

## Minireview

## Cytotoxic ribonucleases: molecular weapons and their targets

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**Abstract** Many ribonucleases (RNases) are highly cytotoxic. In some cases, they attack selectively malignant cells, triggering apoptotic response, and therefore are considered as alternative chemotherapeutic drugs. Factors that determine the cytotoxicity of RNases, primarily of those of microbial origin, are reviewed here. These factors include catalytic activity, ability to escape natural inhibitors, stability, and efficiency of internalization. The latter is, in turn, determined by positive charge on the molecule and interaction with cell membrane. Cellular targets and molecular determinants of RNases decisive for their cytotoxic action are characterized.

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**Key words:** Cytotoxic ribonuclease; Catalytic activity; Structure; Stability; Positive charge; Endocytosis

## 1. Introduction: ribonucleases as killers

Ribonucleases (RNases) play a key role in RNA metabolism. They are involved in host defence and physiological cell death pathways. A single cell can contain as many as 20 distinct RNases with different or overlapping specificities. RNases can be components of supramolecular complexes and function in concert with other enzymes (for a review see [1]).

RNases possess therapeutic opportunities for cancer treatment, as RNA damage caused by RNases could be an important alternative to standard DNA-damaging chemotherapeutics. Certain members of the RNase A superfamily are effective against experimental tumors and show cytotoxicity toward tumor cell lines, among them bovine seminal (BS) RNase, RNase from oocytes of *Rana pipiens* (commercial trademark of Alfacell, Inc., USA, onconase), two closely related frog RNases from *R. catesbeiana* and *R. japonica* (reviewed in [2–4]), human eosinophil-derived neurotoxin with four extra residues SLHV at N-terminus ((–4)EDN) [5],

and eosinophil cationic protein (ECP) [6]. A multicenter phase II trial of onconase demonstrated markedly prolonged survival of patients with unresectable and histologically confirmed malignant mesothelioma [7]. Now onconase has reached phase III of clinical trials. Recent experimental efforts have been aimed at the preparation of immunotoxins (conjugates of RNases with antibodies against tumor antigens) and ‘humanized antitumor RNase’ that would be structurally similar to human enzyme with minimal immunogenicity and side effects. These constructs may avoid problems of unfavorable toxicity and immunogenicity associated with plant or bacterial RNase-containing immunotoxins (reviewed in [8]).

Mammalian cells contain potent cytosolic RNase inhibitor protein (RI). Anticancer activity of several RNases correlates with their resistance to RI. This resistance enables onconase to catalyze degradation of cellular RNA and consequently cause cell death [3]. Mammalian BS RNase exerts selective cytotoxicity toward different types of tumor cells due to its dimeric structure, which makes the enzyme insensitive to RI [9–11]. Thus, a lower intrinsic catalytic activity can be overcome by greater RI evasion [12].

Bacterial and fungal RNases a priori should not be inhibited by RI, because the function of human RI is to protect RNA from the action of adventitious mammalian RNases. RI does not inactivate fungal RNases U<sub>1</sub> from *Ustilago sphaerogena* and T<sub>1</sub> from *Aspergillus oryzae* [13]. Human RI had no effect on the ribonucleolytic activity of *Streptomyces aureofaciens* RNases Sa and Sa3 [14]. Fungal RNases  $\alpha$ -sarcin, restrictocin, Asp-f1, and mitogillin are known as ribotoxins that inactivate eukaryotic ribosomes by cleaving a single phosphodiester bond in 28S rRNA [15–17]. The best characterized member of this family is  $\alpha$ -sarcin, a potent cytotoxin that promotes apoptosis of human rhabdomyosarcoma cells [18]. Colicins representing RNase-type toxins [19] inhibit growth of leukemic cells [20]. *Bacillus intermedius* RNase (binase) preferentially kills mammalian cells expressing *ras*-oncogene [21]. RNase Sa3 exhibits dose-dependent toxicity against the human myelogenous leukemia K-562 cells [14]. Thus, microbial RNases are waiting for their success in clinical applications, still remaining in the shadow of their animal relatives.

In this review, we will discuss molecular determinants and targets of RNase cytotoxic action with a special emphasis on RNases of microbial origin.

## 2. Obvious weapon: catalytic activity

Ribonucleolytic activity of RNases is essential for their cy-

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**Abbreviations:** RNase, ribonuclease; BS, bovine seminal; EDN, eosinophil-derived neurotoxin; (–4)EDN, eosinophil-derived neurotoxin with four extra residues SLHV at N-terminus; ECP, eosinophil cationic protein; RI, ribonuclease inhibitor; ER, endoplasmic reticulum

toxicity [9]. Onconase causes caspase-dependent apoptosis in target cells by damaging cytoplasmic tRNA [22,23]. An alkylated derivative of onconase with 2% residual RNase activity was not toxic [24]. Cationic variants of bovine RNase A and human RNase 1 generated by chemical modification of carboxyl groups exhibited no cytotoxicity if their catalytic activity was less than 0.01% of the non-modified enzyme [25]. So, as anticipated, RNases must retain a certain level of nucleolytic activity to be toxic to cells.

Microbial RNases are not an exception to this rule. Barnase (RNase from *Bacillus amyloliquefaciens*) attached to the transport domain of *Pseudomonas* exotoxin A in order to penetrate the cells was toxic to several tested cell lines due to its RNase activity [26]. Catalytically inactive  $\alpha$ -sarcin mutant was neither toxic nor apoptotic, suggesting that the induced apoptosis is directly related to the damaging effect of the toxin on ribosomes [18]. The SOS response induced by binase and its mutants was shown to correlate with the catalytic activity [27]. A mutant barnase with 2% residual catalytic activity and wild-type binase inactivated by photooxidation were not toxic in isolated perfused kidney system [28].

The catalytic activity is essential but not sufficient to elicit a toxic response in cells. Indeed, mammalian RNases with their positive charge increased by chemical modification and retaining only 1.5% catalytic activity were cytotoxic, whereas non-modified enzymes with 100% activity were not [25]. Mutations of the active site residues in the ECP eliminated the RNase activity, but had no discernible effect on the antibacterial activity of this protein [29]. A charge reversal mutant of RNase Sa, which possessed less than 15% of catalytic activity of the non-toxic wild-type enzyme, was very cytotoxic [30]. It is clear that the level of catalytic activity is not the only factor that defines the cytotoxicity of RNases.

### 3. Killer's image: structure

The number of resolved RNase three-dimensional structures rapidly increases, and most of cytotoxic RNases are 'known by sight'. Although strong structural similarity is a common trait in the RNase A and RNase T<sub>1</sub> families, the differences in their cytotoxic properties are remarkable. Is it possible to identify structural elements responsible for these differences?

Compared to non-toxic RNase A, the cytotoxic onconase shows a very similar topology, with the major differences present in the loop regions and at the C-terminus. An additional disulfide bond (Cys87–Cys104) found only in frog RNases is important for onconase cytotoxicity [31]. Two naturally existing forms of eosinophil-derived neurotoxin (EDN) have identical structures, but only (–)EDN markedly inhibits the viability of Kaposi's sarcoma cells [5,32].

Microbial RNases, members of the RNase T<sub>1</sub> family, provide similar examples. *Streptomyces* RNases Sa (strain BMK), Sa2 (strain R8/26), and Sa3 (strain CCM 3239) resemble each other structurally very much. This is not surprising since the three proteins have identical amino acids at 48 out of 96 positions [33] but only RNase Sa3 is strongly toxic toward a tumor cell line [14]. The cytotoxicity of RNase Sa3 is only 10-fold lower than that of onconase. The three-dimensional structure of RNase Sa3, closely similar to that of its non-toxic homolog RNase Sa [14] (Fig. 1), provides no obvious clues to account for cytotoxicity. On the other hand, structurally close

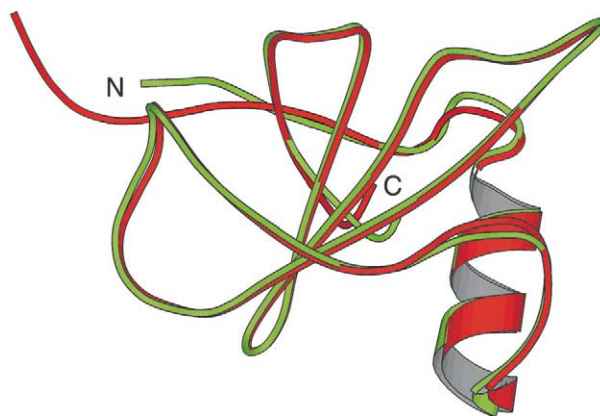


Fig. 1. Superposition of RNase Sa (green, 1RGG) and RNase Sa3 (red, 1MGW) structures based on comparison of C $\alpha$  atoms. The figure was produced with Molscript [34].

homologs, binase and barnase [35], exhibit similar nephrotoxic effects [27]. Hence, a structural key to identify cytotoxic RNases is not yet found.

Only some structural elements and sequence motifs associated with RNase cytotoxicity are known. For instance, the N-terminal  $\beta$ -hairpin of  $\alpha$ -sarcin, absent in other non-toxic structurally related microbial RNases, is responsible for the specific ribosome-inactivating activity of the protein [15]. This hairpin is involved in protein–membrane interaction. Amino acid sequence 131–139 of  $\alpha$ -sarcin (HDKFDSKK) has been shown to be membrane perturbing [36]. Other larger peptides containing this sequence, not found in bacterial enzymes, produced similar effects, which suggests that it may be involved in protein interaction with lipid membranes. Unlike onconase, non-toxic RNase A contains the KFERQ pentapeptide (residues 7–11), known to target cytosolic proteins for lysosomal degradation. This motif is absent from bacterial RNases. This pentapeptide, along with RI, could protect cells against an invading RNase. However, the KFERQ-mediated degradation does not limit the cytotoxicity of RNase A mutants with decreased affinity to RI [37]. Probably, the effect of avoiding RI overweighs that of the KFERQ degradation signal.

### 4. Armour potential of RNases: stability

A definite link exists between stability and metabolic turnover of proteins [38]. There is a large body of data confirming that stability is a determinant of RNase cytotoxicity and that cytotoxicity is connected with the ability of corresponding enzymes to resist proteolysis [6,38,39]. Onconase is an exceptionally stable protein, its denaturation temperature being almost 30°C higher than that of RNase A. The pyroglutamyl residue at the N-terminus and the disulfide bridge at the C-terminus are responsible for this unusual stability [40]. The removal of the Cys87–Cys104 disulfide bond substantially decreases both the conformational stability and cytotoxic activity of onconase [31]. Mutants of human pancreatic RNase 1 with increased stability possess also an increased cytotoxicity [39]. Out of five human pancreatic-type RNases, the most cytotoxic, ECP, is also the most resistant against the guanidine hydrochloride-induced unfolding [6]. By removal or incorporation of a disulfide bond in a cytotoxic mutant of RNase A, a series of cytotoxins with stabilities varying by

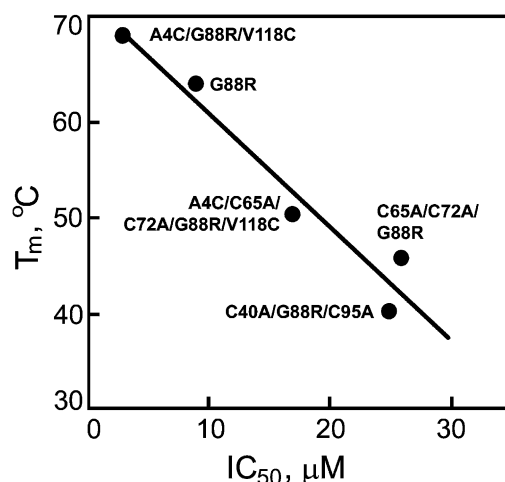


Fig. 2. Melting temperatures values versus  $IC_{50}$  values (the RNase concentration that kills 50% of cells) for variants of RNase A. Reproduced with permission from J. Biol. Chem. [38], © 2000 The American Society for Biochemistry and Molecular Biology, Inc.

nearly 30°C was designed [38]. The introduced mutations have little effect on the enzymatic activity and ability to evade the cytosolic RI. The conformational stability of these RNase A variants correlates directly with their cytotoxicity, as well as with resistance to proteolysis (Fig. 2; [38]).

However, for bacterial RNases this correlation has not been revealed. Incidentally, the stability of RNases Sa and Sa3 is similar, but only RNase Sa3 is cytotoxic [14]. The equally stable RNase Sa and its charge reversal mutant 5K drastically differ in their toxic abilities [30]. In these cases some other features rather than stability determine the cytotoxicity.

### 5. Secret weapon: positive charge

Internalization is probably the limiting step for cytotoxicity. Positively charged protein molecules bind to negatively charged glycolipids and glycoproteins on the outer part of the plasma membrane, and this promotes their internalization. This process is of great importance for therapeutic proteins, because some tumor cells express much more acid phospholipids in the membrane outer leaflet than their non-tumor counterparts [41]. It is known that the protein transduction domains (PTDs) possess a characteristic positive charge due to high content of Arg and Lys residues. Short amino acid sequence with high positive charge can mediate transduction of an enzyme in a broad spectrum of cell lines, including tumor cells. The efficiency of this transduction may exceed by nearly 600-fold the transduction of the same enzymes in which highly cationic sequences are replaced with random amino acid sequences of equal length. When tested *in vivo*, the PTDs were able to facilitate the efficient and rapid protein delivery into mouse solid tumors [42]. Cationic amphipathic peptides, such as defensins and cecropins, induce cell death by increasing membrane permeability, preferably affecting malignant cells [43].

Cationization of a protein is considered to be a powerful strategy to promote its internalization into cells [44]. The toxicity for malignant cells and the cell-binding ability of chemically modified RNase A and RNase 1 correlate well with their net positive charge, indicating that more cationic RNases are

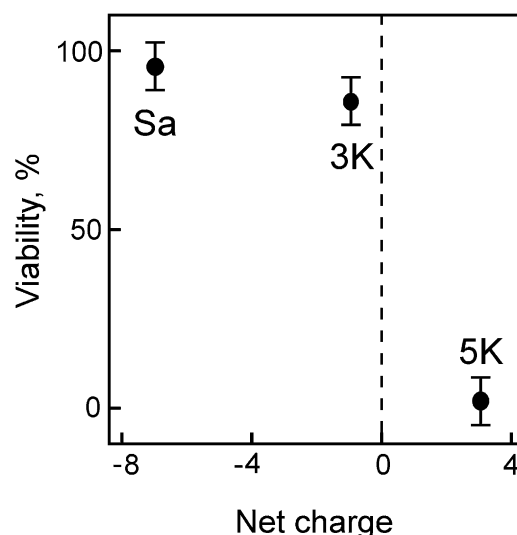


Fig. 3. Viability of *v-ras*-NIH3T3 fibroblasts treated for 24 h with RNase Sa and its 3K (D1K, D17K, E41K) and 5K (3K+D25K, E74K) mutants at a concentration of 500  $\mu$ g/ml versus net charge on the enzymes at pH 7. Based on data published by Ilinskaya et al. (table 1 in [30]).

more cytotoxic [25]. Cytotoxic RNase Sa3 is more cationic than the non-toxic RNase Sa [14]. Replacement of Asp and Glu residues on the surface of RNase Sa molecule with Lys residues made it possible to produce mutants with broad variations in their net charge [45]. By reversing five charges on RNase Sa, it was changed from one of the most acidic proteins to one of the most basic, and it was sufficient to generate

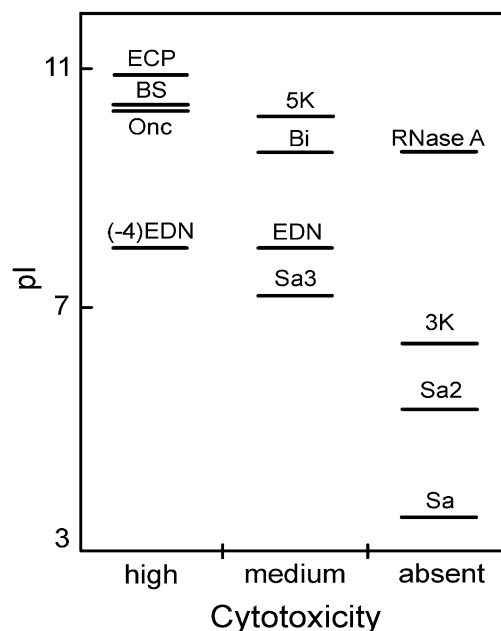


Fig. 4. Cytotoxicity of RNases toward tumor cell lines plotted against isoelectric points (pI): onconase (Onc, [14]), BS RNase (BS, [49]), EDN and (-4)EDN [5], ECP [6], RNase Sa3 (Sa3, [14]), D1K, D17K, E41K mutant of RNase Sa (3K, [30]), 3K+D25K, E74K mutant of RNase Sa (5K, [30]), binase (Bi, [21]), RNase Sa (Sa, [14]), RNase Sa2 (Sa2, Ilinskaya et al., in preparation), RNase A [38]. Enzymes with  $IC_{50}$  values (the RNase concentration that kills 50% of cells) below 5  $\mu$ M form a group of high cytotoxicity, whereas enzymes with  $IC_{50}$  values in the range of 5–30  $\mu$ M constitute a group with medium cytotoxicity.

a cytotoxic RNase [30]. Cytotoxic activity of RNase Sa correlates with the change in net charge from negative to positive (Fig. 3). Fig. 4 shows cytotoxicity of RNases versus their isoelectric points (pI). Despite differences between cell cultures and methods used for determining  $IC_{50}$  (the RNase concentration that kills 50% of cells), positively charged at physiological pH RNases exhibit an evident tendency for cytotoxicity with the exception of non-toxic RNase A (Fig. 4). These results suggest that a net positive charge on the molecule is a key determinant of RNase cytotoxicity. Site-directed mutagenesis allows the creation of positively charged RNases with enhanced toxicity and avoids the unfavorable side effects of chemical modification used for the same purpose. Thus, the development of mutant RNases with increased positive charge may lead to more efficient therapeutics.

## 6. Cellular pathways of RNases

The first step of the extracellular RNase uptake is the protein–membrane interaction that includes the interaction of RNase with intrinsic receptors, membrane lipids, and with the ion transport pathways. Non-specific electrostatic binding of RNase is due to negatively charged carbohydrates on the cell surface. The specificity of this interaction can be due to membrane proteins as well as to lipid clusters. Receptor-like sites on the plasma membrane are known for onconase, angiogenin, and BS RNase [3,9,46]. However, these receptor-like sites for RNases do not contain protein receptors. Indeed, binding studies suggest that there is no specific membrane protein receptor for  $\alpha$ -sarcin, onconase and RNase A [18,47]. Most probably, RNases are internalized via non-protein receptor-dependent mechanisms.

The second step of RNase uptake is internalization via either endocytosis or direct translocation. Direct translocation to the cytoplasm seems to be possible for unchaperoned positively charged and misfolded proteins that exhibit hydrophobic regions. These proteins ( $\beta$ -amyloid protein, prion, calcitonin, etc.) are able to interact with lipid membranes, thereby inducing membrane damage and cell malfunction mediated by the formation of ion channels (reviewed in [48]). For instance,  $\alpha$ -sarcin translocates into artificial lipid vesicles [36]. Results of in vivo experiments, on the contrary, point to endocytosis as the mechanism responsible for the  $\alpha$ -sarcin internalization, probably through acidic endosomes [18]. The pathway of BS RNase from extracellular matrix into the cell also includes endosomes as an essential cellular station [46]. Onconase and RNase A are internalized via acidic vesicles [47]. Thus, RNases appear to enter cells via endocytosis mediated by endosomes [18,46,47,49]. As it was shown for  $\alpha$ -sarcin, onconase, and toxic G88R RNase A, this mechanism is clathrin independent [18,47]. Moreover, for onconase and G88R RNase A it is dynamin independent [47].

The third possible step of RNase pathway is its transport from endosomes to the Golgi network and the endoplasmic reticulum (ER). It was found that in tumor cells BS RNase progresses from the endosomal compartment to the Golgi complex, but in normal cells it does not [46]. A nucleolar localization of BS RNase was also found only for malignant cells [46]. Agents that distort the Golgi apparatus and disrupt retrograde transport from the Golgi complex to ER usually increase the cytotoxic activity of RNases as was found for  $\alpha$ -sarcin [18], angiogenin, RNase 1, BS RNase [49], onconase,

and G88R RNase A [47]. The entry of RNases into the Golgi and their retrograde transport to the ER are not essential for RNase translocation to the cytosol. Probably, RNases are translocated to the cytosol from the pre-ER compartment. Once an RNase reaches the cytosol, it degrades its substrate, RNA. Yet, no direct evidence was found that any cytotoxic RNase crosses the membrane of endosome or of both associated compartments of the Golgi apparatus and the ER.

External RNases target different species of cellular RNA. It seems that nucleolytic cleavage of RNA becomes possible after electrostatic binding of external RNase to membranes, because plasma membranes and ER contain RNA as an integral component and as a structural part of membrane-associated ribosomes. It can be hypothesized that leaving membrane compartments is not necessary for RNase to initiate its cytotoxic action.

Toxic consequences of RNA cleavage may be due both to the inactivation of protein synthesis and to the regulatory effects of substrates and products of RNA hydrolysis. Effects mediated by non-specific (or still non-identified specific) binding of RNases to some cellular components suggest the existence of additional targets of cytotoxic RNases, besides elements of protein synthesis machinery. For example, signal recognition particles, which target proteins to ER, contain RNA as a component of highly conserved RNA–protein core [50]. A regulatory role of small non-coding RNAs [51] indicates that any targeting of RNA-containing cellular components, as well as possible cleavage of micro-RNAs, could lead to very broad alteration of gene expression.

Evidence suggests that the interaction of binase with the ion transport pathways could be implicated in the control of cell proliferation and differentiation phenotypes. The enzyme blocks  $Ca^{2+}$ -activated  $K^{+}$  channels and inhibits the proliferation of *ras*-transformed fibroblasts, whereas these effects do not occur in normal cells and in cells transformed by *src* or *fms* oncogenes [21]. Thus, cells expressing *ras* oncogene were more sensitive to binase than the non-expressing cells. Similar

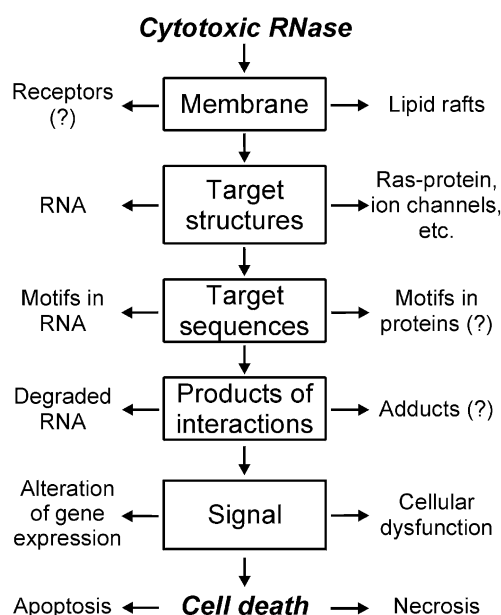


Fig. 5. A scheme of potential specific and non-specific interactions of a cytotoxic RNase with cellular components.



selectivity was observed for the cytotoxic effect of the cationic 5K mutant of RNase Sa [30]. Likewise, onconase exhibits cytotoxic activity towards *ras*-transformed mouse fibroblasts [24]. These data suggest the key role of a positive mediator (Ras) of the proliferative signal transduction in the cytotoxic response in fibroblasts. The Ras-targeting RNases provide therapeutic possibilities, because Ras proteins have become important targets in pharmacological approaches to cancer therapy [52]. Indeed, it is known that Ras mutations contribute to at least 25% of all human cancers. In experimental animals, the frequency of mutations in *ras* genes in preneoplastic lesions is about 20–60% (reviewed in [53]). It has been demonstrated that *ras* expression is significantly suppressed by small interfering RNAs generated in vitro and transfected into mammalian cells [54]. Evidently, degradative potential of RNases could contribute to the alteration of *ras* expression.

The hypothetical scheme in Fig. 5 reflects a complicated net of specific and non-specific interactions of cytotoxic RNases with cellular components. It illustrates our idea that the enzyme–substrate interaction as well as other direct and mediated effects of cytotoxic RNases are important for understanding the mechanisms of RNase cytotoxicity. Attempts to identify cellular targets of cytotoxic RNases encounter the problem of distinguishing between their direct ribonucleolytic action and indirect effects. Currently, it is unclear which of these targets is more effective in triggering apoptotic events and, therefore, more promising for cancer treatment.

In conclusion, the killer strategy of cytotoxic RNases includes hitting of a main target, RNA, but does not exclude additional ravages leading to the cell death. Now, when major biological activities of RNases have been exposed, time has come to translate this knowledge into new therapeutic applications.

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