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Compensating effects on the cytotoxicity of ribonuclease A variants

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Abstract

Ribonuclease (RNase) A can be endowed with cytotoxic activity by enabling it to evade the cytosolic ribonuclease inhibitor protein (RI). Enhancing its conformational stability can increase further its cytotoxicity. Herein, the A4C/K41R/G88R/V118C variant of RNase A was created to integrate four individual changes that greatly decrease RI affinity (K41R/G88R) and increase conformational stability (A4C/V118C). Yet, the variant suffers a decrease in ribonucleolytic activity and is only as potent a cytotoxin as its precursors. Thus, individual changes that increase cytotoxicity can have offsetting consequences. Overall, cytotoxicity correlates well with the maintenance of ribonucleolytic activity in the presence of RI. The parameter $(k_{cat}/K_m)_{cyto}$, which reports on the ability of a ribonuclease to manifest its ribonucleolytic activity in the cytosol, is especially useful in predicting the cytotoxicity of an RNase A variant.

Keywords: Cancer; Conformational stability; Cytotoxin; Disulfide bond; Enzymatic activity; Ribonuclease A; Ribonuclease inhibitor; Onconase

Onconase (ONC)[1] [1] is a homologue of bovine pancreatic ribonuclease (RNase A; EC 3.1.27.5) [2]. Isolated from the Northern leopard frog *(Rana pipiens),* ONC is now in Phase III clinical trials (USA) for the treatment of malignant mesothelioma [3]. Although ONC is a potent antitumor agent, it has demonstrated dose-dependent renal toxicity [4,5]. RNase A does not possess antitumor activity, but certain variants of RNase A [6–8] and its human homologue [9] are toxic to tumor cells in vitro. Unlike ONC, mammalian ribonucleases are not retained in the kidney [10] and can therefore serve as the basis for new cancer chemotherapeutics [11].

RNase A and ONC possess 30% amino acid identity [12] and have similar tertiary structures [1,13]. Both RNase A and ONC catalyze the cleavage of the P–$O^{5'}$ bond of RNA on the 3' side of pyrimidine nucleotides [14]. Two biochemical properties of ONC that are known to contribute to its cytotoxic activity are its conformational stability and its evasion of the cytosolic ribonuclease inhibitor protein (RI).

Three of the four disulfide bonds in RNase A are conserved in ONC. ONC possesses a fourth, synapomorphic disulfide bond that tethers the C terminus to a central β strand. Removal of this disulfide bond compromises the conformational stability and the cytotoxic activity of ONC [15]. Incorporating a fifth disulfide that tethers the N and C termini of RNase A (Fig. 1) increases its conformational stability and cytotoxicity [8].

To date, the known property of secretory ribonucleases that correlates most closely with cytotoxicity is the ability to evade RI. ONC binds weakly to RI (estimated $K_d^{app} \geqslant 10^{-6}$ M [18]), but RNase A binds strongly to the inhibitor ($K_d = 6.7 \times 10^{-14}$ M [19]). The difference in RI affinity can be attributed to subtle differences in sequence and structure. For example, many of the RNase A residues that contact RI are replaced by dissimilar residues in ONC [6,20]. RNase A variants have been created that, like ONC, evade RI. For example, Gly88 of RNase A forms a close contact with Trp257 and Trp259 of RI (Fig. 1). Incorporating the large, hydrophilic amino acid arginine at position 88 results in a 10^4-fold decrease in affinity for RI [6]. Similarly, Lys41 of RNase A interacts with Tyr430 and Asp431 of RI (Fig. 1). Replacing Lys41 with arginine results in an additional 20-fold decrease in RI affinity [7].

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[1] *Abbreviations used:* ONC, Onconase, RNase, ribonuclease, RI, ribonuclease inhibitor protein. PBS, phosphate-buffered saline.

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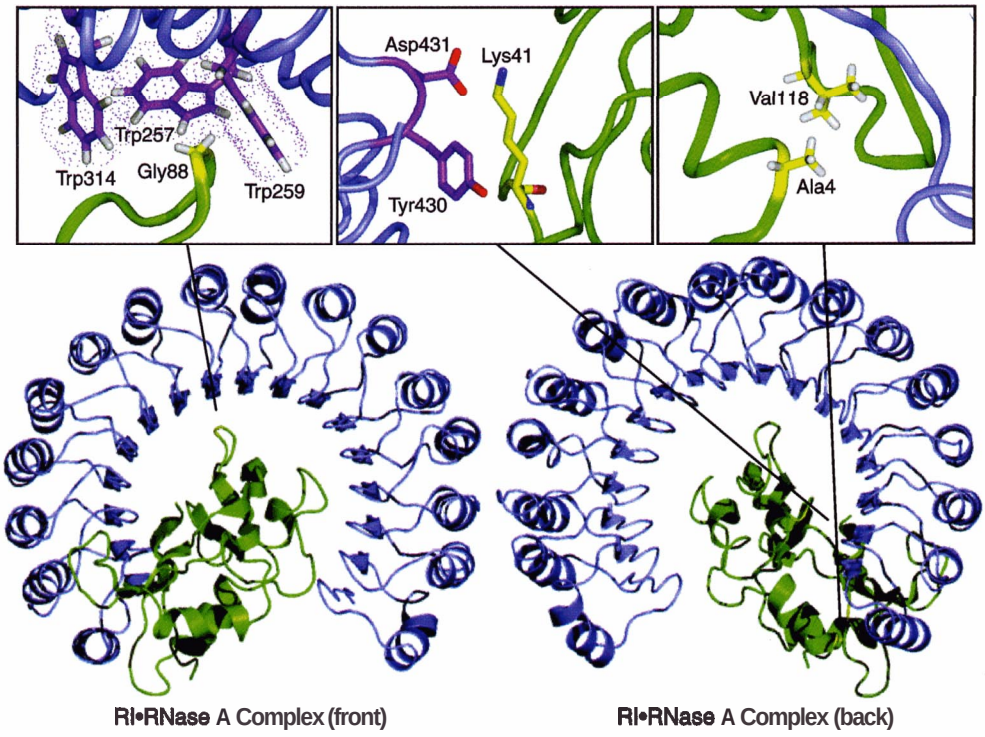

Fig. 1. Interactions in the complex of ribonuclease inhibitor (blue mainchain; purple sidechains) and ribonuclease A (green mainchain; yellow sidechains). Images were created by using the atomic coordinates from Protein Data Bank entry 1DFJ [16] and the program PyMOL [17].

Catalytic activity must be maintained to retain cytotoxicity. Lys41 of RNase A plays an important role in catalysis by donating a hydrogen bond to a nonbridging phosphoryl oxygen in the transition state during RNA cleavage [14]. The K41R substitution disrupts the RI·RNase A complex, but also reduces k_{cat}/K_m by 30-fold relative to G88R RNase A [7]. Still, the 20-fold increase in its K_d value for binding to RI is sufficient to produce a more cytotoxic ribonuclease. These data imply that cytotoxicity can be retained in an RNase A variant with decreased catalytic activity if there is a concomitant decrease in affinity for RI.

Here, we attempt to maximize the cytotoxic potency of RNase A by enhancing both its ability to evade RI and its conformational stability. Specifically, we combine the K41R and G88R substitutions intended to disrupt the RI·RNase A complex with a fifth disulfide bond that tethers the N and C termini. The results reveal that an interplay between these two biochemical properties exists and provide guidance for the development of new cytotoxic ribonucleases.

Materials and methods

Materials

Escherichia coli strain BL21(DE3) and the pET22b(+) expression vector were from Novagen (Madison, WI). E. coli strain TOPP 3 (Rif^r [F' *proAB lacI*^{q}ZΔ*M15* Tn10 (Tet^r) (Kan^r)]), which is a non-K-12 strain, was from Stratagene (La Jolla, CA). Enzymes for DNA manipulations were from Promega (Madison, WI) or New England BioLabs (Beverly, MA). Oligonucleotides and 6-FAM~(dA)rU(dA)$_2$~6-TAMRA, where 6-FAM refers to 6-carboxyfluorescein and 6-TAMRA refers to 6-carboxytetramethylrhodamine, were from Integrated DNA Technologies (Coralville, IA). K-562 cells, which were derived from a human chronic myelogenous leukemia, were from the American Type Culture Collection (Manassas, VA). [*methyl*-^{3}H]Thymidine (6.7 Ci/mmol) was from NEN Life Sciences (Boston, MA). All other chemicals and biochemicals were of commercial grade or better and were used without further purification.

Instruments

Absorbance measurements were made with a Cary 3 double-beam spectrophotometer equipped with a Cary temperature controller (Varian, Palo Alto, CA). Fluorescence measurements were carried out on a QuantaMaster 1 photon-counting fluorometer equipped with sample stirring (Photon Technology International, South Brunswick, NJ). Radioactivity was measured with a Beckman Model LS 3801 liquid scintillation counter from Beckman Instruments (Fullerton, CA).

Preparation of proteins

Plasmids that direct the production in E. *coli* of wild-type RNase A [21], its G88R [6], A4C/G88R/V118C [8], and K41R/G88R variants [7], and ONC [6] were described previously. Site-directed mutagenesis of the plasmid encoding A4C/G88R/V118C RNase A with oligonucleotide GTGCACAAAGGTGTTMCTGGA CGGCATCTATCTTTGGT was used to replace the AAG codon of Lys41 with a codon for arginine (reverse complement in boldface).

Proteins were prepared as described previously [6–8,21], except that RNase A variants possessing the G88R substitution were refolded in the presence of 0.5 M arginine instead of 0.1 M NaCl. Ribonucleases were dialyzed extensively against phosphate-buffered saline (PBS) for use in all cytotoxicity and RI-binding assays.

Ribonuclease concentrations were determined by UV spectroscopy using $\varepsilon = 0.72\,\mathrm{ml \cdot mg^{-1}\,cm^{-1}}$ at 277.5 nm for RNase A [22] and its variants and $\varepsilon = 0.87\,\mathrm{ml \cdot mg^{-1}\,cm^{-1}}$ at 280 nm for ONC [6].

Porcine RI was produced as described previously [23]. The concentration of active RI was determined by its titration with RNase A.

Assays of conformational stability

The reversible thermal denaturation of A4C/K41R/G88R/V118C RNase A was monitored by using UV spectroscopy [24]. Specifically, the A_{287} of a 0.4 mg/ml solution of ribonuclease was monitored as the temperature of the solution was increased from 25 to 80 °C in 1-°C increments. The data were fitted to a two-state model for denaturation using the program THERMAL (Varian, Palo Alto, CA). The value of T_m is the temperature at the midpoint of the thermal transition between the native and the unfolded states.

Assays of ribonucleolytic activity

The catalytic activity of ribonucleases was measured with the fluorogenic substrate 6-FAM~dArUdAdA~6-TAMRA [25]. Cleavage of this substrate results in a ~200-fold increase in fluorescence intensity (excitation at 492 nm; emission at 515 nm). Assays were performed at 23 °C in 2.0 ml of 0.10 M 4-morpholineethanesulfonic acid–NaOH buffer (pH 6.0) containing NaCl (0.10 M), 6-FAM~dArUdAdA~6-TAMRA (50 nM), and enzyme (5–500 pM). Data were fitted to the equation $k_{cat}/K_m = (\Delta I/\Delta t)/\{(I_f - I_0)[E]\}$, where $\Delta I/\Delta t$ is the initial velocity of the reaction, I_0 is the fluorescence intensity prior to the addition of enzyme, I_f is the fluorescence intensity after complete hydrolysis with excess wild-type enzyme, and [E] is the ribonuclease concentration.

Assays of ribonuclease inhibitor binding

The fluorescence of fluorescein-labeled A19C/G88R RNase A (fluorescein~G88R RNase A) decreases by nearly 20% upon binding to RI [26]. A competition assay exploiting this property was used to determine the affinity of each (unlabeled) RNase A variant for RI. Briefly, fluorescein~G88R RNase A (50 nM [26]) and an RNase A variant (1 nM–2 μM) were incubated in 2.0 ml of PBS for 30 min at 23 °C. The fluorescence intensity (excitation at 491 nm; emission at 511 nm) was measured before and after the addition of RI (to 50 nM). Values of K_d for the complex between RNase A variants and RI were determined as described previously [26].

Table 1
Properties of ribonuclease A, its variants, and Onconase

Ribonuclease	T_m[a] (°C)	k_{cat}/K_m[b] (10^6 M^{-1} s^{-1})	K_d[c] (nM)	$\Delta\Delta G$[c] (kcal/mol)	$(k_{cat}/K_m)_{cyto}$ (10^3 M^{-1} s^{-1})	IC_{50}[d] (μM)
Wild-type RNase A	63.0[e]	43 ± 3[h]	6.7×10^{-5}[i]	0.0	0.00072	>50
G88R RNase A	64.0[e]	14 ± 2	0.57 ± 0.05[j]	5.3	2.0	10 ± 1
A4C/G88R/V118C RNase A	68.8[f]	2.6 ± 0.2	1.34Z0.3	5.8	0.84	4.1 ± 0.6
K41R/G88R RNase A	63.0[g]	0.6 ± 0.06	7.5%1.8	6.8	1.1	5.2 ± 0.7
A4C/K41R/G88R/V118C RNase A	67.0	0.13 ± 0.03	27 ± 3.7	7.6	0.87	7.6 ± 0.9
ONC	90.0[e]	0.00035● 0.000l0[h]	⩾ 1 x 10^6[k]	>14	>0.35	0.49 ± 0.065

[a] Values of T_m were determined by UV (RNase A and its variants) or CD (ONC) spectroscopy.
[b] Values of k_{cat}/K_m (±SE) are for catalysis of 6-FAM~dArU(dA)$_2$~6-TAMRA cleavage at pH 6.0 and 23 °C.
[c] Values of K_d (±SE) and AAG = $RT \ln(K_d/K_d^{\text{wild-type RNase A}})$ are for the complex with porcine RI at 23 °C.
[d] Values of IC_{50} (±SE) are for cytotoxicity to human chronic myelogenous leukemia line K-562.
[e] Ref. [6].
[f] Ref. [8].
[g] Ref. [7].
[h] Ref. [27].
[i] Ref. [19].
[j] Ref. [26].
[k] Ref. [18].

Assays of cytotoxicity

The cytotoxicity of ribonucleases was determined by monitoring the incorporation of [*methyl*-^{3}H]thymidine into the newly synthesized DNA of K-562 cells [6]. Briefly, cells were maintained at 37 °C in RPMI media containing fetal bovine serum (10% v/v), penicillin (100 units/ml), and streptomycin (100 μg/ml). Ribonucleases were incubated with K-562 cells for 44 h at 37 °C. [*methyl*-^{3}H]Thymidine (0.4 μCi/well) was added for 4 h, after which cells were harvested onto a glass fiber filter and counted. [*methyl*-^{3}H]Thymidine incorporation in cells incubated in the absence of ribonuclease was used to define 100% ^{3}H incorporation. IC_{50} values were calculated by fitting the data to the equation $S = 100 \times IC_{50}/(IC_{50} + [\text{ribonuclease}])$, where S is the percentage of [*methyl*-^{3}H]thymidine incorporated after a 48-h incubation with a ribonuclease [27].

Results

Conformational stability

Values of T_m for RNase A, its variants, and ONC are listed in Table 1. The T_m of A4C/K41R/G88R/V118C RNase A was 67.0 °C. Thus, the increase in T_m achieved by installing a fifth disulfide bond in G88R RNase A and K41R/G88R RNase A is similar.

Ribonucleolytic activity

Values of k_{cat}/K_m for RNase A, its variants, and ONC are listed in Table 1. The k_{cat}/K_m of A4C/K41R/G88R/V118C RNase A was $1.3 \times 10^5\ M^{-1}\ s^{-1}$, which is 330-fold lower than that of wild-type RNase A. The k_{cat}/K_m values for wild-type RNase A, its other variants, and ONC are similar to those reported previously [7,8].

Ribonuclease inhibitor binding

Values of K_d for complexes of RI with RNase A and its variants are listed in Table 1. The complex of RI with A4C/K41R/G88R/V118C RNase A has $K_d = 27$ nM. This value is the highest yet reported for a variant of RNase A and is nearly 10^6-fold greater than that for wild-type RNase A [19]. The K_d values for wild-type RNase A, its other variants, and ONC are similar to those reported previously [7,8].

The K_d values were used to calculate the change in the free energy of association (ΔΔG) for RI with each of the RNase A variants. These ΔΔG values are listed in Table 1.

Cytotoxicity

Data on the cytotoxicity of RNase A, its variants, and ONC are shown in Fig. 2, and the resulting IC_{50}

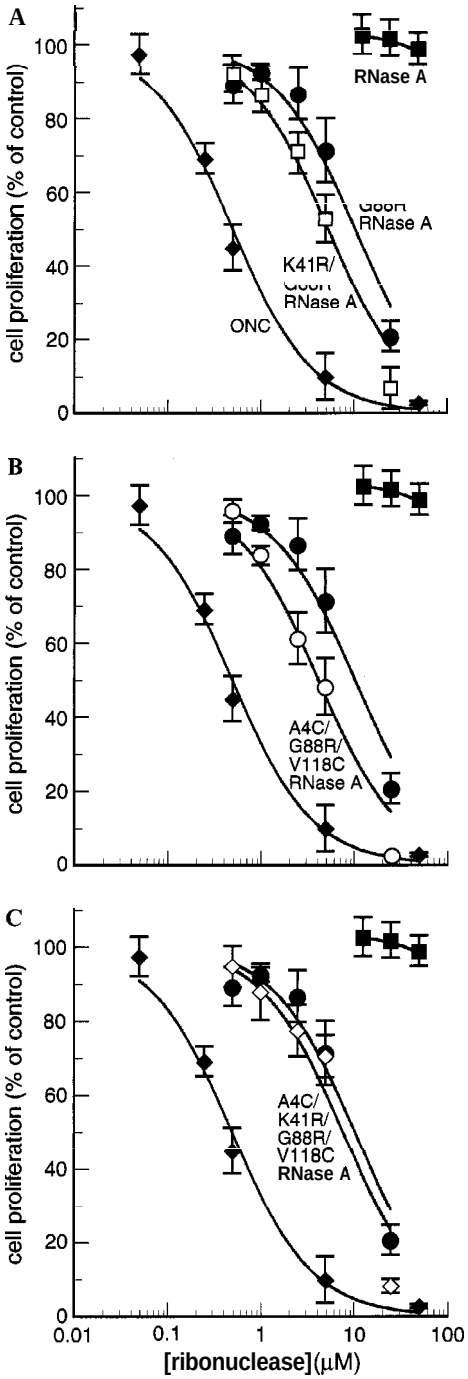

Fig. 2. Effect of ribonucleases on the proliferation of K-562 cells. Cell proliferation was measured by [*methyl*-^{3}H]thymidine incorporation into cellular DNA after a 44-h incubation at 37 °C with a ribonuclease. Values are the mean (± SE) of at least three independent experiments with triplicate samples and are expressed as a percentage of control cultures lacking an exogenous ribonuclease. For comparison, data for wild-type RNase A, its G88R variant, and Onconase (closed symbols) are depicted in each panel.

values are listed in Table 1. The IC_{50} values for all four toxic variants of RNase A vary by only 2.4-fold. Surprisingly, A4C/K41R/G88R/V118C RNase A was less cytotoxic ($IC_{50} = 7.6\,\mu M$) than either K41R/G88R RNase A or A4C/G88R/V118C RNase A. The IC_{50} values for the other RNase A variants and ONC are in gratifying agreement with those reported previously [7,8].

$(k_{cat}/K_m)_{cyto}$

The ability of a ribonuclease to manifest its catalytic activity in the cytosol is related to its values of k_{cat}/K_m and K_d. This ability can be described by the parameter $(k_{cat}/K_m)_{cyto}$ [7,28–30]:

$$(k_{cat}/K_m)_{cyto} = (k_{cat}/K_m)/[1 + ([RI]_{cyto}/K_d)]. \quad (1)$$

The value of $[RI]_{cyto}$ was estimated to be $4\,\mu M$ by assuming that RI constitutes 0.08% of cytosolic protein [30] and that the total concentration of protein in the cytosol is 250 mg/ml [31]. The resulting values of $(k_{cat}/K_m)_{cyto}$ for RNase A, its variants, and ONC are listed in Table 1.

Discussion

Ribonucleases exert their cytotoxic activity in the cytosol of the cell. To be cytotoxic, a ribonuclease must maintain its ribonucleolytic activity (and hence its conformational stability) so as to degrade cellular RNA, even in the presence of RI. We have created an RNase A variant (A4C/K41R/G88R/V118C RNase A) that combines changes that confer conformational stability and the ability to evade cellular RI.

Many of the residues in RNase A that are most important for substrate binding and catalysis participate in intermolecular interactions with RI [20]. Our data provide direct experimental support for this dichotomy. We find that values of k_{cat}/K_m for RNase A variants decrease in the order G88R > A4C/G88R/V118C > K41R/G88R > A4C/K41R/G88R/V118C (Table 1). Values of K_d demonstrate exactly the opposite trend. Hence for these variants, ribonucleolytic activity is related inversely to the ability to evade RI. In other words, amino acid substitutions that disrupt the RI . RNase A complex compromise catalytic activity.

The conformational stability afforded by the incorporation of the fifth disulfide bond in G88R RNase A leads to a more cytotoxic variant [8]. Yet, the addition of the same disulfide bond to K41R/G88R RNase A results in a less cytotoxic variant (Table 1). The fifth disulfide bond also decreases the value of k_{cat}/K_m by fivefold. Apparently, the benefit of enhanced conformational stability cannot always overcome a decrease in k_{cat}/K_m to generate a more cytotoxic ribonuclease.

The value of $(k_{cat}/K_m)_{cyto}$ reports on the ribonucleolytic activity of a ribonuclease in the cytosol [7,28–30]. Values of $(k_{cat}/K_m)_{cyto}$ vary by <3-fold for the four cytotoxic RNase A variants (Table 1). Likewise, values of IC_{50} vary by <3-fold for these four variants. These small variations are in marked contrast to the nearly 10'-fold variation in the values of both k_{cat}/K_m and K_d. Thus, $(k_{cat}/K_m)_{cyto}$ correlates much more closely with IC_{50} than does either k_{cat}/K_m or K_d. Only the parameter $(k_{cat}/K_m)_{cyto}$, which takes into account both (k_{cat}/K_m) and K_d, provides a reliable forecast for the cytotoxicity of an RNase A variant.

What is the limit to the cytotoxicity of an RNase A variant? Suppose a variant could maintain the k_{cat}/K_m value of wild-type RNase A and have $K_d \gg 4\,\mu M$. Then according to Eq. (1) and the data in Table 1, its $(k_{cat}/K_m)_{cyto} = 4.3 \times 10^7\,M^{-1}\,s^{-1}$. This value is 5 x lo^4-fold greater than that of the A4C/G88R/V118C, K41R/G88R, or A4C/K41R/G88RV118C variants. If IC_{50} is truly inversely proportional to $(k_{cat}/K_m)_{cyto}$, then such an RNase A variant would have an $IC_{50} = 5\,\mu M/5 \times 10^4 = 0.1\,nM$ for K-562 cells. This IC_{50} value is much less than that of ONC (Table 1). Of course, this analysis is simplistic. Many other factors, including conformational stability [8], are known to affect cytotoxicity. Still, this analysis provides both an inspiration for the design of new ribonuclease-based cytotoxins and a benchmark with which to gauge the success of those designs.

Overcoming inhibition by RI in the cytosol is an even more formidable task than is apparent from the K_d values listed in Table 1. The cytosol is crowded with macromolecules [31,32]. The relatively low concentration of water there favors the formation of intermolecular complexes, thereby effectively lowering values of K_d. For example, the K_d value for the dimerization of a typical 40-kDa spherical protein is reduced by four- to eightfold in the E. coli cytosol [32]. Because far more surface area is buried in the RI . RNase A complex than in a typical protein–protein interaction [16], the RI · RNase A complex is likely to be significantly more stable in the cytosol than in the dilute solution of in vitro assays.

In conclusion, A4C/K41R/G88R/V118C RNase A has an enhanced ability to evade RI and greater conformational stability than its precursors. These attributes are offset by diminished catalytic activity. These compensating effects endow A4C/K41R/G88R/V118C RNase A with cytotoxic activity that differs by <3-fold from that of the G88R, A4C/G88R/V118C, and K41R/G88R variants, despite a nearly lo^3-fold variation in the values of both k_{cat}/K_m and K_d. Increasing the cytotoxicity of RNase A (or its human homologue) requires diminishing its affinity for RI without compromising its conformational stability or catalytic activity.

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References

[1] R.J. Youle, G. D'Alessio, in: G. D'Alessio, J.F. Riordan (Eds.), Ribonucleases: Structures and Functions, Academic Press, New York, 1997, pp. 491–514.
[2] R.T. Raines, Chem. Rev. 98 (1998) 1045–1065.
[3] S.M. Mikulski et al., J Clin. Oncol. 20 (2002) 274–281.
[4] S.M. Mikulski, A.M. Grossman, P.W. Carter, K. Shogen, J.J. Costanzi, Int. J. Oncol. 3 (1993) 57–64.
[5] S.M. Mikulski, H.G. Chun, A. Mittelman, T. Panella, C.A. Puccio. K. Shogen, J.J. Constanzi, Int. J. Oncol. 6 (1995) 889–897.
[6] P.A. Leland, L.W. Schultz, B.-M. Kim, R.T. Raines, Proc. Natl. Acad. Sci. USA 98 (1998) 10407–10412.
[7] L. Bretscher, R.L. Abel, R.T. Raines, J. Biol. Chem. 275 (2000) 9893–9896.
[8] T.A. Klink, R.T. Raines, J. Biol. Chem. 275 (2000) 17463–17467.
[9] P A. Leland, K.E. Staniszewski, B.-M. Kim, R.T. Raines, J. Biol. Chem. 276 (2001) 43095–43102.
[10] V.M. Vasandani, Y.-N. Wu, S.M. Mikulski, R.J. Youle, C. Sung, Cancer Res. 56 (1996) 4180–4186.
[11] P.A. Leland, R.T. Raines, Chem, Biol. 8 (2001) 405–413.
[12] W. Ardelt, S.M. Mikulski, K. Shogen, J. Biol. Chem. 266 (1991) 245–251.
[13] S.C. Mosimann, W. Ardelt, M.N.G. James, J. Mol. Biol. 236 (1994) 1141–1153.
[14] J.M. Messmore, D.N. Fuchs, R.T. Raines, J. Am. Chem. Soc. 117 (1995) 8057–8060.
[15] P.A. Leland, K.E. Staniszewski, B.-M. Kim, R.T. Raines, FEBS Lett. 477 (2000) 203–207.
[16] B. Kobe, J. Deisenhofer, Nature 374 (1995) 183–186.
[17] W.L. DeLano, The PyMOL User's Manual, DeLano Scientific, San Carlos, CA, 2002.
[18] E. Boix, Y. Wu, V.M. Vasandani, S.K. Saxena, W. Ardelt, J. Ladner, R.J. Youle, J. Mol. Biol. 257 (1996) 992–1007.
[19] A.M. Vicentini, B. Kieffer, R. Mathies, B. Meyhack, B.A. Hemmings, S.R. Stone, J. Hofsteenge, Biochemistry 29 (1990) 8827–8834.
[20] B. Kobe, J. Deisenhofer, J. Mol. Biol. 264 (1996) 1028–1043.
[21] S.B. delCardayré, M. Ribó, E.M. Yokel, D.J. Quirk, W.J. Rutter, R.T. Raines, Protein Eng. 8 (1995) 261–273.
[22] M. Sela, C.B. Anfinsen, W.F. Harrington, Biochim. Biophys. Acta 26 (1957) 502–512.
[23] T.A. Klink, A.M. Vicentini, J. Hofsteenge, R.T. Raines, Protein Express. Purif. 22 (2001) 172–179.
[24] E.S. Eberhardt, P.K. Wittmayer, B.M. Templer, R.T. Raines, Protein Sci. 5 (1996) 1697–1703.
[25] B.R. Kelemen, T.A. Klink, M.A. Behlke, S.R. Eubanks, P.A. Leland, R.T. Raines, Nucleic Acids Res. 27 (1999) 3696–3701.
[26] R.L. Abel, M.C. Haigis, C. Park, R.T. Raines, Anal. Biochem. 306 (2002) 100–107.
[27] M.C. Haigis, E.L. Kurten, R.L. Abel, R.T. Raines, J. Biol. Chem, 277 (2002) 11576–11581.
[28] R.T. Raines, in: P.A. Frey, D.B. Northrop (Eds.), Enzymatic Mechanisms, IOS Press, Washington, DC, 1999, pp. 235–249.
[29] J. Futami, E. Nukui, T. Maeda, M. Kosaka, H. Tada, M. Seno, H. Yamada, J. Biochem. (Tokyo) 132 (2002) 223–228.
[30] M.C. Haigis, E.L. Kurten, R.T. Raines, Nucleic Acids Res. 31 (2003) 1024–1032.
[31] R.J. Ellis, Trends Biochem. Sci. 26 (2001) 597–604.
[32] S.B. Zimmerman, A.P. Minton, J. Mol. Biol. 222 (1991) 599–620.