to calculate the value of $T_m$ (Pace *et al*., 1998), which is the temperature at the midpoint of the transition between the folded and unfolded states.

### *Assay of ribonuclease-inhibitor binding*

The affinity of RNase A variants for human RI (hRI) was determined by using a competitive binding assay reported recently (Lavis *et al*., 2007). Unlike other assays (Abel *et al*., 2002), this assay exhibits negligible protein loss by non-specific binding, allowing the determination of $K_d$ values for the highly cationic $R_9$-tagged variants of RNase A.

### *Assay of cytotoxicity*

The effect of ribonucleases on the proliferation of K-562 cells was determined by measuring the incorporation of [methyl-$^3$H]thymidine into cellular DNA as described previously (Leland *et al*., 1998; Rutkoski *et al*., 2005).

## Results and discussion

### *Design of cationic RNase A variants*

The ability of polycations to effect the cellular internalization of a macromolecule has been known for over 40 years (Ryser and Hancock, 1965; Fuchs and Raines, 2006). Recently, we used an arginine 'graft' to endow the green fluorescent protein from the jellyfish *Aequorea victoria* with cell permeability (Fuchs and Raines, 2007). We have now applied the arginine grafting strategy to RNase A, a mammalian protein that can be cytotoxic.

We used the cytotoxic activity of an RNase A variant as a measure of its cellular internalization. This measure, though indirect, requires the internalized ribonuclease to retain its catalytic activity (and, thus, its three-dimensional structure) to elicit cytotoxicity (Ardelt *et al*., 1991; Kim *et al*., 1995). Accordingly, most of our variants contained the G88R substitution, which makes RNase A cytotoxic by raising the $K_d$ value for the hRI·RNase A complex (Leland *et al*., 1998).

We sought to increase the cationicity of RNase A without disrupting catalytic activity. In an electrostatic potential map of RNase A, we noted the presence of two acidic residues on its molecular surface (Fig. 1A and B). These residues, Glu49 and Asp53, are in close proximity to one another but remote from the active site. We hypothesized that replacing these acidic residues with basic ones would create a cationic patch without compromising catalytic activity. We chose to replace Glu49 and Asp53 with arginine, which is the most effective residue for facilitating cellular internalization (Mitchell *et al*., 2000; Suzuki *et al*., 2002). Recently, we showed that appending an $R_9$ tag to the C-terminus of RNase A increased its internalization (Fuchs and Raines, 2005). Hence, we also determined if an arginine graft and an $R_9$ tag have an additive effect on cytotoxic activity.

### *Biochemical properties of RNase A variants*

The E49R/D53R-containing variants of RNase A exhibited wild-type catalytic activity (Table I), as expected. The conformational stability of the E49R/D53R and E49R/D53R/G88R variants ($T_m = 54$°C) was, however, significantly lower than that of either wild-type RNase A ($T_m = 64$°C) or the G88R variant ($T_m = 60$°C). Appending an $R_9$ tag to the E49R/D53R or E49R/D53R/G88R variant decreased stability

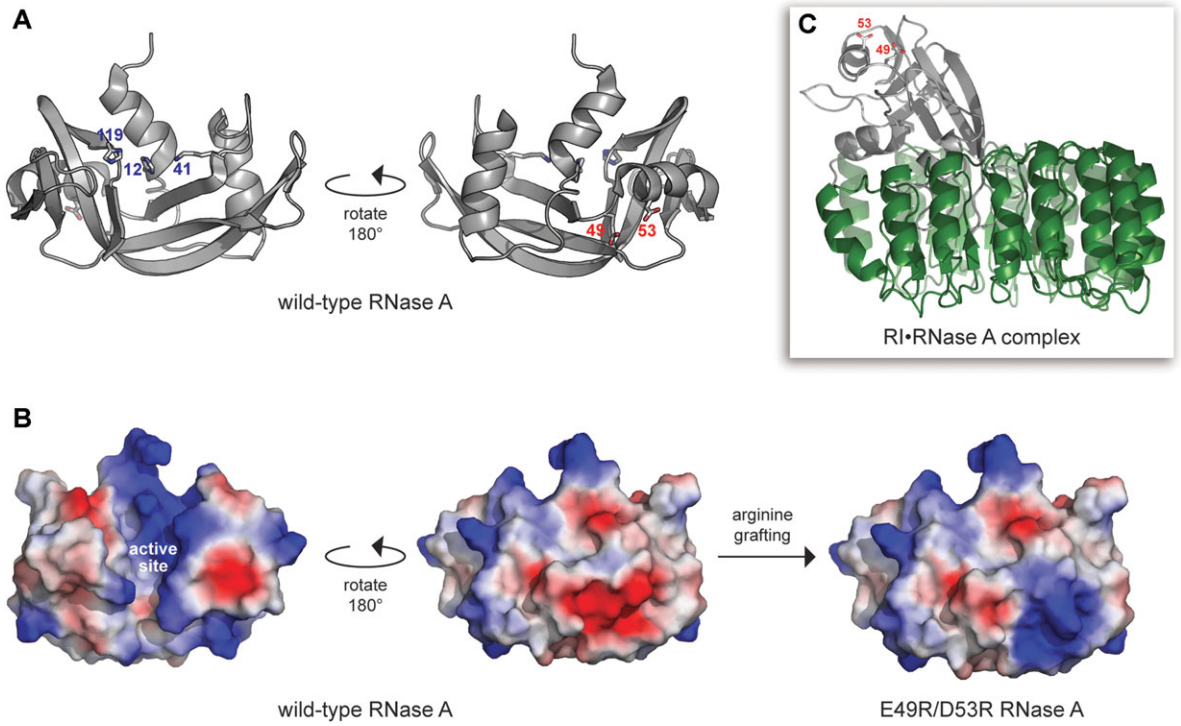


**Fig. 1.** Arginine grafting of RNase A. (**A**) Ribbon diagram of RNase A based on PDB entry 7RSA. The side chain of residues in the active site (His12, Lys41 and His119) and Glu49 and Asp53 are shown explicitly. (**B**) Electrostatic potential map of wild-type RNase A and a model of its E49R/D53R variant (blue, cationic; red, anionic). (**C**) Ribbon diagram of the porcine RI·RNase A complex based on PDB entry 1DFJ.

even further. Still, all variants were >99% folded at physiological temperature.

The ability to evade the RI protein within mammalian cells is a strong determinant of ribonuclease cytotoxicity (Leland *et al*., 1998; Rutkoski *et al*., 2005; Johnson *et al*., 2007). Glu49 and Asp53 are remote from the molecular interface in the porcine RI·RNase A complex (Fig. 1C). Nevertheless, we measured the affinity of both E49R/D53R/G88R RNase A and E49R/D53R/G88R RNase A–$R_9$ for RI (Table I), finding these affinities ($K_d$ = 2.6 nM and $K_d$ = 3.0 nM, respectively) to be comparable to that of G88R RNase A ($K_d$ = 2.8 nM).

### *Cytotoxicity of RNase A variants*

The toxicity of a ribonuclease increases as its cellular internalization becomes more efficient (Fuchs and Raines, 2005). We monitored the internalization of the RNase A variants by measuring their toxicity to K-562 cells (Fig. 2; Table I). Wild-type RNase A is not toxic to mammalian cells due to

**Table I.** Biochemical parameters of RNase A and its variants

| Ribonuclease | p*I*[a] | $T_m$ (°C)[b] | $k_{cat}/K_M$ ($10^6$ $M^{-1}s^{-1}$)[c] | $IC_{50}$ (μM)[d] | $K_d$ (nM) |
|---|---|---|---|---|---|
| RNase A | 8.6 | 64[e] | 2.34 ± 0.09 | >25 | $44 \times 10^{-6}$[f] |
| G88R RNase A | 8.8 | 60[e] | 2.9 ± 0.1 | 6.2 ± 0.5 | 2.8 ± 0.1 |
| E49R/D53R RNase A | 9.2 | 54 | 2.21 ± 0.02 | >25 | <2.8[g] |
| E49R/D53R/G88R RNase A | 9.3 | 54 | 2.5 ± 0.1 | 1.9 ± 0.2 | 2.6 ± 0.8 |
| RNase A–$R_9$ | 9.6 | 56[e] | 1.0 ± 0.2[e] | >25[e] | ND |
| G88R RNase A–$R_9$ | 9.8 | 54[e] | 9.6 ± 0.2[e] | 1.9 ± 0.1[h] | ND |
| E49R/D53R RNase A–$R_9$ | 10.0 | 48 | 1.55 ± 0.03 | 6.0 ± 0.3 | <2.8[g] |
| E49R/D53R/G88R RNase A–$R_9$ | 10.1 | 49 | 3.59 ± 0.05 | 0.58 ± 0.02 | 3.0 ± 1.1 |

ND, not determined.
[a]Values of p*I* were estimated from amino-acid composition (Bjellqvist *et al*., 1993; Bjellqvist *et al*., 1994).
[b]Values of $T_m$ (±2°C) were determined in PBS by ultraviolet spectroscopy.
[c]Values of $k_{cat}/K_M$ (±SE) are for the catalysis of 6-FAM–dArU$(dA)_2$–6-TAMRA cleavage at 25°C in 10 mM Bis-Tris–HCl buffer, pH 6.0, containing NaCl (0.50 M).
[d]Values of $IC_{50}$ (±SE) are for the incorporation of [methyl-$^3$H]thymidine into the DNA of K-562 cells (Fig. 2).
[e]Value from Fuchs and Raines (2005).
[f]Value from Lee *et al*. (1989).
[g]Value was below the lower limit of detection for the assay.
[h]Raw data from Fuchs and Raines (2005) fitted in the manner of this work.

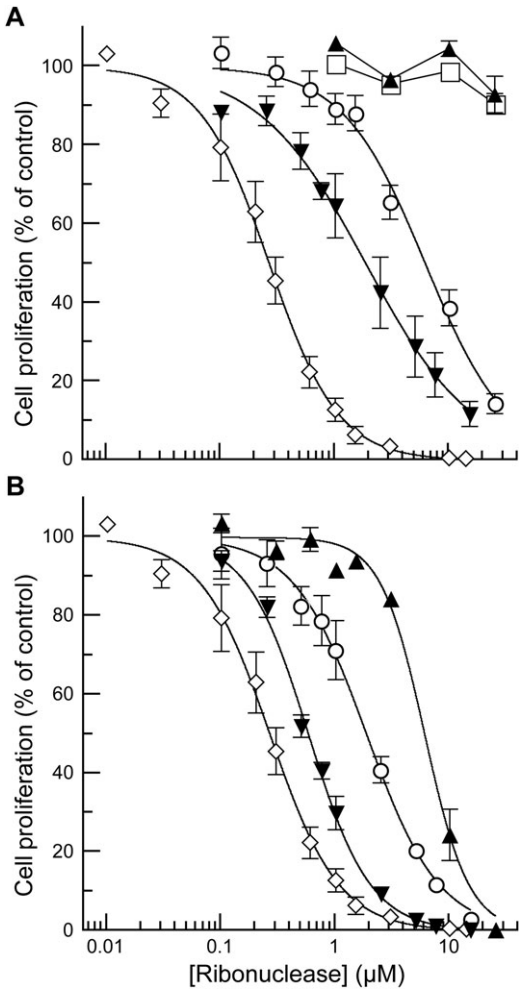


**Fig. 2.** Cytotoxicity of Onconase (◇), wild-type RNase A (□), and its G88R (○), E49R/D53R (▲), and E49R/D53R/G88R (▼) variants. Data in panel B are for RNase A variants with a C-terminal $R_9$ tag. Data for Onconase are depicted in both panels to facilitate comparisons. Cell viability was measured by monitoring [methyl-$^3$H]thymidine incorporation into the DNA of K-562 cells after a 44 h incubation with a ribonuclease. Data points are for the mean (±SE) of three experiments; values of $IC_{50}$ (±SE) are listed in Table I.

its high affinity for the cytosolic RI protein. The RI-evasive variant, G88R RNase A, had an $IC_{50}$ value of 6.2 μM. Arginine grafting increased the cytotoxicity of this variant by 3-fold. In contrast, E49R/D53R RNase A was not toxic to K-562 cells.

Finally, the addition of an $R_9$-tag to either G88R RNase A or E49R/D53R/G88R RNase A increased cytotoxicity by an additional 3-fold, with G88R RNase A–$R_9$ and E49R/D53R/G88R RNase A–$R_9$ exhibiting $IC_{50}$ values of 1.9 and 0.58 μM, respectively (Fig. 2B; Table I). Thus, the cumulative effect of arginine grafting and the addition of an $R_9$ tag increased cytotoxic activity by an order of magnitude. E49R/D53R/G88R RNase A–$R_9$ is one of the most toxic of known variants for K-562 cells (Rutkoski *et al*., 2005).

Surprisingly, E49R/D53R RNase A–$R_9$, which retains high affinity for RI, exhibited cytotoxicity ($IC_{50} = 6.0$ μM) comparable to that of G88R RNase A. This result was unexpected because RNase A–$R_9$ ($Z = +13$) does not exhibit cytotoxic activity (Fuchs and Raines, 2005). We suspect that the highly cationic E49R/D53R RNase A–$R_9$ ($Z = +17$) is internalized so efficiently that its concentration in the cytosol overwhelms endogenous RI. A similar explanation has been put forth to explain the cytotoxic activity of otherwise RI-sensitive ribonucleases (Leich *et al*., 2006) of which there have been several reports (Notomista *et al*., 2006; Bosch *et al*., 2004; Naddeo *et al*., 2005).

## Summary

The cytotoxicity of a ribonuclease arises from its degradation of cellular RNA. Herein, we demonstrated that this cytotoxic activity can be enhanced by the installation of an arginine graft. The increased efficiency of internalization presumably afforded by the arginine graft can be used in conjunction with an $R_9$ protein transduction domain to achieve additive enhancements in cytotoxicity. In the absence of an $R_9$ tag, evasion of RI is necessary for a permeant variant to be cytotoxic. Accordingly, the potency of a ribonuclease correlates with its ability to manifest unfettered ribonucleolytic activity in the cytosol. These findings inform the design and engineering of ribonucleases with enhanced cytotoxic activity and clinical utility.

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