**Modifications of Ribonucleases** **in Order to Enhance Cytotoxicity in Anticancer Therapy**

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**Abstract**

Ribonucleases (RNases) are a superfamily of enzymes that have been extensively studied since the 1960s. For a long time, this group of secretory enzymes was studied as an important model for protein chemistry such as folding, stability and enzymatic catalysis. Since it was discovered that RNases displayed cytotoxic activity against several types of malignant cells, recent investigation has focused mainly on the biological functions and medical applications of engineered RNases. In this review, we describe structures, functions and mechanisms of antitumor activity of RNases. They operate at the crossroads of transcription and translation, preferentially degrading tRNA. As a result, this inhibits protein synthesis, induces apoptosis and causes death of cancer cells. This effect can be enhanced thousands of times when RNases are conjugated with monoclonal antibodies. Such combinations, called immunoRNases, have demonstrated selective antitumor activity against cancer cells both in vitro and in animal models. This review summarizes the current status of engineered RNases and immunoRNases as promising novel therapeutic agents for different types of cancer. Also, we describe our experimental results from published or previously unpublished research and compare with other scientific information.

**Keywords:** Apoptosis, cancer therapy, clinical trials, cytotoxicity, immunoRNases, ribonuclease

**1. INTRODUCTION**

Cancer is one of the deadliest diseases in both developed and developing countries [1]. Conventional methods such as chemotherapy, radiotherapy and surgery are used to fight cancer. These methods have saved many lives; however, they also have serious side effects [2]. Hence, identification of better ways to combat cancer is a continuing challenge for cancer researchers. The discovery of monoclonal antibody (mAb) for cancer therapy opened a promising window. 2It led to the approval of many mAbs by FDA to treat a wide variety of malignancies [3]. Targeting cancer cells via antibodies specific for tumor-associated surface proteins establishes cellular selectivity that is not available with radiotherapy and chemotherapy. On the other hand, immunotherapy that endeavors to simulate a host response in order to effectuates long-lived tumor destruction is attracting a lot of attention and may potentially become a leading approach in the treatment of cancer. The chimeric-antigen receptor T-cell (CAR-T) is one of adoptive immunotherapy that showing remarkable efﬁcacy in the treatment of several B-cell. CAR T-cell therapy is a way to get T cells (a type of immune cells) to fight cancer by changing them in the lab so they can find and destroy cancer cells [4].

Recently, anticancer immunotherapy strategies have emerged that are based on immunoconjugates employing toxins, drugs or ribonuclease (RNase) molecules [5-7]. The recent strategy in cancer therapy is to discover, characterize, and validate the most promising cancer-related molecular targets for which new drugs can be designed [8, 9]. Over the past two decades, a number of highly potent plant and bacterial protein-based immunotoxins have been applied to cancer therapy [10, 11]. However, combining these toxins and antibodies is limited, due to their high immunogenicity and inherent and non-specific toxicity [8].

An alternative source of reagents to overcome the problem of immunogenicity and non-specific toxicity is the RNase A super family. These enzymes have shown potent cytotoxic activity upon cell internalization, but as there is not any RNase receptor in the surface of the cell [12-15]. RNase can degrade the transcriptome of a cell after internalization into cytosol; therefore, it can be applied as a biological tool to induce cell death. For example, injection of bovine pancreatic RNase A and B into oocytes was at least as potent as ricin at abolishing protein synthesis [16].

Several studies showed the RNA-hydrolyzing action of RNase could induce apoptosis and cell death in cancer cells. This could be enhanced thousands of times when RNase is linked to antibodies [9, 17-19]. However, for this important property to be applied in the cell, the RNase must be able to evade cytosolic RNase inhibitor (RI) so that it can remain functional [20-22].

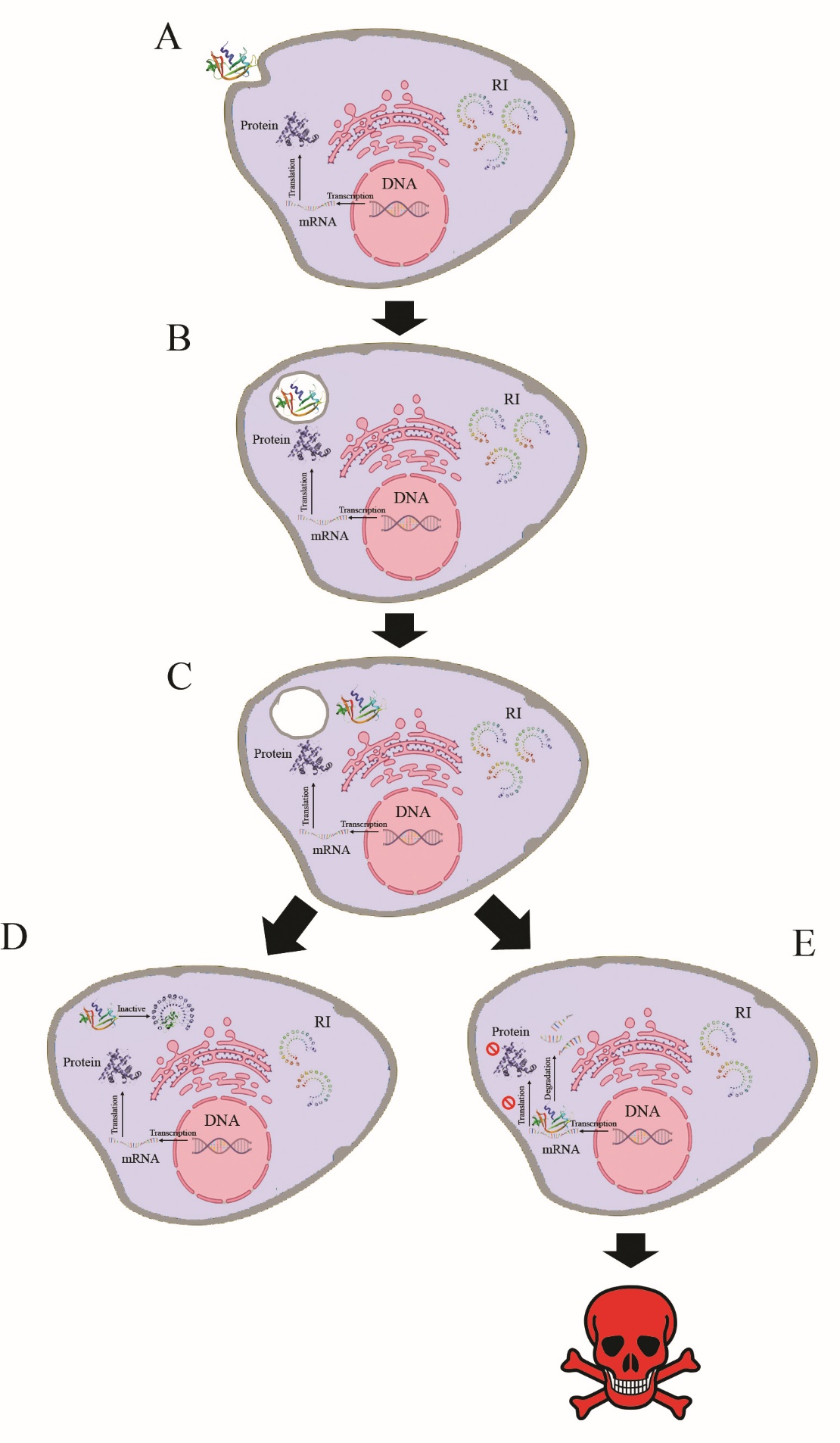
In this review, we are going to show our experimental results on immuno-RNase as we published or unpublished yet, and comparison with other scientific materials.

**2. RNASES AND RIBONUCLEASE INHIBITOR (RI)**

RNases are heterogeneous enzymes that catalyze the cleavage of phosphodiester bonds in RNA [6, 7]. Their catalytic activity is indispensable for causing cytotoxicity to cancer cells [15, 18, 19]. The mechanism of ribonuclease-mediated cytotoxicity is depicted in Fig. **1**. All of the natural RNases that cause toxicity are grouped in the pancreatic-type RNase superfamily [5]. Pyrimidine-specific RNases are the main feature of the members of this family. These RNases are found in reptiles, amphibians and birds and are highly homologous to those of bovine pancreatic ribonuclease A (RNase A) [13, 14].

RNases play an essential role in RNA metabolism in host defense and physiological cell death pathways. There are approximately 20 RNases with divergent or overlapping specificities [23, 24]. The variety of specificities suggests these enzymes might be useful as therapeutic factors for various types of cancer, possibly as important alternatives to standard DNA-damaging chemotherapeutics.

RNase A, the most important pancreatic-type of RNase in the superfamily, was the first enzyme to have its complete amino acid sequence determined [12, 13, 16]. This enzyme has the ability to cleave RNA specifically on the 3′ side of pyrimidine bases. Human pancreatic ribonuclease (RNase 1) is secretory in nature and has many similarities to RNase A [25]. The structures of RNase 1 and RNase A are similar (70%), as are key structural and catalytic residues; however, RNase 1 shows some unique features [26]. It has remarkable activity against double-stranded RNA because of a higher proportion of basic residues. RNase 1 activity is substantially affected by ionic strength and divalent ions in comparison with RNase A. It has a carboxyl-terminal extension of four residues [27].



**Fig. (1).** Mechanism of ribonuclease-based cytotoxicity. **A**: ribonucleases bond to the cell membrane and **B**: inter to cell via endocytosis and **C**: translocate into the cytosol. **D**: In the cytosol, ribonucleases are bonded and inhibited by RI and then inactive or **E**: evade RI, degrade cellular RNA and ultimately cause cell death.

Mammalian pancreatic-type ribonucleases (ptRNases) have the necessary catalytic activity and non-immunogenicity to warrant consideration as potential chemotherapeutic agents, but their success is limited by two substantial barriers. The first is [internalization](https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/internalization) into the cytosol of target cells, and the second is inhibition by RI. RI is a mammalian protein found exclusively in the cytosol. Several biological roles have been suggested for RI, including preserving cellular redox homeostasis and protecting cells from internalized secretory ptRNases [20-22]. Although the precise physiological role(s) of RI is unclear, it attaches to members of the mammalian ptRNase superfamily with a 1:1 stoichiometry, leading to complete inhibition of its catalytic activity by steric occlusion of the enzyme active site.

RI binds with femtomolar affinity to most ptRNases [6, 7], but not BS-RNase (Fig. **2**) [28]. Therefore, the dimeric BS-RNase and an amphibian ortholog, Onconase® (ONC), have proven to be natural cytotoxins for human cancer cells [29- 31].

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| **A** | **B** |

**Fig. (2).** Three-dimensional structure of the RI·RNase complexes. **A**: human RI·RNase 1 complex (PDB ID:1z7x), **B**: porcine RI·RNase A complex (PDB ID:1DFJ).

Adapted from Ref. [27]

Success in applying ptRNase to induction of cellular apoptosis by cytotoxic activity depends on evading RI [32]. However, because of the tight junction between RI and its ligands, evading RI is a difficult task. Nevertheless, modifying the appropriate ptRNase residues can reduce their interaction [33]. To recognize the residues that exist in the binding interfaces, the residues containing most contacts with RI were identified by computational analysis [22]. The identified residues were then exposed to site-directed mutagenesis. Liu et al. [34] found that electrostatic and steric incompatibilities in these regions could weaken RI•RNase binding, and this destabilization could best be achieved if small neutral or anionic residues in ptRNase replaced arginine. Hence, arginine, as the most polar and second largest amino acid, is responsible for steric strain and electrostatic repulsion with a high level of positive charge. The engineered protein was able to maintain greater RNase activity in the cytosol. An efficient ptRNase-based cytotoxin must enter the cytosol, evade RI, and retain its catalytic activity throughout the process [35]. The second-order rate constant (kcat/KM = 3.3 × 109 M−1 s −1) of mammalian ptRNases is the highest rate for any enzyme-catalyzed reaction [36]. Cellular apoptosis is activated after this cleavage, which is vital for ptRNase-mediated cytotoxicity [31]. Proteolytic degradation of proteins with high thermo-stability tends to be lower [37]. Adding a disulfide bond to RNase A significantly enhanced its resistance to proteolysis, improved thermo-stability, and increased cytotoxicity [13, 14]. Altering residues within ptRNases may also have negative consequences. Changes that interfere with active-site residues or other residues critical to structure and function must be avoided [38].

Protein engineering may also be useful for increasing the internalization of RNase. The ErbB-2 transmembrane tyrosine kinase receptor is an appealing target for immunotherapy because of its key role in the development of malignancy [39, 40]. The activated ErbB-2, which can be imitated by an antibody directed toward the receptor, is readily internalized; therefore, RNase can be delivered through anti- ErbB-2 antibody into ErbB-2-overexpressing tumor cells. This strategy has been tested using a murine scFv attached to a human RNase [41].

**3. RNASE FAMILY ENGINEERING**

In the cytosol of normal cells, RNase must be regulated so that only the correct amount of RNA is degraded. RI is important in this regulation. However, in cancer cells, if RNase is to be an effective cytotoxin, enough RNA must be destroyed that the cells’ ability to reproduce is decreased. In this situation, it is important to decrease the formation of the RI-RNase complex that inactivates RNase.

RI can capture and inactivate RNase by forming hydrogen bonds, actually, hydrogen bonding plays a crucial role in the formation of the complex [21]. Therefore, it is important to identify the amino acids that participate in the formation of the RI•ptRNase complex and inactivate RNase [21]. For RNase to function as cytotoxic agents, information is needed about several important steps. They are internalization**,** escape from lysosome degradation**,** release and relocation into the cytosol**,** evading the cytosolic RNase inhibitor**,** hydrolyzing target RNAs, and inhibiting protein synthesis. RNases can then be engineered to maximize their cytotoxic effects. The next part of this review will examine the effect of amino acid substitution in RNases (RNase engineering) and how this substitution affects bonding in the RI-RNase complex.

**3.1. Human Pancreatic Ribonuclease (RNase 1) Engineering**

Based on our genetic and protein engineering knowledge of RNase 1, we have hypothesized that the substitution of different amino acids in key positions might reduce interaction with RI and improve cytotoxic activity. We expected the amino acid substitutions in the HP-RNase 1 surface loop to not only preserve conformational stability and ribonucleolytic activity but also to reduce the inhibitory efficacy of RI in cytosol. Hydrogen bonds are important for the stability of a protein and for the interaction between two proteins: the number of hydrogen bonds has a direct relationship with stability [19]. Consequently, substituting amino acids for those that participate in the formation of hydrogen bonds is a useful strategy to decrease affinity of a protein-protein interaction [42]. There are 124 amino acids in RNase. Eleven of these can form hydrogen bonds with RI [43] (Table **1**). Bretscher et al. [44] engineered human RNase and substituted lysine at 41 and glycine at 89 for two arginines. Their results demonstrated that the engineered enzyme could evade RI, but compared to wild type, its activity was low. The low activity was related to lysine 41 substitution, because this residue is located in an active site. In order to engineer human RNase, Gaur et al. [45] substituted lysine 7, asparagine 71 and glutamine 111 with three alanines. Three other locations can form hydrogen bonds between RNase and RI [30]. The results of this project showed that engineered RNase can maintain its activity and structure along with the ability to evade RI [45]. In related research, D'Avino et al. [46] engineered human RNase for conjugation with Trastuzumab. In this case, arginine 39, asparagine 68, asparagine 88, glycine 99 and arginine 91 were substituted with aspartic acid, aspartic acid, alanine, aspartic acid and aspartic acid, respectively. The engineered enzyme was more cytotoxic than the wild enzyme [46]. It is important to note that arginine 39 of RNase can bond with glutamic acid 397 of RI, and asparagine 67 of RNase can bond with valine 495 of RI [30], providing adequate hydrogen bonding.

The history of RNase 1 is summarized in Table **2**. During this decade, our research group did some RNase engineering by substituting suitable amino acids. Since 2010, we have also focused on monoclonal antibody engineering. In 2014, our group identified several amino acids with the potential to influence the interaction between RI and RNase 1 and subjected them to substitution mutagenesis [47]. We expected the amino acid substitutions in the HP-RNase 1 surface loop to not only preserve its conformational stability and ribonucleolytic activity but also to reduce the efficacy of RI in mammalian cytosol. To reduce hydrogen bonding in RNase, amino acids were substituted for lysine 7, asparagine 71, asparagine 88, arginine 91, and glutamic acid 111. Also, to increase heat resistance, the last four amino acids of RNase were deleted. It is important to note that alanine was the main amino acid substituted, because it has no side chain, and it is also small. After the substitu tions were confirmed, both engineered and wild enzymes were purified. RNase activity was evaluated by the RNase precipitation assay. The engineered RNase retained its activity better than the wild RNase in the presence of RI. These data suggest the engineered recombinant HP-RNase 1 as a potential new immunotherapeutic agent for application in human cancer therapy [47].

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| **Table 1. The important hydrogen bonds in RNase 1.hRI complex (PDB ID 1Z7X)** | |
| **RNase 1** | **hRI** |
| ARG4 | TRP438 |
| LYS7 | SER460 |
| GLN11 | SER460 |
| ARG31 | GLN10 |
| ASP35 |
| ARG32 | ASP36 |
| ARG39 | TRP434 |
| GLU401 |
| LYS41 | ASP435 |
| ASN67 | LEU409 |
| TYR437 |
| ASN88 | LYS320 |
| GLY89 | TRP261 |
| ARG91 | GLU287 |
| Hydrogen bonds were identified using pyMOL software by measuring N-O distances between RNase 1 and hRI in hRI. RNase1 complex.  Adapted from Ref. [43] | |

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| **Table 2. Human pancreatic RNases engineered and their characteristics.** | | | | |
| **Ribonuclease** | **IC50a (µM)** | **Kdb**  **(nM)** | **Conclusion** | **Refs.** |
| Wild-type RNase 1 | >25 | 29🞨10-8 |  | [48] |
| K41R | no toxicity was observed at a ribonuclease concentration of 10 µM | ND | Similar binding affinity for ribonuclease inhibitor to wild type, 60 fold reductions in enzymatic activity. | [28, 49] |
| N88R/G89S |  | ND | Similar affinity for hRI with wild type. | [50] |
| Q11A  K7A  N71A  E111A | 41  46  57  26 | ND | Higher cytotoxic activity than the wild type HPR, higher resistance toward inactivation by RI, hydrolytic activity similar to wild type. | [45] |
| K7A/N71A/E111A | 18 | 0.5 | 10 4 fold Higher resistance toward inactivation by RI than wild type RNase 1 | [45] |
| L86E/N88R/G89D/R91D | 7 | 0.21 | Conformational stability and ribonucleolytic activity similar to the wild type RNase 1 and >103 fold lower affinity for RI | [28] |
| N88C |  |  | Reduced binding affinity for ribonuclease inhibitor, Similar ribonuclease activity with wild type. | [51] |
| R4C/L86E/N88R/G89D/R91D/V118C | 3 | 2.6 |  | [28] |
| Truncation of seven amino-acid residues at the amino terminal sequence of RNase 1 |  |  | reduced ribonucleolytic activity (approximately nine times lower than RNase 1 towards yeast RNA), reduced binding ability to PRI | [50] |
| G89R, S90R | ranging from 0.3 to 3 µM |  | 5-15 times more cytotoxic than Onconase, weakly evaded the RI | [52] |
| K41R/Q69A/N88R/G89D/R91D | 7.4 | <250 |  | [53] |
| K7A  Q69A | >10 |  | Significant changes in catalytic activity versus wild type, exhibited a slight increase in activity in the presence of RI versus wild type. | [53] |
| T24L/Q28L/R31L/R32L |  |  | Decrease in stability, the leucine incorporation did not affect the enzymatic activity of RNase 1. | [54] |
| aValues of IC50 are for the incorporation of [*methyl*-3H]thymidine into the DNA of human cancerious cells.  bValues of the equilibrium dissociation constant (or inhibition constant) are for the complex with human RI. | | | | |
| **Table 2 (Continued). Human pancreatic RNases engineered and their characteristics.** | | | | |
| **Ribonuclease** | **IC50a (µM)** | **Kdb**  **(nM)** | **Conclusion** | **Refs.** |
| R39D/N67D/N88A/ R91D | >25 | 278±50 | 3.3 fold decrease in activity. | [30] |
| R4C, V118C, N88L, G89R, R91G |  |  | Retains ribonucleolytic activity, exhibits enhanced cytotoxic activity relative to wild type, have a lower binding affinity for hRI than wild type | [55] |
| R39D |  |  | Decreases the affinity of hRI for RNase 1,cytotoxicity lower than other variants with similar RI evasion, 3-fold decrease in the catalytic activity | [55] |
| G38R/R39G/N67R/N88R | >25 | 0.032± 0.016 | Retains ribonucleolytic activity, lower affinity for hRI than wild type, exhibits enhanced cytotoxic activity relative to wild type | [56] |
| R91L |  |  | 110 fold lower affinity | [56] |
| N88R |  |  | Not useful, steel high affinity for RI. | [56] |
| R4C/G38R/R39G/N67R/R91G/V118C | 15 | (5.5±1.6) 10-8 M | Retains ribonucleolytic activity, exhibits enhanced cytotoxic activity relative to wild type and has a lower binding affinity for hRI than wild type. These substitutions could electrostatically repel and/or sterically hinder binding of human ribonuclease inhibitor (hRI). | [57] |
| R39D/N67D/N88A/G89D/R91D  (DDADD) | 13.3+\_1.7 | (1.7±0.5) 103 | 10 7 fold lower affinity and 2700 fold lower association rate for hRI than wild type RNase 1, not potent cytotoxin because its anionicity deters cellular uptake. | [58, 59] |
| K7A/N71A/N88A/ R91D/E111A  (AAADA) |  | 463±22 | keep ribonucleolytic activity in the presence of RI, lower affinity for hRI than wild type, exhibits enhanced cytotoxic activity relative to wild type | [47] |
| R4C/V118C |  |  | The enzymatic activity nearly of the native RNase 1  Evades hRI, Exhibits significant cytotoxic activity. | [60] |
| R39L/N67L/N88A/G89L/R91L | >25 | 30 ± 1 | Native ribonucleolytic activity  Lower binding affinity for RI  Exhibit enhanced cytotoxic activity | [60] |
| G89 |  |  | Failed to produce variants with lower affinity and cytotoxic variant. | [60] |
| N67D |  |  | Destabilize the complex by 1.9 Kcal/mol  Plays a role in complex formation | [60] |
| aValues of IC50 are for the incorporation of [*methyl*-3H]thymidine into the DNA of human cancerious cells.  bValues of the equilibrium dissociation constant (or inhibition constant) are for the complex with human RI. | | | | |

**3.2. Bovine Pancreatic Ribonuclease (RNase A) Engineering**

In 2020, our group engineered RNase A for conjugation with Cetuximab [61]. Based on a literature review [30, 45, 46], six amino acid residues of wild-RNase A including, lysine 7, arginine 39, [asparagine](https://en.wikipedia.org/wiki/Asparagine) 67, [asparagine](https://en.wikipedia.org/wiki/Asparagine) 71, [glycine](https://en.wikipedia.org/wiki/Glycine) 88 and glutamic acid 111 were substituted with [alanine](https://en.wikipedia.org/wiki/Alanine), [aspartic acid](https://en.wikipedia.org/wiki/Aspartic_acid), [aspartic acid](https://en.wikipedia.org/wiki/Aspartic_acid), [alanine](https://en.wikipedia.org/wiki/Alanine), [arginine](https://en.wikipedia.org/wiki/Arginine) and [alanine](https://en.wikipedia.org/wiki/Alanine), respectively. After the substitution, the most important features of engineered-RNase A were investigated. In order to investigate formation of disulfide bonds, PROTSITE, as a part of ExPASy, was employed to predict 2D and 3D structures, SOMPA and I-TASSER software were applied, respectively. The results showed that the engineered-RNase A could not only maintain its structure and other features, but it could also evade RI [61]. To measure and compare the activity of wild-RNase A and engineered-RNase A, RNA fragmentation assay was applied. To perform this assay, both enzymes were incubated with equal concentrations and volumes of yeast RNA for 15, 30, 60, 90 and 120 s, with RI used to inhibit. Then, incubated products were loaded on 1% agarose gel to detect the rate of RNA fragmentation based on brightness of bands. The function of wild RNase A in RNA degradation was greater than the engineered RNase A; therefore, amino acid substitution decreased the engineered RNase A activity. Other research reported results of amino acid substitution in RNase A to decrease affinity to RI. To decrease affinity between bovine pancreatic RNase and RI, arginine was substituted for glycine 88 [62]. The side chain of glycine 88 of RNase can make a strong hydrogen bond with tryptophan 257 of RI. Because of the lack of a side chain in arginine, this amino acid not only could eliminate formation of a hydrogen bond but also could maintain activity of RNase [61]. This RNase was named G88R/RNaseA: affinity between this RNase and RI was 10,000 fold lower than with wild RNase [62]. Their result proved that glycine 88 has an important role in interaction between RNase A and inhibitor, which is consistent with our research. Most important hydrogen bonds in RNase A-RI are mentioned in Table **3** [43]. These researchers also replaced Asp-38 and Ala-109 residues of RNase A with arginine. Their results indicated that replacing Gly-88 with aspartate (G88D RNase A) decreased affinity for RI and increased cytotoxic activity. When Asp-38 and Ala-109 were replaced by arginine, RNase A remained highly sensitive to hRI, with no effect on the RI–RNase interaction. Also, these variants were not cytotoxic.

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| **Table 3. The important hydrogen bonds in RNase A.pRI complex (PDB ID 1DJF)** | |
| **RNase A** | **RI** |
| LYS1 | GLU440 |
| GLU2 | ARG443 |
| LYS7 | SER456 |
| GLN11 | SER456 |
| GLN28 | ASN89 |
| LYS31 | GLN6 |
| ASP38 | ARG453 |
| ARG39 | GLU397 |
| GLN426 |
| LYS41 | ASP431 |
| ASN67 | LEU405 |
| TYR433 |
| SER89 | ASP228 |
| TRP259 |
| LYS91 | GLU283 |
| GLU111 | TRP433 |
| GLU436 |
| Hydrogen bonds were identified using pyMOL software by measuring N-O distances between RNase A and pRI in pRI. RNase A complex Adapted from Ref. [43] | |

Rutkoski et al. [63] designed RNase A variants that evade RI in order to disrupt the RI-RNase A interaction. They replaced three RNase A residues (Asp-38, Asp-67, and Gly-88) that form multiple contacts with RI in RI-RNase A with arginine, and arginine39 in this complex with aspartate. Their results indicated these substitutions increased the *K*d value of the pRI·RNase A complex by 20×106-fold (to 1.4 μM). In addition, changes to [catalytic activity](https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/enzymatic-activity) and conformational stability were small. This variant of RNase A was more toxic than ONC to human cancer cells. In order to produce dimeric and trimeric conjugates of four pancreatic-type ribonucleases, Rutkoski et al. [64] used eight distinct thiol-reactive cross-linking reagents. Their results indicated that a monomeric RI-evasive variant of RNase A could inhibit the growth of human prostate and lung tumors in mice. An RI-evasive trimeric conjugate inhibited tumor growth at a lower dose than the monomer. They attributed this effect to enhanced persistence of the trimers in circulation.

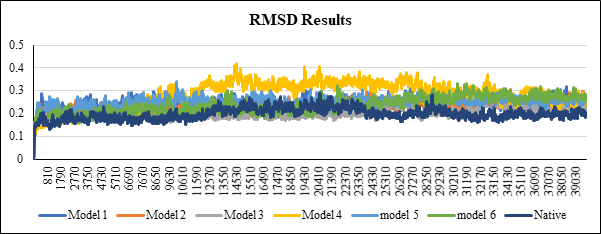
Fuchs et al. [65] proved that residues Glu49 and Asp53 form an anionic patch on the surface of RNase A. They discovered that replacing these two residues with arginine did not affect affinity of cytosolic RI or catalytic activity while it could increase toxicity towards human cancer cells. These results correlate the potency of a RNase with its deliverance of ribonucleolytic activity to the cytosol and demonstrate a rational means to increase the efficacy of RNases and other cytotoxic proteins. Some engineered RNase A enzymes are shown in Table **4**.

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| **Table 4. Salient Modifications of bovine pancreatic RNases that enhance cytotoxicity.** | | | | | | |
| **Ribonuclease** | ***T*m (°C)a** | **Ribonucleolytic Activity (%)b** | ***K*i or *K*d (n*M*)c** | **IC50 (μ*M*)d** | ***Z***[**e**](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3304445/table/T1/?report=objectonly#TFN5) | **Refs.** |
| Wild-type RNase A | 64 | 100 | 44 × 10−6 | >25 | +4 | [63] |
| G88R RNase A | 60 | 142 | 2.8 | 6.2 | +5 | [62] |
| A4C/G88R/V188C RNase A | 69 | 94 | 0.65 | 3 | +5 | [66] |
| D38R/R39D/N67R/G88R RNase A | 56 | 75 | 1.4 × 103 | 0.19 | +6 | [63] |
| E49R/D53R/G88R RNase A | 54 | 5 | 2.6 | 1.9 | +9 | [65] |
| E49R/D53R/G88R RNase A–R9 | 49 | 7 | 3.0 | 0.58 | +18 | [65] |
| (RNase A)2 [SGRSGRSG linker] | 61 | 1.2 | ND | 12.9 | +10 | [16] |
| (D38R/R39D/N67R/G88C RNase A)3 | ND | 17 | ND | 1.0 | +16 | [64] |
| K7A/R39D/N67D/N71A/G88R/E111A RNase A | ND | 65 | ND | 0.5 | ND | [61] |
| aValues of *T*m are the temperature at the midpoint of thermal denaturation, which can be monitored by ultraviolet or circular dichroism spectroscopy.  bValues of ribonucleolytic activity are relative to the wild-type enzyme.  cValues of the equilibrium dissociation constant (or inhibition constant) are for the complex with human RI.  dValues of IC50 are for the incorporation of [*methyl*-3H]thymidine into the DNA of K-562 human leukemia cells.  eValues of *Z* refer to the net molecular charge: Arg + Lys − Asp − Glu − Pyr (where “Pyr” refers to a pyroglutamate residue, which is found at the N-terminus of Onconase®) | | | | | | |

**3.3. Onconase Engineering**

The antitumoral activity of pancreatic-type RNases was initially observed with onconase (ONC), an amphibian endoribonuclease homologue of RNase 1 from the oocytes of the northern leopard frog (Rana pipiens) [67]. ONC is the smallest (104 amino acid residues) member of the RNase A superfamily that is toxic to tumor cells both in vitro and in vivo [8]. These two proteins share 30% amino-acid sequence identity and a similar three-dimensional structure. The absence of a cis prolyl peptide bond and four disulfide bonds contribute to this protein’s remarkable conformational stability (Tm= 90 °C). This enzyme exhibits anticancer properties mediated by degradation of cellular RNA and induction of apoptosis [68]. ONC is also able to evade cellular RI. The ability to retain ribonucleolytic activity in the presence of RI is the basis of the cytotoxicity of ONC. Evasion of this enzyme is based on divergent tertiary structure, which has severe truncations in its surface loops [69]. Despite the apparent absence of a cell‐surface receptor protein, internalization of ONC to the cytosol of cancer cells is essential for its cytotoxic activity [70]. The ribonucleolytic activity of ONC is three to five orders of magnitude lower than that of RNase A, due in large part to low affinity for its substrate. Nonetheless, the catalytic activity of ONC is necessary for cytotoxicity [29, 71].

In 2020, we published a paper on ONC engineering as an RNase family member. In this study, we modified Ranpirnase and assessed engineered Ranpirnase characteristics such as cellular uptake, cytotoxicity, the ability to evade RI, and protein functionality [72]. Six models of Ranpirnase enzyme were designed, based on protein docking and molecular dynamic (MD) simulation results. Then, based on MD results, five mutations (K31A, K55A, S72C, R73A and K76A) were selected, because the results confirmed that the combination would be effective in achieving the main objective of the study (increasing the cellular uptake and evading RI with no significant adverse effect on the structural stability of Ranpirnase). Molecular dynamic simulation results for stability of engineered Ranpirnase are shown in Fig. **3**. These results demonstrate that RMSD values of the recombinant proteins (Sample 1–Sample 5) are rather unstable in comparison to the native protein. Nevertheless, the native protein was stabilized at around 3 ÅRMSD value. The majority of RMSD values of the recombinant proteins were higher compared to the native protein, particularly Model 4 which showed the highest value. Also, Fig. **3** indicates that the amino acid replacements in Model 6 had no significant destabilizing effects on the protein structure.



**Fig. (3).** RMSD analysis of six models designed for engineering of ONC enzyme. Model 6 had a similar pattern with the native model.

Adapted from Ref. [72]

Our experimental results revealed that the activity of the recombinant Ranpirnase produced in this study was 213 ± 19 U/mol, which is 3.5 times lower than RNase A (746 ± 13 U/nmol); however, this enzyme is resistant to RI. Also, our recombinant Ranpirnase was able to induce apoptosis in HeLa cancer cells at 1.99 μg/uL, whereas RNase A had no cytotoxic effects on these cells under similar conditions [72]. Since it appears there is a correlation between endocytosis and cytotoxicity due to the net positive charge of RNases, Turcotte et al. [73], in order to dissect the contribution made by the endogenous arginine and lysine residues of ONC to its cytotoxicity, created 22 variants in which cationic residues were replaced by alanine. They demonstrated that variants with the same net charge (+2 to +5) and equivalent catalytic activity and conformational stability exhibited large differences (>10‐fold) in toxicity to cells of a human leukemia line. In addition, they discovered that, depending on the distribution of cationic residues, a more cationic ONC variant could be either much more or much less cytotoxic than a less cationic variant. Lee [67] reported the first atomic structures of ONC-nucleic acid complexes which were a T89N/E91A ONC-5′-AMP complex at 1.65 Å resolution and a wild-type ONC-d(AUGA) complex at 1.90 Å resolution. They used the latter structure and site-directed mutagenesis to determine the atomic basis for substrate recognition by ONC. Since Glu91 of ONC forms two hydrogen bonds with guanine, the nucleobase in d(AUGA), and Thr89 is in close proximity to that nucleobase, they installed a neutral or cationic residue at position 91 and an asparagine residue at position 89. Their results indicated that replacing T89 with an asparagine residue increased the value of *k*cat/*K*M for UpA cleavage by 3-fold. In addition, this substitution (T89N) decreased the guanine preference of ONC by 50-fold. Moreover, the E91A, E91Q, and E91N variants of ONC cleaved UpG 2- to 9-fold more quickly than UpA, indicative of substantially lower guanine preferences than that of the wild type ONC.

ONC is a homologue of RNase A that possesses a conserved active-site catalytic triad composed of His10, Lys31, and His97 [29]. The three-dimensional structure of this enzyme suggests that two additional residues, Lys9 and an N-terminal lactam formed from a glutamine residue (Pca1), could also contribute to catalysis [67]. To determine the role of Pca1, Lys9, and Lys31 in the function of ONC, Lee and Raines [74] replaced each of them with alanine by site-directed mutagenesis. The results of this project revealed that K9A and K31A variants display 103-fold lower kcat/KM values than the wild-type enzyme, and a K9A/K31A double variant has a >104-fold decrease in catalytic activity. In addition, replacing Lys9 or Lys31 eliminates the antitumoral activity of ONC. Their results also indicated that replacing Pca1 with an alanine residue decreased the catalytic activity of ONC by 20-fold. Yet, replacing Pca1 in K9A variant enzyme did not further reduce catalytic activity, which indicates that the function of the N-terminal pyroglutamate residue is to secure Lys9. Based on binding isotherms with a substrate analogue, this research indicated that Lys9 and Lys31 contribute little to substrate binding and that the low intrinsic catalytic activity of ONC originates largely from the low affinity of the enzyme for its substrate [74].

Majchrzak et al. [75] assessed cytotoxicity of ONC in combination with currently used anticancer drugs on a human diffuse large B-cell lymphoma (DLBCL)-derived cell line (Toledo cells). They measured cytotoxic activity by the exclusion of propidium iodide assay while apoptosis was assessed by the annexin-V binding method. Additionally, they used flow cytometry to assess the decline of mitochondrial potential and to determine activation of caspases 3, 8 and 9. Their findings indicated that in vitro treatment with ONC in combination with rituximab, mafosfamide, vincristine, doxorubicin, and dexamethasone (drugs corresponding with elements of R-CHOP regimen) resulted in increased cytotoxicity. In conclusion, ONC showed marked cytotoxicity against Toledo cells. Their results also showed that the main mechanism responsible for intensifying cytotoxicity when ONC was combined with drugs imitating the R-CHOP regimen was induction of apoptosis along a mitochondrial-dependent pathway [75].

**4. IMMUNOTOXINS AND IMMUNORNASES**

Immunotoxins (ITs) have been used as anticancer drugs for the past 2 decades. They consist of the antibody or mini-antibody moiety and the toxin moiety [11, 53]. These molecules can bind to specific target cells, cross the cell membrane, enter the target cell and selectively kill them. The specificity of the reaction with the target cells through these chimeric proteins is dependent on the antibody part, and the toxicity effect is due to the toxin moiety [10, 11].

The toxin moiety is typically of plant or bacterial origin, but the high toxicity of these moieties has considerably narrowed the therapeutic potential of ITs, in particular because of the occurrence of vascular leak syndrome, immunogenicity and nonspecific toxicity [76, 77]. These effects have been lessened by developing humanized antibodies, but the toxins themselves remain a problem. In order to solve the non-specific toxicity and immunogenicity of the toxin moiety, researchers have suggested human effector enzymes as alternatives to heterologous toxins, because they are much less immunogenic than xenogeneic proteins [78]. Therefore, members of the RNase A family, as important human effector enzymes, warrant further investigation. The first immuno-RNase (IR) was made of pancreatic RNase A that was attached to an anti-transferrin receptor antibody and was cytotoxic to tumor cells expressing the transferrin receptor in the 200-500 nM range [15].

ErbB2 transmembrane receptor, an attractive target for IR, is expressed on numerous tumor cells, especially in breast, ovary and lung carcinomas, and is proposed in the development of malignancy [79]. Also, ErbB2 expresses in normal cells only in certain epithelial cell types and at low levels [80]. It has been proved that Herceptin acts as an effective drug in clinical anticancer therapy [81].

Erbicin-human-RNase (ErbhRNase) is the fusion of RNase 1 with a human single chain variable fragment (scFv) [82]. Erb-hRNase can distinguish between target and non-target cells and especially hindered the proliferation of ErbB2-positive cells in an in vitro test. The antitumor activity of native HP-RNase has been proved by this chimeric protein in vivo in mice implanted with ErbB2- positive tumors [82, 83].

Erb-hcAb-RNase, a novel human IR, was constructed by fusing compact anti-ErbB2 antibody and HP-RNase with anti-cancer activity against the Her2 receptor [84]. This study indicated that Erb-hcAb-RNase preserves the enzymatic activity of HP-RNase and explicitly binds to ErbB2-positive cells with high affinity, compared to that of the parental Erb-hcAb. This type of IR acts effectively and selectively for ErbB2-positive tumor cells both in vitro and in vivo, and it has more antitumor activity than the parental Erb-hcAb, ERB–HP-DDADD-RNase, as the second-generation anti-ErbB2 IR, is constructed by fusing Erbicin, a human ErbB2-directed scFv, with an inhibitor-resistant variant of RNase 1 (HPDDADD- RNase) [85]. This novel IR (ERB–HPRNase) has the enzymatic activity of RNase 1 and the specific binding of the parental scFv to ErbB2-positive cells.

Other results indicated that the conjugation of RNase (including wild, engineered and HP-RNase) with the antibody as the IR can improve cytotoxicity to cancer cells [82, 84, 86]. In 2014, our group conjugated engineered HP-RNase 1 with Trastuzumab as an IR in order to induce death in Her2 positive cell lines [47]. The results of a cytotoxicity dose response assay revealed that this immunotoxin could induce death on different types of Her2 positive cell lines at an IC50 of 250 nM. It was concluded that the engineered enzyme had evaded the cytoplasmic RI of Her2 positive cell lines [47]. Our results indicated that the scFv of Trastuzumab with HP-RNase 1 was more potent than the Trastuzumab alone due to the smart release and delivery to the three HER2 over-expressing breast cancer cell lines and Herceptin-resistant cell line. These results indicated that the engineered scFv-Fc-HPR antibodies could be a promising human anti-cancer agent for breast cancer therapy [47].

Genetically engineered molecules have the following advantages: ability to induce both ADCC (antibody dependent cellular immunity) and CDC (complement-dependent cytotoxicity), RNase-based cytotoxicity, prolonged half-life due to its higher molecular size, smart delivery, increased avidity due to the presence of two scFv moieties, and resistance to the action of RI. Forouharmehr et al. [61] conjugated engineered-RNaseA with Cetuximab for inducing death to Her1-positive cell lines. This immunotoxin induced cell death with an IC50 around 50nM. We also designed a new immunotoxin based on Ranpirnase [72]. We engineered *Rana pipiens* RNase (Ranpirnase) with 5 mutations (M23L, L45R, L49R, L55R and E91A) to bind to the heavy chain of human anti-epidermal growth factor receptor antibody. The molecular dynamic simulations confirmed protein stability and the ability of Ranpirnase to bind to the epidermal growth factor receptor protein. The immunotoxin function was assessed in A431 cancer cells and HEK293 normal cells, and the IC50 was estimated at 39.50 and >2559 nM, respectively. The results indicated that the immunotoxins produced in this study against EGFR receptor could be used as anticancer drugs [72].

**5. CLINICAL IMPACT OF RIBONUCLEASES IN CANCER THERAPY**

Recombinant variants of RNases are effective in mouse xenograft models of multiple cancer types without exhibiting any significant toxicity [87, 88]. Therefore, a novel approach to antitumor treatment is to use RNases to target RNA for cancer therapy. The main focus is to find an enzyme selectively damaging cancer cells without affecting the surrounding normal cells. We have observed the anticancer potential of RNases derived from bacteria (binase), fungi (streptomycete ribonuclease), mammals (bovine pancreatic and seminal ribonuclease) and, most importantly, from reptiles (ONC, Amph).

The first enzyme of this group that was tested in vitro and in vivo for possible anti-cancer activity was RNase A [13, 14]. Results were inconsistent. Some scientists did not observe any positive effects [16], while others reported anti-cancer activity only when large amounts of enzyme were employed e.g., milligrams injected into solid tumors [28]. Ardelt et al. [8] conducted a clinical study with RNase A as a potential anti-cancer drug. No therapeutic effects were observed in most of the 23 patients. In some cases, a regression or disappearance of cancer tissue occurred when the enzyme was applied directly to the tumors in large amounts (up to 3 mg/kg of BW/day). Therefore, after such studies, interest in the therapeutic potential of RNases waned for some time. Later, when engineered RNases displayed cytotoxic activity against several types of malignant cells, interest was revived. These proteins were considered as novel chemotherapeutic agents that could be cytotoxic to cancer cells at low concentrations.

Bovine seminal ribonuclease (BS-RNase) was the first homolog in the RNase superfamily that reduced transplanted Crocker tumors in mice [29]. This enzyme, as a dimeric variant of RNase A that evades hRI, was toxic to cancer cells in vitro. The toxicity of BS-RNase was not limited to tumor cells: it was also toxic to embryonic cells, oocytes, and testicular tissue. The cytotoxic effects of BS-RNase were associated with the induction of apoptosis. Sinatra et al. [89] presented ultrastructural and flow cytometry evidence of apoptotic death following BS-RNase treatment in normal cells and phytohemagglutinin-stimulated lymphocytes. The results obtained from transmission and scanning electron microscopy, and supported by flow cytometry data, showed typical features of apoptosis, such as decreased cell size, fragmentation in micronuclei, chromatin condensation, and the presence of apoptotic bodies.

Among recently described cytotoxic RNases, amphibian RNases, including Ranpirnase (Onconase; ONC) and Amphinase (Amph), have been widely investigated in clinical trials, due to the discovery of their remarkable and complex biological activities [90]. The cytotoxic effect of in vitro ONC alone, and in combination with other antitumor agents, was investigated in several clinical studies [29, 71, 75]. These results proved that these two enzymes are more toxic to cancer cells than to non-cancer cells in cell culture systems, animal models, and human clinical trials. Hodge et al. [91] performed a study in order to test the effectiveness of ONC against Ebola virus infection in vitro. The results indicated that most of the mice were protected from the consequences of Ebola infection by being treated with ONC, with weight loss the only observed adverse effect. ONC was efficient against the Rabies virus in vitro; however, this action was not confirmed in vivo [91]. As a result of ONC effectiveness against human papillomavirus (HPV) infection in vitro, phase I of a study was performed with 42 patients [73]. A total of 30 subjects were evaluated after 8 weeks of topical use of 1 mg ONC. Twenty five patients were clinically healed, and the rest showed a 50% reduction of lesions. The median time to curing was significantly shorter in comparison to spontaneous healing (30 days vs. 6 to 7 months).

Clinical applications of ONC indicated that this enzyme acts strongly when combined with numerous well-known chemotherapeutic drugs [68, 73]. These drugs are characterized by entirely different mechanisms of action; however, some of them are toxic at low doses. The results of clinical trials for unresectable malignant mesothelioma revealed that ONC has antitumor activity, particularly in cases that failed a prior chemotherapy regimen [70, 92]. Such patients, when treated with ONC plus doxorubicin, had a significantly longer median survival time than patients treated with doxorubicin alone. Lee et al. reported a sensitizing effect of ONC to radiotherapy on human lung cancer cells (A549) in vitro. They also confirmed this in mouse models, with the best results when ONC was given 2h prior to radiation. The increase in oxygen concentration in tumor cells and the decrease in tumor intestinal fluid pressure explain this sensitizing action of ONC [93].

A strong synergism was reported by Mikulski et al. [94] when ONC was combined with tamoxifen, trifluoroperazine (Stelazine), or lovastatin to treat pulmonary carcinoma A549 or pancreatic adenocarcinoma ASPC-1 cells. Other studies also revealed a synergism of ONC with agents such as vincristine [95], interferons [96], differentiation-inducing agents [71], tumor necrosis factor α [97], cepharanthine [98], ionizing radiation [99], and, in vivo, with tamoxifen [93].

In 2006, Alfacell released interim data from the company’s ongoing Phase IIIb randomized clinical trial of ranpirnase and doxorubicin for the treatment of malignant mesothelioma [100]. The first interim analysis stated that 105 events (patient deaths) of the total 316 patients enrolled. The results also indicated that the overall median survival time favored the ranpirnase plus doxorubicin treatment group (12 months) over the doxorubicin group (10 months). Ranpirnase is cytotoxic to cancer cells because of its enzymatic activity against RNA [67]. It is internalized by endocytosis and released into the cytosol of the cancerous cell, where it selectively degrades tRNA. In addition, Ranpirnase induces apoptosis by inhibiting protein synthesis and terminating cell cycle proliferation. Therefore, using Ranpirnase as an adjunct drug to increase the effectiveness of treating various tumors with radiotherapy or chemotherapy appears to be the most promising direction in its clinical applications [72].

Despite the effectiveness of ONC in clinical trials, it has a serious shortcoming, When RNases are injected into mice, 50% of ONC is found in kidneys after 3 h, compared to only 1% of RNase 1 [101]. Therefore, a chemotherapeutic agent based on a human protein is likely to be preferable to one that is based on an amphibian protein. Indeed, the creation and testing of potent cytotoxic variants of human RNase 1 is an important development [30]. Therefore, researchers are still doing clinical trials on human pancreatic-type ribonuclease endowed with toxicity for cancer cells (QBI-139; Rains et al., data unpublished) to evaluate the toxicity, tolerability, and maximum tolerated dose of QBI-139 in patients with advanced and refractory solid tumors.

**CONCLUSION**

Although modern treatments for cancer such as radiation, chemotherapy, and surgery are improving, cancer is still one of the main causes of death in most developed countries. The use of monoclonal antibodies (mAb) is a new method of cancer treatment. Today, fifty mAbs have been recognized for therapeutic applications, and several other types are under investigation. One approved example is rituximab (Rituxan), and another is trastuzumab (Herceptin). In order to improve their clinical potential, antibodies also have been joined to cytotoxic agents or radionuclides. Some antibodies have also been coupled with engineered proteins to enhance cytotoxicity. Currently the preferred mode of therapy for cancer treatment is target therapy. Immunotoxins are a kind of target therapy that has made significant strides to treat cancer. They can be made more potent when a cytotoxic agent, such as RNase, is fused to an antibody. Minimal side effects, long half-life, and high specificity are three characteristics contributing to therapeutic success of immunotoxins.

The RNases, as antitumor drugs or therapeutic agents, have the ability to eliminate tumor cells. They are not toxic to cells normally and just become cytotoxic after internalization into the cytosol. PtRNases seemingly self-target and preferentially enter cancer cells. An immunotherapeutic based on a human source has several advantages such as decreased renal toxicity and less immunogenicity. Some RNases such as ONC, bovine seminal ribonuclease and RNase 1 hold great promise as cancer immunotherapeutic agents by causing a significant reduction in the protein synthesis of tumor cells after internalization into cytosol. Uncovering new biological roles for RNases is a crucial step toward exploiting their unique biology therapeutically. However, currently several key mechanisms associated with these enzymes are unclear. In order to create new anticancer drugs, it is imperative to understand the underlying mechanisms of action of RNases to exploit their functionalities.

By genetic engineering and chemical modification of a ligand/receptor the interaction between RNases and RI can be disrupted. This results in more of the cytotoxic RNase molecules to work in the tumor cells. A review of the literature and our experimental results demonstrated that engineered RNases from RNase 1, RNase A, and Rana pipiens could be conjugated with promising anti-cancer therapeutics agents (immuno-RNase) for inducing death to cancer cells and enhance cytotoxicity. As ptRNases are excellent candidates for protein-drug conjugation, it is important to learn the functions of these proteins in vivo in order to improve existing therapeutics or derive novel drugs.

Pancreatic-type ribonucleases, RNase 1 in particular, have been well characterized structurally. However, many mysteries remain regarding the biological functions of this enzyme family. Hence, the development of future generations of mammalian RNase-based anticancer agents is underway. The immunoRNases have been under investigation in several malignant diseases, both in vitro and in animal models. There are preclinical evidences that immunoRNases might be a new class of adjunct drugs that will significantly increase the therapeutic activity of chemotherapeutic treatments of various cancer cells [86]. This review also indicated that immuno-RNases and monoclonal antibody studies will be a prerequisite for development of effective drugs against tumor cells and workable anticancer therapeutics.

**AUTHOR CONTRIBUIONS**

Mohammadreza Nassiri and Vinod Gopalan designed and supervised the work. Masoume Vakili-Azghandi and Mohammadreza Nassiri wrote the manuscript. All authors read and approved the final manuscript.

**LIST OF ABBREVIATIONS**

Amph = Amphinase

BS-RNase = Bovine seminal [ribonuclease](https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/ribonuclease)

DLBCL = Diffuse large B-cell lymphoma

ErbhRNase = Erbicin-human-RNase

HPR = Human pancreatic ribonuclease

HPV = Human papillomavirus

IR = Immuno-RNase

ITs = Immunotoxins

mAb = Monoclonal antibody

ONC = Onconase

Pca1 = Glutamine residue

ptRNases = Mammalian pancreatic-type ribonucleases

RI = Ribonuclease inhibitor

RNase = Ribonuclease

RNase A = Bovine pancreatic ribonuclease A

scFv = Human single chain variable fragment

TIFP = Tumor intestinal fluid pressure

**CONSENT FOR PUBLICATION**

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest, financial or otherwise

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