KFERQ Sequence in Ribonuclease A-mediated Cytotoxicity*

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Onconase® (ONC) is an amphibian ribonuclease that is in clinical trials as a cancer chemotherapeutic agent. ONC is a homolog of ribonuclease A (RNase A). RNase A can be made toxic to cancer cells by replacing Gly88 with an arginine residue, thereby enabling the enzyme to evade the endogenous cytosolic ribonuclease inhibitor protein (RI). Unlike ONC, RNase A contains a KFERQ sequence (residues 7–11), which signals for lysosomal degradation. Here, substitution of Arg10 of the KFERQ sequence has no effect on either the cytotoxicity of G88R RNase A or its affinity for RI. In contrast, K7A/G88R RNase A is nearly 10-fold more cytotoxic than G88R RNase A and has more than 10-fold less affinity for RI. Up-regulation of the KFERQ-mediated lysosomal degradation pathway has no effect on the cytotoxicity of these ribonucleases. Thus, KFERQ-mediated degradation does not limit the cytotoxicity of RNase A variants. Moreover, only two amino acid substitutions (K7A and G88R) are shown to endow RNase A with cytotoxic activity that is nearly equal to that of ONC.

Onconase® (ONC)1 is a homolog of ribonuclease A (RNase A; EC 3.1.27.5) from the Northern leopard frog, Rana pipiens (1, 2). ONC demonstrates both antitumor and antiviral activity, and is in Phase III clinical trials for the treatment of malignant mesothelioma (for reviews, see Refs. 3–8). The most damaging side effect from ONC treatment is renal toxicity, which is dosage-dependent and reversible (9, 10). Studies in mice have demonstrated that ONC is retained in the kidneys, in contrast to mammalian ribonucleases (11). Hence, mammalian ribonucleases could provide a ribonuclease-based anticancer therapy without renal toxicity.

ONC and RNase A share 30% amino acid sequence identity and have similar tertiary structure (2, 12), yet differ in catalytic activity and affinity for the cytosolic ribonuclease inhibitor protein (RI). RNase A demonstrates 104-fold more ribonucleolytic activity than does ONC (2, 13). In cells, the high ribonucleolytic activity of RNase A is blocked by its high affinity for RI (14–16) (Fig. 1). ONC has low affinity for RI (13, 17).

Several studies have focused on understanding the contribution of ribonucleolytic activity and affinity for RI to cytotoxicity. RNase A itself does not have marked antitumor activity, but variants of RNase A are toxic to cancer cells. For example, substituting the glycine residue at position 88 with arginine decreases the affinity for RI and endows RNase A with cytotoxic activity (16). Replacing Lys14 with an arginine residue results in a decrease in catalytic activity that is compensated by a decrease in affinity for RI (18). K41R/G88R RNase A has enhanced toxicity to K-562 cells as compared with G88R RNase A.

Some endosomal pathways end in the lysosomal degradation of proteins. The KFERQ pentapeptide sequence targets cytosolic proteins for lysosomal degradation via an alternative pathway (for reviews, see Refs. 19–21). Dice and co-workers (22) found that microinjected RNase A associates with lysosomes upon cellular fractionation. Subsequent studies found that the KFERQ pentapeptide, which comprises residues 7–11 of RNase A, regulates lysosomal degradation (23, 24). The KFERQ sequence of RNase A is recognized by a cytosolic heat shock cognate (hsc) protein of 73 kDa (hsc73) (25). ONC does not contain a KFERQ sequence (Fig. 2). The significance of KFERQ-targeted lysosomal decay in ribonuclease-mediated cytotoxicity is unknown. This sequence, along with RI, could serve to protect cells against an invading ribonuclease.

Here, we determine the effect of the KFERQ sequence on G88R RNase A-mediated cytotoxicity. Replacing Lys2 of the KFERQ sequence with an alanine residue has little effect on the conformational stability or catalytic activity of G88R RNase A. K7A/G88R RNase A does, however, have a marked decrease in affinity for RI compared with G88R RNase A and is the most cytotoxic variant of RNase A reported to date. Using other RNase A variants with substitutions in the KFERQ sequence that do not disrupt RI binding, we find that targeted lysosomal degradation via the KFERQ sequence does not modulate ribonuclease toxicity. Moreover, the toxicity of ribonucleases is not diminished in serum-deprived cells, which have enhanced KFERQ-mediated lysosomal degradation (22).

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). Cell culture medium and supplements were from Invitrogen.

ONC (16) and porcine RI (26) were prepared as described. Enzymes used for DNA manipulation were from Promega (Madison, WI) or New England Biolabs (Beverly, MA).

[methyl-3H]Thymidine was from PerkinElmer Life Sciences (Boston, MA). 6-Carboxyfluorescein—dArU(dA)2—6-carboxyfluorescein-methylsulfone (6-FAM—dArU(dA)2—6-TAMRA) was from Integrated DNA Technologies (Coralville, IA). Yeast RNA (16 S and 23 S) was from Roche Molecular Biochemicals. All other chemicals were of commercial reagent grade or better and were used without further purification.

Analytical Instruments—Ultraviolet and visible absorption was measured with a Cary model 50 spectrophotometer from Varian (Saugus, MA). Fluorescence was measured with a QuantaMaster1 photomultiplier from Photon Technology International.
ribonucleases used in biological assays were dialyzed exhaustively
very thoroughly. The dialysis buffer contained KCl (0.20 g), KH₂PO₄ (0.20 g), NaCl (8.0 g), and Na₂HPO₄ (2.16 g).

**Assay of Catalytic Activity**—Ribonuclease activity was measured by using a fluorogenic substrate (33). Assays were performed at 23 °C in 2.00 ml of 0.10 M MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M). Solutions contained 6-FAM−dArU(dA)₂−6-TAMRA (50 nM) and enzyme (10 units, per ml). Fluororescence was monitored by using 493 and 515 nm for the excitation and emission wavelengths, respectively. Kinetic parameters were determined by a linear least-squares regression analysis of the initial velocity using Equation 1 (33),

\[ V/K = (\Delta F/\Delta t) = \frac{F_{\text{max}} - F_0}{k_{\text{cat}}/K_m} \]  
(Eq. 1)

where \( V/K \) is the first-order rate constant, \( \Delta F/\Delta t \) is the slope from the linear regression, \( F_{\text{max}} \) is the final fluorescence intensity after the reaction has reached completion, and \( F_0 \) is the initial fluorescence intensity before enzyme is added. The value of \( k_{\text{cat}}/K_m \) was calculated by dividing \( V/K \) by the enzyme concentration.

**Fluorescence Assay of Ribonuclease Inhibitor Binding**—The value of \( K_d \) for the complex between porcine RI and RNase A variants was determined by using a competitive binding assay. It has been shown that the fluorescence of fluorescein-labeled A19C/G88R RNase A (fluorescein−G88R RNase A) decreases by ~15% upon binding to RI.**

Thus, fluorescence spectroscopy can be used to evaluate the ability of an unlabeled ribonuclease to compete with fluorescein−G88R RNase A for binding to RI. Specifically, cuvettes of PBS containing fluorescein−G88R RNase A (50 nM), an unlabeled RNase A variant (1 nM–2 µM), and dithiothreitol (1 mM) were incubated at room temperature (23 ± 2 °C). After 15 min, the initial fluorescence intensity was measured with 490 and 511 nm for the excitation and emission wavelengths, respectively. Next, RI was added with stirring (to 50 nM), and the average fluorescence intensity was measured after an additional incubation of 4 min. The maximum fluorescence decrease upon RI binding was measured with samples that lacked unlabeled ribonuclease. The concentration of the RI−fluorescein−G88R RNase A complex was determined by comparing the fluorescence of a sample with the fluorescence decrease observed when all of the fluorescein−G88R RNase A was bound by RI. The \( K_d \) value was determined as described.**

**Gel Assay of Ribonuclease Inhibitor Binding**—The effect of RI binding on catalytic activity was monitored directly, but qualitatively, by an agarose gel-based assay as described previously (16). Briefly, 0.6-ml siliconized microcentrifuge tubes of 0.10 M MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M), dithiothreitol (1 mM), yeast RNA (4 µg), and a RNase A variant (10 ng) were mixed with RI (0, 10, 20, or 40 units, where 1 unit is the amount required to inhibit the activity of 5 ng of RNase A by 50%). After a 15-min incubation at 37 °C, 10 mM Tris-HCl buffer (pH 7.5) containing EDTA (50 mM), glycerol (30%, w/v), xylene cyanol FF (0.25%, w/v), and bromphenol blue (0.25%, w/v) was added. Samples were analyzed by electrophoresis through an agarose gel (1%, w/v) containing ethidium bromide (0.4 µg/ml). Control samples were incubated in the absence of a ribonuclease or RI (or both).

**Assay of Cytotoxicity**—The effect of RNase A, its variants, and ONC on cell proliferation was determined by measuring the incorporation of [methyl-³H]thymidine into cellular DNA. K-562 cells were grown in RPMI 1640 medium. Unless indicated otherwise, all culture medium contained fetal bovine serum (10%, v/v), penicillin (100 units/ml), and streptomycin (100 µg/ml). Cells were cultured at 37 °C in a humidified incubator containing CO₂ (g; 5%, v/v). All toxicity studies were performed using asynchronous log-phase cultures. For toxicity assays, cells (95 µl of a solution of 5 × 10⁴ cells/µl) were incubated with a 5 µl solution of a ribonuclease or PBS in the wells of a 96-well plate. Cells were incubated for 44 h at 37 °C in a humidified incubator containing CO₂ (g; 5%, v/v). Next, the proliferation of cells was measured with a 4-h pulse of [methyl-³H]thymidine (0.4 µCi/well). Cells were harvested onto glass fiber filters using a PHD Cell Harvester (Cambridge Technology; Watertown, MA). Filters were washed with water and dried with methanol, and their ³H content was measured with liquid scintillation counting.

**Fig. 1. Molecular interactions between porcine ribonuclease inhibitor (red) and ribonuclease A (blue).** This figure was created by using atomic coordinates derived by x-ray diffraction analysis (39) and the program MOLSCRIPT (34). A, crystalline structure of the RNase A complex. B, close-up of the interaction between Lys⁷ (RNase A) and Ser⁴⁵⁶ (RI).

**Fig. 2. Amino acid sequence of residues 1–20 of ribonuclease A and the corresponding residues of Onconase.**

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(A) KETTA AAKFERQHMDSSSATA

(B) RNase A — QDWLT — FQKHI TNTRDV

Onconase — QDWLT — FQKHI TNTRDV

**Note:** R. A. Abel, M. C. Haigis, C. Park, and R. T. Raines (2002), submitted manuscript.
Serum-deprived cells have enhanced KFERQ-mediated lysosomal degradation of RNase A (22). To discern the effect of RNase A, its variants, and ONC on the proliferation of cells with enhanced KFERQ-mediated degradation, K-562 cells were grown in RPMI medium without fetal bovine serum for 18 h prior to the addition of a ribonuclease. Ribonuclease-mediated cytotoxicity was measured as described above. Cytotoxicity data were analyzed with the programs SIGMAPLOT (SPSS Science, Chicago, IL) and DELTAGRAPH (DeltaPoint, Monterey, CA). Each data point represents the mean (± S.E.) of at least three experiments, each performed in triplicate. The IC₅₀ value of each variant was calculated by using Equation 2,

\[ S = \left( \frac{IC_{50}}{IC_{50} + [\text{ribonuclease}]} \right) \times 100 \]

(Eq. 2)

where \( S \) is the percent of total DNA synthesis after the incubation period (48 h).

**RESULTS**

**Design of Ribonuclease A Variants**—RNase A variants were designed with the primary goal of discerning a role for the KFERQ sequence (residues 7–11) in cytotoxicity. Because RNase A itself is not cytotoxic, the cytotoxic G88R RNase A variant was used as a basis for this work. In addition, amino acid substitutions were combined with the secondary goal of producing variants that have high ribonucleolytic activity, low affinity for RI, and thus (presumably) high cytotoxicity.

**Lys⁷ and Arg¹⁰**—Lys⁷ and Arg¹⁰ comprise the enzymic P2 subsite, which interacts with a phosphoryl group of a RNA substrate (35–37). Cuchillo and co-workers (35) found that replacing either Lys⁷ or Arg¹⁰ alone with a glutamine residue has only a minor effect on catalysis of RNA cleavage, but that replacing both Lys⁷ and Arg¹⁰ decreases catalytic activity by 60-fold. Likewise, replacing both Lys⁷ and Arg¹⁰ with alanine residues results in a \( k_{cat}/K_m \) value that is 60-fold lower than that of wild-type RNase A (37).

The interaction between Lys⁷ of RNase A and porcine RI was investigated previously by using semisynthetic variants (38). Neumann and Hofsteenge (38) found that replacing Lys⁷ with an S-methyl cysteine residue resulted in a 50-fold decrease in affinity for RI. This result is consistent with the structure of the RI-RNase A complex in which the side-chain nitrogen of Lys⁷ donates a hydrogen bond to the C-terminal carboxyl group of RI (Fig. 1) (39). Arg¹⁰ makes no contact with RI in the complex.

Accordingly, we replaced Lys⁷ and Arg¹⁰ of RNase A independently with an alanine residue. The resulting K7A and R10A variants are designed to disrupt the KFERQ sequence, without decreasing ribonucleolytic activity. By systematically incorporating these changes in a cytotoxic variant, G88R RNase A, we were able to investigate the contribution of Lys⁷ and Arg¹⁰ to cytotoxicity.

**Phe⁶, Glu⁹, and Gln¹¹**—Phe⁶, Glu⁹, and Gln¹¹ are important to the structure and function of RNase A. Replacing Glu⁹ with an alanine, glutamine, or histidine residue enables the enzyme to bind a substrate in a nonproductive manner (40). The contributions of Phe⁶ and Glu⁹ have been determined in RNase S (or RNase S'), which is a noncovalent complex of residues 1–20 (or 1–15) and 21–124. In this complex, replacing Phe⁶ (41) or Glu⁹ (42) with other residues decreases its conformational stability or catalytic activity (or both). Because these residues of the KFERQ sequence play roles other than in lysosomal degradation, we left them intact.

Lys⁴¹—The side chain of Lys⁴¹ of RNase A donates a hydrogen bond to the transition state during catalysis of RNA cleavage (29). Changing Lys⁴¹ to an arginine residue results in a 10²-fold decrease in catalytic activity. Although K41R/G88R RNase A has low catalytic activity, it binds RI with less affinity than does G88R RNase A. Moreover, K41R/G88R RNase A is more cytotoxic than G88R RNase A (19). Hence, we used the K7A/K14R/G88R RNase A variant to explore the additivity of single substitutions that disrupt RI binding, as well as the relationship between catalytic activity, RI affinity, and cytotoxicity.

**Conformational Stability**—The conformational stability of the RNase A variants was measured to ensure that the proteins were folded properly during all assays. Values of \( T_m \) for RNase A, its variants, and ONC are listed in Table I. The \( T_m \) values of both wild-type RNase A and G88R RNase A in PBS were determined to be 63 °C, respectively; this value is similar to those reported previously (16, 43). K7A RNase A, K7A/G88R RNase A, R10A RNase A, and R10A/G88R RNase A were found to have \( T_m \) values of 63, 62, 60, and 62 °C, respectively. K41R/G88R RNase A and K7A/K41R/G88R RNase A were both found to have a \( T_m \) value of 63 °C. Hence, all RNase A variants were essentially completely folded during assays at 37 °C or room temperature. The \( T_m \) value of ONC in PBS was reported previously to be 90 °C (44).

**Catalytic Activity**—The cytotoxicity of ribonucleases relies on their ribonucleolytic activity (17, 45, 18). Ribonucleolytic activity was measured by using a fluorogenic substrate, 6-FAM–dArU(dA)₂–6-TAMRA, which exhibits a nearly 200-fold increase in fluorescence upon cleavage of the P–O₅ bond on the 3’ side of the single ribonucleotide-embedded residue

### Table I

| RNase A | \( T_m \) °C | \( IC_{50} \) μM | \( k_{cat}/K_m \) (10⁶ μM⁻¹ s⁻¹) | \( K_m \) μM | \( ΔG \) kcal/mol |
|---------|--------------|----------------|-----------------------------|------------|-----------------|
| RNase A | 63           | >25            | 43 ± 0.3                    | 67 × 10⁻⁶  | 0.0             |
| G88R RNase A | 63 | 7.3 ± 0.4 | 15 ± 3 | 0.54 ± 0.07 | 5.3 |
| K7A RNase A | 63 | >25 | 9.2 ± 1.6 | 0.07 ± 0.01 | 4.1 |
| K7A/G88R RNase A | 62 | 1.0 ± 0.1 | 8.8 ± 2.6 | 7.2 ± 4 | 6.8 |
| R10A RNase A | 60 | >25 | 9.2 ± 0.8 | ND | ND |
| R10A/G88R RNase A | 62 | 5.9 ± 0.8 | 12 ± 1 | 0.56 ± 0.07 | 5.3 |
| K41R/G88R RNase A | 63 | 3.9 ± 0.4 | 0.41 ± 0.05 | 2.9 ± 0.4 | 6.3 |
| K7A/K14R/G88R RNase A | 63 | 12.3 ± 1.9 | 0.07 ± 0.01 | 47 ± 4 | 7.9 |
| ONC | 90 | 0.54 ± 0.04 | 0.00035 ± 0.00001 | ≥10³ | ≥9.7 |

*Values of \( T_m \) (± 1 °C) were determined in PBS by UV spectroscopy. The \( T_m \) value of ONC is from Ref. 44 and was determined by CD spectroscopy.

*Values of \( IC_{50} \) are for incorporation of [methyl-³H]thymidine into the DNA of K-562 cells.

*Values of \( k_{cat}/K_m \) (± S.E.) are for the catalysis of 6-FAM–dArU(dA)₂–6-TAMRA cleavage at 25 °C in 0.10 M MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M).

* Values of \( K_m \) were determined at (23 ± 2) °C in PBS. The \( K_m \) value for RNase A is from Ref. 15. The \( K_m \) value for ONC is an estimate from Ref. 13.

* Values of ΔG were calculated with the equation: ΔG = –RTln(K₅₀ [RNase A]/K₅₀).

     ND, not determined.
Fig. 3. Agarose gel-based assay for inhibition of ribonucleolytic activity by porcine ribonuclease inhibitor. Inhibition was assessed by visualizing the cleavage of rRNA by 10 ng of a ribonuclease in the absence or presence of RI (10, 20, or 40 units).

Fig. 4. Effect of ribonucleases on the proliferation of K-562 cells. Cell proliferation was measured by incorporation of [methyl-3H]thymidine into cellular DNA after a 48-h incubation at 37 °C with a ribonuclease. Each value is the mean (± S.E.) of at least three independent experiments with triplicate samples and is expressed as a percentage of the PBS control. A, assays in the presence of fetal bovine serum (10%, v/v). B, assays on cells deprived of fetal bovine serum for 18 h prior to the addition of a ribonuclease.

DISCUSSION

Secretory ribonucleases can be potent cytotoxins by virtue of their ability to enter the cytosol and catalyze the cleavage of cellular RNA (3–8). The ribonucleolytic activity within the cytosol is regulated by two factors: (i) the concentration of enzyme within the cytosol, and (ii) how much of that enzyme is bound by RI. The cytosolic concentration of a ribonuclease is known to depend on its ability to enter the cytosol and catalyze the cleavage of rRNA (3–8). The ribonucleolytic activity within the cytosol is regulated by two factors: (i) the concentration of enzyme within the cytosol, and (ii) how much of that enzyme is bound by RI. The cytosolic concentration of a ribonuclease is known to depend on its ability to enter the cytosol and catalyze the cleavage of rRNA (3–8).
ONC is more cytotoxic in the presence of protease inhibitors (48). In addition, the degradation of fluorescein-labeled RNase A in murine L cells can be blocked by preincubation with lysosomal protease inhibitors (30).

The sequence-specific, lysosome-targeted degradation of cytosolic proteins can also lower the cellular concentration of a protein. RNase A, unlike ONC, contains a KFERQ sequence (2). This sequence is required for the targeted lysosomal degradation of cytosolic RNase A (22, 23). The targeted degradation of RNase A or toxic RNase A variants would lead to a decrease in cytosolic ribonucleolytic activity, and hence a decrease in cytotoxicity. Dice et al. (23) found that certain fragments of the RNase A KFERQ sequence (residues 1–10 and 2–8) are not degraded in a serum-dependent manner. In addition, although the order of the KFERQ residues is unimportant (20), the biochemical nature of the side chains must be conserved. Thus, replacing residues of the KFERQ sequence could enhance the cytotoxicity of an RNase A variant.

Arg10 of RNase A is located in the KFERQ sequence, but does not form any interaction with RI. Hence, we used the R10A variant to isolate the consequence of lysosomal degradation from RI evasion. R10A RNase A is not toxic to cells. In addition, R10A/G88R RNase A has an IC50 value similar to that of G88R RNase A. We also investigated the toxicity of a G88R RNase A variant with Gln11 replaced by a histidine or alanine residue. The cytotoxicity of the Q11H/G88R and Q11A/G88R variants does not differ from that of G88R RNase A (data not shown). Hence, disrupting the KFERQ sequence has no effect on ribonuclease-mediated cytotoxicity.

We measured the cytotoxicity of ribonuclease in cells with up-regulated lysosomal degradation. This experiment was based on the hypothesis that if KFERQ-mediated degradation limits the concentration of cytosolic ribonuclease, then enhancing this pathway would result in decreased toxicity. Cells cultured in the absence of serum show enhanced degradation of cytosolic RNase A (22). The data demonstrate that toxic variants of RNase A do not have lowered potency in serum-deprived cells (Fig. 4B and Table II). These results indicate that the KFERQ-mediated degradation of cytosolic ribonuclease does not limit their potency.

RI binds to members of the RNase A superfamily in a 1:1 stoichiometry (49). The interaction between RNase A and porcine RI buries 2550 Å2 of protein surface and forms one of the tightest noncovalent complexes known, with Kd values in the fs range (14, 39, 50). Upon binding to RI, the activity of RNase A is abolished completely.

We have shown that Lys7 of RNase A is an important residue in the RI-RNase A interaction. In the crystalline structure of the RI-RNase A complex, Lys7 is proximal to Ser146 of porcine RI (Fig. 1B) (39), which corresponds to Ser140 of human RI. The distance between the side chain nitrogen of Lys7 and the side chain oxygen of Ser146 is 3.1 Å. The side chain nitrogen is 3.5 and 4.1 Å away from the two oxygens of the C-terminal carbonyl group of RI. Replacing Lys7 with an alanine residue removes any hydrogen bonds and favorable Coulombic interactions with Ser146 of RI. The value of Kd for the RI-K7A RNase A complex is 70 pM (Table I). The corresponding value of Kd for the double variant, K7A/G88R RNase A, is 7.2 nM. Moreover, K7A/G88R RNase A is endowed with enhanced cytotoxicity.

Surprisingly, we find that the interactions of Lys7, Lys11, and Gly188 with RI are not additive. Single substitutions at Lys7 or Gly188 result in decreases of binding free energy of 4.1 or 5.3 kcal/mol, respectively (Table I). The double variants, K7A/G88R RNase A and K41R/G88R RNase A, have lost 6.9 and 6.3 kcal/mol of binding free energy, respectively. Yet, the triple variant, K7A/K41R/G88R RNase A, has lost only 8.0 kcal/mol of binding free energy. If the interactions had been additive, then the effect of single substitutions would contribute fully to the loss of binding free energy. Such conservation in binding free energy loss would suggest rigidity between the interface of the complex. For example, the effect of single substitutions at Lys4 and Lys7 of RNase A on RI binding is indeed additive, suggesting that changing Lys1 does not affect RI binding interactions at Lys7 (38). In contrast, our data show that the interface between RI and RNase A is not rigid. Rather, compensatory changes occur upon perturbation of key contacts. A similar conclusion was reached by Shapiro et al. (51) who measured the affinity of RI variants for wild-type RNase A. Thus, the dynamic nature of the RI-RNase A interface must be addressed when engineering new ribonuclease variants to evade RI.

In conclusion, we have shown that the KFERQ sequence does not contribute to a decrease in ribonuclease-mediated cytotoxicity. We find that Lys7 of RNase A contributes to a key interaction that tethers the N terminus of RNase A with the C terminus of RI. The K7A/G88R RNase A variant has >10-fold lower affinity for RI than does wild-type RNase A. The ribonucleolytic activity of K7A/G88R RNase A is, however, within 10-fold of that of the wild-type enzyme. Together, its high ribonucleolytic activity and low affinity for RI make K7A/G88R RNase A the most cytotoxic known variant of RNase A, with an IC50 value within 2-fold of that of ONC. Finally, we have found that the RI-RNase A interface is dynamic; disruption of one contact can alter other contacts.

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| Examination of KFERQ Sequence in Ribonuclease A-mediated Cytotoxicity |  |  |
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| **Table II** |  |  |
| Toxicity of ribonuclease A, its variants, and Onconase® for serum-deprived cells |  |  |
| Ribonuclease | IC50 (nM) |  |
| RNase A | >25 |  |
| G88R RNase A | 5.0 ± 2.4 |  |
| K7A/G88R RNase A | 0.62 ± 0.04 |  |
| ONC | 0.48 ± 0.03 |  |

* Values of IC50 are for incorporation of [methyl-3H]thymidine into the DNA of K-562 cells grown in the absence of fetal bovine serum for 18 h prior to the addition of a ribonuclease.
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