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Review Article

Cytotoxic ribonuclease-based cancer therapies

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CONTENTS

Abstract

Cytotoxic ribonucleases (RNases) represent promising therapeutic agents for cancer. Here, we present a general description of the most representative examples of this drug class, bovine seminal RNase (BS-RNase) and the amphibian-derived RNases, along with their advantages and limitations in terms of specificity, toxicity and delivery efficiency. The arsenal of potential modifications and variants that can improve their function as anticancer drugs, together with their specificity for cancer cells, is also described. As our knowledge of the structure and function of this RNA-based strategy advances, a new potential class of chemotherapeutic agents emerges.

Introduction

Over the past 20 years, research has uncovered many of the molecular alterations occurring during the development and progression of cancer. The ability to target these molecules is a powerful approach to cancer treatment. Here, we provide a review of the currently available RNA inactivation technologies using ribonucleases (RNases) and their therapeutic potential in cancer.

RNases are nucleases that catalyze the destruction of RNA. They can be divided into endoribonucleases, which cleave phosphodiester bonds within a polynucleotide chain, and exoribonucleases, distinguished by cleaving nucleotides one at a time from the end of a polynucleotide chain (1) (Table I). RNases comprise several subclasses within the EC 3.1 enzyme class. Widely found in both plant and animal species, although RNases are best known for their ability to cleave RNA, evidence also suggests other important functions for these enzymes (2). For example, it has been suggested that several RNases may function in RNA metabolism and gene expression (3), although the role of many is still unknown. Also, RNases appear to have physiological roles in host defense against cancer, viruses and other parasites (4).

Exogenous cytotoxic endoribonucleases, such as amphibian RNases and bovine seminal RNase (BS-RNase), can be employed to target RNA for cancer therapy (Fig. 1). Also, endogenous RNases can be activated indirectly to target specific RNA employing technologies such as: 1) antisense oligonucleotides (Fig. 2A) (reviewed in Ref. 5); 2) antisense molecules with an additional (guide) sequence that targets the messenger RNA (mRNA) for degradation by endogenous RNases, *i.e.,* RNAse P-associated external guide sequence (RNase P-EGS) (reviewed in Ref. 5); and 3) RNA-induced silencing complexes (RISC) or RNA interference (RNAi) (Fig. 2B) (reviewed in Ref. 6).

RNases may hold the key to promising therapeutic agents for human diseases (5, 7-10). Here, we present a general description of the exogenous cytotoxic endoribonucleases, along with their advantages and limitations in term of specificity, toxicity and delivery efficiency. These RNA-based strategies provide exciting tools for multiple clinical applications, including therapeutic intervention in cancer.

Table I: Major ribonucleases (RNases).

Endoribonucleases	Exoribonucleases
RNase A	Polynucleotide phosphorylase
RNase P	RNase PH
RNase H	RNase II
RNase III	RNase R
RNase T1	RNase D
RNase T2	RNase T
RNase U2	Oligoribonuclease
RNase V1	Exoribonuclease I
RNase I	Exoribonuclease II
RNase L	
RNase PhyM	
RNase V	
Dicer	
Argonaute 2	

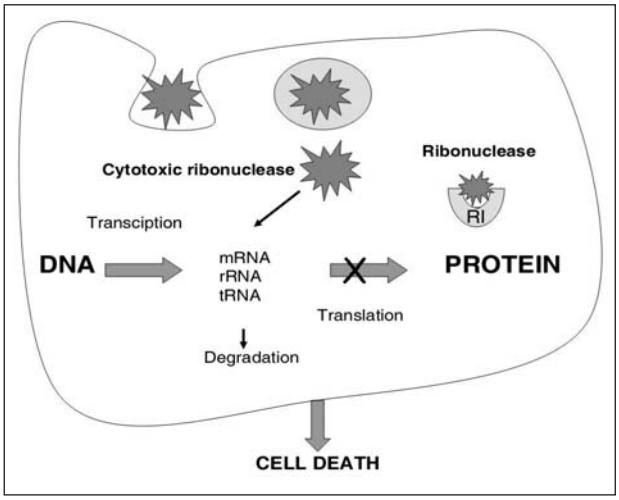

Fig. 1. Schematic diagram illustrating the biochemical basis for the cytotoxicity of exogenous ribonucleases (RNases). The RNase first interacts with the plasma membrane surface. The RNase is internalized, apparently by endocytosis. The mechanism of bilayer transversal is unknown. In the cytosol, RNases face potential degradation by RNase inhibitors (RIs). Those RNases that avoid RIs catalyze the degradation of RNA, which may lead to cell death.

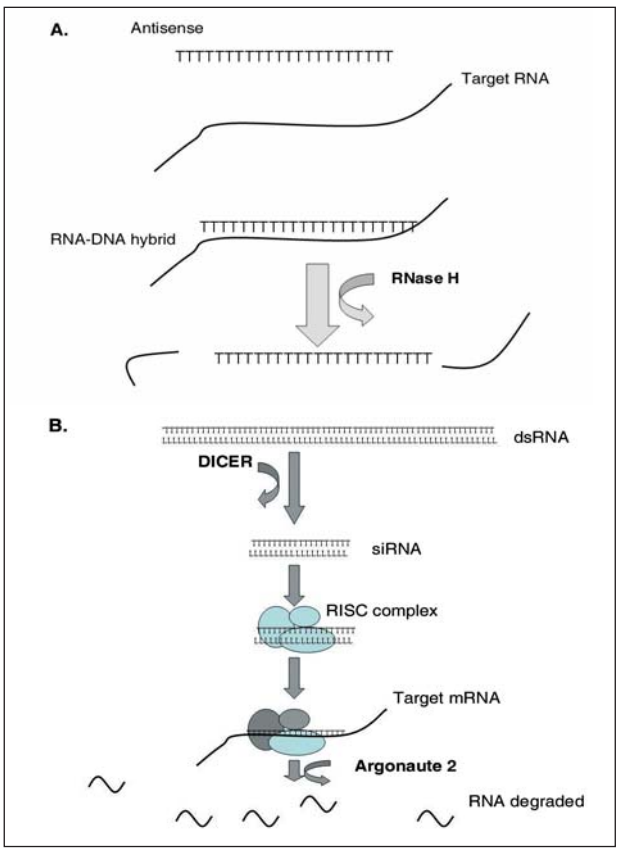

Fig. 2. Schematic diagram illustrating the indirect activation of endogenous ribonucleases (RNases) by oligonucleotides. **A.** Degradation of RNA by antisense techniques with the involvement of RNase H. **B**. Degradation of RNA by RNAi pathways involving the endoribonucleases Dicer and Argonaute 2.

Cytotoxic RNases

History

Five decades ago, bovine pancreatic RNase A was the first RNase to be studied. Ledoux *et al*. first reported the antitumor activity of RNase A (11-13). Soon thereafter, Aleksandrowicz administered RNase A to patients with myelocytic leukemias (0.5-1 mg/day) and reported general improvement in these patients (14). Subsequent studies did not corroborate these findings, however. The disappointing results with RNAse A opened up an opportunity to test another RNase (α-sarcin), isolated from *Aspergillus giganteus* (15). However, further clinical research showed very low cytotoxicity for this RNase.

More recently, two homologues of RNase A, BS-RNase and a group of RNases isolated from amphibian eggs and embryos, were found to have greater cytotoxic activity than RNase A itself. In 1963, D'Alessio and Leone discovered RNase activity in bovine seminal plasma (10), and later, three independent laboratories successfully isolated BS-RNase from bovine seminal vesicles (16-19). The first observation of antitumor activity for BS-RNase was published in 1973 (20). The discovery of BS-RNase was followed by the isolation of other homologues of RNase from eggs and embryos of the frogs *Rana catesbeiana, Rana japonica* and *Rana pipiens* (21-24). The RNase A homologue present in the oocytes and early embryos of *R. pipiens*, the Northern leopard frog, was named Onconase®. Onconase® is a trademark of Alfacell, which has received 9 U.S. and 4 European patents for this amphibian RNase (25). This particular RNase is undergoing clinical testing in the U.S. and Europe.

BS-RNase

BS-RNase (EC 3.1.27.5) is a 27-kDa protein consisting of two identical monomers with a subunit amino acid sequence 83% identical to bovine pancreatic RNase A and covalently linked through 10 disulfide bonds (26-29). The most structurally critical disulfide bonds bridge Cys31 and Cys32 of one subunit with the corresponding Cys32' and Cys31' of the partner subunit (5, 26). BS-RNase is the only dimeric protein among the pancreatic-like RNases (Fig. 3). Native homodimeric BS-RNase exists in two quaternary forms. The major isoform is characterized by the swap between subunits of its *N*-terminal ends (MxM isoform), whereas the minor isoform (M=M) shows no swap (30, 31). The functional characterization of these isoforms revealed that the lack of swapping negatively effects the cytotoxic activity of BS-RNase (30). After selective reduction and alkylation of the two intrachain disulfide bridges, the dimeric protein can be transformed into a monomeric derivative with RNase activity higher than the parent dimeric protein, but devoid of the special biological functions. The structure of the recombinant monomeric form of BS-RNase, as determined by three-dimensional (3D) heteronuclear NMR, shows close similarity to RNase A in all regions characterized by regular elements of secondary structure. However, significant dif-

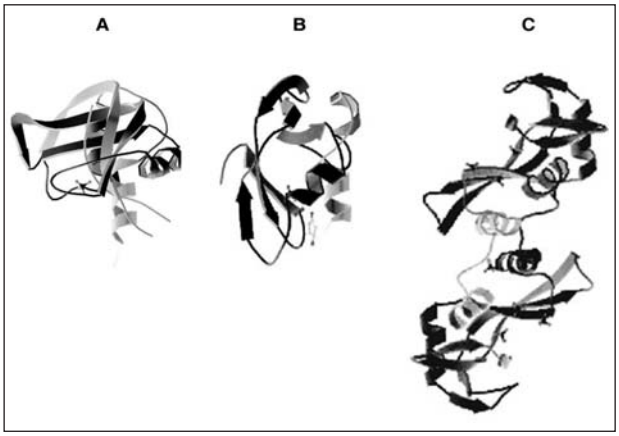

Fig. 3. Three-dimensional structure of: **A**, ribonuclease A (RNase A; RCSB PDB protein data base entry 1SRN [107]); **B**, Onconase® (PDB entry 1ONC [53]; and **C**, bovine seminal RNase (BS-RNase; PDB entry 1BSR [108]) showing the dimeric nature of BS-RNase.

ferences exist in the flexible regions, which could account for the different behavior of the two proteins (32).

1. Mechanisms of BS-RNase cytotoxicity

BS-RNase is a cytotoxic protein that targets ribosomal RNA (rRNA). This cytotoxicity can lead to immunosuppression, antitumor activity, embryotoxicity and aspermatogenic activity (17, 33, 34).

The cytotoxicity of BS-RNase has been related to its interaction with RNase inhibitor proteins, its RNase activity and its structure. RNases are recognized by specific RNase inhibitors (RIs). The binding of RIs to RNases results in the complete loss of ribonucleolytic activity. However, there are a few exceptions, such as amphibian Onconase® and BS-RNase, which are endowed with cytotoxic activity (35). Studies have found that RIs neutralize monomeric BS-RNase, but not dimeric BS-RNase (36). This may partially explain the importance of BS-RNase's structure for its cytotoxicity. Also, RNase variants created by mutagenesis to partially evade the RI acquire cytotoxic activity. These findings suggest that RIs constrain the cytotoxicity of RNases (37). However, other investigations cast doubt on the conclusion that the cytotoxic action of RNase depends mainly on the ability to evade RIs (38, 39). For example, Bosch *et al.* reported a human pancreatic RNase variant featuring an Arg triplet introduced onto one of its surface-exposed loops (named PE5), which still retained sensitivity to RIs but exhibited cytotoxicity. This variant reached the nucleus, leading these researchers to conclude that targeting an RNase to the nucleus resulted in cytotoxicity.

RNase cytotoxicity has also been related to structure. RNase A and monomeric BS-RNase are not cytotoxic. In contrast, artificially dimerized RNase A is cytotoxic, but less so than BS-RNase (40-43). Moreover, recent evidence indicates that the MxM quaternary isoform of BS-RNase (explained above) is responsible for its cytotoxicity (34, 44, 45). The cytosol of cells is a reducing environment, which may destroy the two disulfide bonds that cross-link the monomers of dimeric BS-RNase. In this event, the M=M form will be converted to monomers susceptible to inhibition by RIs. In contrast, noncovalent interactions in the MxM form enable it to remain dimeric, retaining its lethal enzymatic activity without its intersubunit disulfide bonds. It has been proposed that BS-RNase evolved its MxM form to evade RIs, thereby retaining its enzymatic activity (34, 45, 46).

2. Antitumor activity of BS-RNase

Cytotoxic BS-RNase possesses potential for use in cancer therapy, as shown in both *in vitro* and *in vivo* studies (reviewed in Ref. 10). BS-RNase is selectively cytotoxic to malignant cells. To date, the underlying functional mechanism of the antitumor action of BS-RNase remains obscure. Apparently, BS-RNase enters the cytosol, binds to rRNA and inhibits protein synthesis, resulting in cell death (47). Using immunofluorescence, Bracale *et al.* found that BS-RNase bound both normal and malignant cells, and was internalized by both cell types in endosome vesicles. Noncytotoxic RNases, such as RNase A and a monomeric derivative of BS-RNase, did not bind to the cell surface and were not internalized. They also found that the pathway of BS-RNase in malignant cells from the extracellular matrix to the cytosol had two essential intracellular stations: endosomes and the trans-Golgi network. In normal cells, however, the protein did not progress from the endosomal compartment to the Golgi complex (48).

3. Modifications and synthetic alternatives of BS-RNase

BS-RNase exerts potent antitumor activity when administered intratumorally to nude mice bearing human tumors. The lack of efficacy following i.v. or i.p. administration, however, led Soucek *et al*. to synthesize polymeric conjugates of BS-RNase to prevent degradation in the bloodstream. Hydrophilic poly[*N*-(2-hydroxypropyl)methacrylamide] was used for BS-RNase modification. In sharp contrast to the total lack of efficacy of free BS-RNase administered at a daily i.v. dose of 10 mg/kg, application of the conjugate caused significant inhibition of the growth of human melanoma in nude mice. Treated groups survived an average of 75.5 days compared to an average of 32.7 days in the control group. BS-RNase conjugated to water-soluble polymers was the first BS-RNase preparation to produce anticancer activity following i.v. administration (49).

Synthetic BS-RNase and its corresponding recombinant molecules have also been prepared. Kim and Raines (1993) designed a gene encoding BS-RNase based on criteria expected to maximize the translational efficiency of its mRNA in *Escherichia coli*. This gene constructed from 12 synthetic oligonucleotides was expressed with the phage T7 system. Under optimal conditions, the insoluble protein produced accumulated 15% of total cellular protein, or 200 mg/l of culture. RNase activity was generated by air oxidation of the reduced and denatured protein. Three forms of active BS-RNase

were isolated by gel filtration chromatography: the well-characterized dimer, the monomer and a previously uncharacterized form that migrated as a trimer. The ribonuclease activities of all three forms were equivalent to or greater than dimeric BS-RNase isolated from bull seminal plasma (50).

4. Potential chemotherapeutic application of BS-RNase

The antitumor effect of BS-RNase has been examined in several models. For example, cytotoxic activity was observed in both human neuroblastoma cell lines possessing the multidrug resistance (MDR) phenotype and in cells without MDR. BS-RNase was equally toxic to all neuroblastoma cells at concentrations of 0.2-100 μg/ml, and it was nontoxic to normal fibroblasts and epithelial cells. The antitumor effects of BS-RNase were also demonstrated *in vivo* using established subcutaneous xenografts in athymic (nude) mice. Intratumoral injections (12.5 mg/kg) of BS-RNase over 4 weeks resulted in complete tumor regression, without tumor regrowth over a 2-week observation period after treatment ended (51).

Another study demonstrated the cytotoxicity of BS-RNase against anaplastic thyroid carcinoma cells. All tumor cell lines exhibited marked sensitivity to BS-RNase as compared to nonmalignant cells. The cytotoxic action of BS-RNase was associated with induction of apoptosis by downregulation of Bcl-2 in treated cells. *In vivo* treatment for 20 days induced significant tumor regression (28). Another study demonstrated that BS-RNase, but not monomeric RNase A, induced apoptosis of human thyroid carcinoma cell lines. RNase-induced apoptosis was associated with activation of caspase-8 and -9. This was followed by activation of caspase-3, leading to the proteolytic cleavage of poly(ADP-ribose) polymerase (PARP). RNase-triggered apoptosis and caspase activation were accompanied by reduced survival signals to cancer cells. BS-RNase antitumor effects in nude mice were accompanied by caspase activation and apoptosis (52).

BS-RNase therefore appears to be a good antitumor candidate for the treatment of neuroblastoma and thyroid carcinoma, but remains in preclinical studies. Side effects that may limit its use as an anticancer agent include degeneration of testicular tissue in males and immunosuppressive activity in human blastic transformed T- and B-lymphocytes (10).

Amphibian RNase A homologues

Onconase® is the smallest member of the RNase A superfamily, with 104 residues as compared to the 124 in the primary structure of RNase A (23), and a molecular mass of 11.8 kDa (23). It shares 30% identity with the RNase A sequence. Despite the low degree of identity between their primary structures, the 3D structure is similar to that of RNase A (53). Major differences are present in the loop regions and at the *C*-terminus, where Onconase® has an additional disulfide bond (Cys87-Cys104) found only in amphibian RNases (53, 54). This extra disulfide bond is believed to contribute to its high thermal stability and low catalytic activity (2, 5). Without this extra disulfide bond, the thermal stability of Onconase® is reduced and its cytotoxicity is affected (2, 55). Also, the amino terminus of Onconase® is blocked by a pyroglutamyl residue (Pca1), formed by the cyclization of an amino-terminal glutamine residue (2, 53). This particular residue appears to be related to its enzyme activity (56, 57), cytotoxicity (57) and resistance to cellular RIs (57).

RNases with biochemical properties similar to Onconase® have also been isolated from oocytes of *R. catesbeiana* (bullfrog) and *R. japonica* (Japanese rice paddy frog) (58). These two RNases share approximately 50% amino acid sequence homology with Onconase® (2, 59, 60). *R. catesbeiana* RNase shares several features with Onconase®, including the four main disulfide bonds and high thermal stability (2). *R. japonica* RNase, the structure of which is unknown, shares the cysteine residues and stability of Onconase® (2).

1. Mechanisms of amphibian RNase cytotoxicity

The cytotoxicity of Onconase® is dependent on its ribonucleolytic activity (23), as is the cytotoxicity of *R. catesbeiana* (2). Upon entry into mammalian cells, Onconase® targets mainly transfer RNA (tRNA) (61, 62). In cell-free systems, Onconase® can target rRNA and mRNA (58). It has been hypothesized that protein subunits associated with rRNA and mRNA protect these nucleic acids from Onconase®-induced degradation (5). Like RNase A, Onconase® and the RNases from *R. catesbeiana* and *R. japonica* catalyze the cleavage of the P-O 5' bond of RNA on the 3'-side of pyrimidine nucleosides. The main catalytic residues of RNase A are conserved in Onconase®, including two histidine residues (His10, His97) and a lysine residue (Lys31) (2, 62, 63). Other residues found in the active site of Onconase® are Lys9 and Pca1 (64). The most important structural differences between Onconase® and RNase A occur in the length and composition of the loop regions, at the chain termini (the *N*-terminal pyroglutamate residue of Onconase®), and a *C*-terminal disulfide bond, both of which contribute to the stability of the Onconase® molecule (53, 55, 65, 66). Additionally, the hydrophobic cluster formed by Val17, Ile22, Met23, Leu27 and Phe36 in Onconase® (corresponding to Tyr25, Met29, Met30, Leu35 and Phe46 in RNase A) is extended by replacing Thr36 in RNase A with Phe28 in Onconase® (67).

Compared to RNase A and BS-RNase, Onconase® is less ribonucleolytically active but more cytotoxic (64). Its greater cytotoxicity appears to be related to its high thermal stability (Tm = 90 °C, nearly 30 °C higher than that of RNase A) (62). The *C*-terminal disulfide bond is one factor contributing to the thermal stability of Onconase®, as the deletion of this bond has been shown to dramatically decrease the thermodynamic stability (55, 65, 67). Also, the increased cytotoxicity of Onconase® compared to RNase A is attributed to its high resistance to degradation by RIs. Except for resistance to RIs, RNase A has all the necessary properties to be a potent cytotoxin (2).

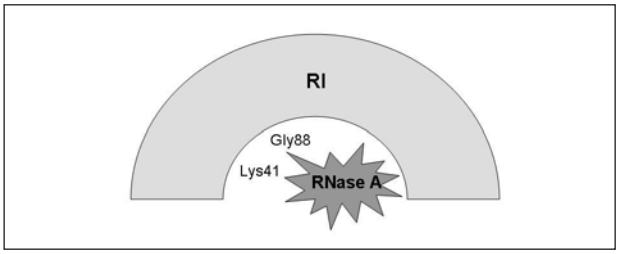

Fig. 4. Schematic representation of the interacting residues (Gly88 and Lys41) critical in the association between ribonuclease A (RNase A) and an RNase inhibitor (RI). Modified from Ref. 2.

Several studies have addressed this hypothesis. For example, Leland *et al.* substituted critical RI-RNase A complex interaction amino acid residues, and determined the relationship between RI degradation and cytotoxicity. In the RI-RNase A complex (Fig. 4), residue Gly88 of RNase A is important in the interaction of these two proteins. By replacing Gly88 with Arg (G88R), Leland *et al.* showed a reduced RNase A susceptibility to inactivation by RIs. Most significantly, they also found increased RNase A cytotoxicity (62). A double variant of G88R with another important residue Lys41 to Arg (K41R) increases RNase A resistance to RIs even more, but diminishes catalytic activity (68). Interestingly, as the conformational stability of an RNase A variant increased, the enzyme became less susceptible to proteolytic degradation (68). The cytotoxicity of RNase A homologues therefore appears partially dependent on RI resistance.

RNase-mediated cell death consists of two major steps: cytosolic internalization and RNA cleavage (69). The cytotoxic pathway of Onconase® is initiated by its binding to the plasma membrane of the target cell (37, 61, 70, 71). Onconase® binds to specific sites on the plasma membrane of cultured glioma cells (70), although specific receptors have not been identified. Whether these sites are common to other cancer cells is unknown. *R. catesbeiana* and *R. japonica* RNases also bind cancer cells. In this case, the binding site has been identified as sialic acid-binding lectins that specifically agglutinate cancer cells (72-74). Onconase® does not appear to share the tumor cell-agglutinating capability of the other amphibian ribonucleases (23).

RNases must reach the cytosol to degrade cellular RNA. RNases microinjected into the cytosol are more toxic than those added to cells externally (75), indicating that internalization is a rate-limiting step in their cytotoxicity. After binding to the plasma membrane, Onconase® is internalized, probably through endocytosis (2, 62, 63, 69, 76). Consistent with this theory, studies using small molecules inhibiting ATP synthesis, and therefore energy-dependent processes, also abolished the cytotoxicity of Onconase® (70). After internalization by non-receptor-mediated endocytosis, it is routed through the Golgi apparatus to the cytosol (77). Similar to BS-RNase, Onconase® appears to pass more efficiently into the cytosol through the Golgi apparatus disrupted by monensin or retinoic acid (71).

Regardless of its internalization mechanism, the transbilayer movement of Onconase® into the cytosol is unknown (2). This is a major obstacle in the internalization of cytotoxic RNases, including Onconase®, given their highly cationic nature and the need to maintain structural integrity during transport to the cytosol (2).

Once Onconase® has reached the cytosol, it degrades mainly transfer RNA (tRNA) (61), contributing to cell death via inhibition of protein synthesis (see Fig. 1) (77). Evidence suggests that tRNA irreparably damaged by Onconase® may constitute a direct signal for apoptosis through the caspase-9/caspase-3 cascade (78). Significantly, Onconase®-related apoptosis appears not to depend on functional p53 protein (77-79). This is an important feature, since many tumors carry inactivated p53, known to suppress or delay apoptosis (80).

2. Antitumor activity of amphibian ribonucleases

The anticancer effect of Onconase® has been documented *in vitro* (24, 81-86) and *in vivo* (82, 83, 85, 87). The amphibian RNases are more toxic to cancer cells than to normal cells, although the mechanisms responsible for this difference are unclear (2). Some critical distinctions which may influence this outcome could include the following:

- The fact that protein synthesis is increased in malignant cells compared to normal cells. By targeting tRNA, Onconase® targets the critical proteins necessary for cancer cell proliferation and survival.
- Endocytosis, or cellular routing, rates of Onconase® may be different for cancer and normal cells.
- Changes at the plasma membrane level may allow Onconase® to more readily enter cancer cells.

Onconase® is more efficient at killing proliferating cells than quiescent cells (88), arresting the cells in the G1 phase of the cell cycle (24, 84). Therefore, agents that increase proliferation activate Onconase® and *vice versa* (2).

The antitumor activity of Onconase® is enhanced by several therapeutic agents, including tamoxifen, cisplatin, lovostatin, trifluoroperazine and vincristine (83, 89-92). Reducing tumor interstitial pressure, one of the major causes of inadequate drug delivery into solid tumors, is one potential mechanism by which Onconase® synergizes with other therapeutic drugs (82). Onconase® can produce cytotoxic effects directly or in combination with other therapeutic agents, and may enhance radiation sensitivity. *In vitro* studies using androgen-insensitive prostate cancer cells showed that Onconase® could decrease cellular oxygen consumption. It was thought that the diffusion of oxygen into previously hypoxic tumor cells probably increased radiation sensitivity (93).

The *R. catesbeiana* and *R. japonica* RNases are cytotoxic to several cancer cells *in vitro*, with LD_{50} values close to those of Onconase® (0.1 μM). *R. catesbeiana* RNase has also been shown to effectively increase survival time in animals injected with tumor cells (94).

3. Production, modifications and synthetic alternatives of amphibian RNases

Recombinant production of Onconase® has been limited, since the critical pyroglutamic acid at the *N*-terminus can not be expressed by bacteria, creating the need for intricate strategies to overcome this limitation (95). It has also been difficult to create and retain the structural integrity in Onconase® fusion proteins (http://www.pharmcast.com/Patents100/).

Some successful Onconase® combinations with other drugs and modifications have been produced. For example, Onconase® potency has been enhanced by adding drugs that alter cellular routing (71). Likewise, conjugation of Onconase® to delivery molecules has enhanced specificity and potency. For example, a human monoclonal antibody against CD22 (B-cell-specific cell-surface receptor), known as LL2, has been covalently linked to Onconase® in an effort to specifically target B-cell lymphoma. Targeting CD22 on human B-cells with a monoclonal antibody conjugated to a cytotoxic RNase causes potent and specific killing of the lymphoma cells *in vitro*, as well as antitumor effects in human lymphoma models in SCID mice (85, 96).

The most serious side effect from Onconase® treatment is a reversible renal toxicity, which appears to be caused by the unusual stability of the enzyme (97). To improve the performance of the protein as an antitumor agent, recombinant technology has been used to create Onconase® variants. These mutants exhibited reduced thermal stability and/or increased catalytic activity (54, 65, 95). In particular, the mutant in which methionine-23 has been replaced by leucine (M23L-ONC) is 5-fold more active than the native enzyme, while being fully toxic toward tumor cells (54). On the other hand, mutants in which the disulfide bond Cys87-Cys104 has been eliminated, *e.g.*, (C87S,des104)-ONC and (C87S,des103–104)-ONC, exhibit greatly reduced thermal stability (65), whereas catalytic action and antitumor activity are practically unaffected (98).

Glycosylation of Onconase® has also proven to improve its conformational stability and toxicity in cancer cells (99). Despite the fact that Onconase® contains an *N*-linked glycosylation site (-N69-V70-T71-), only the non-glycosylated form of the protein has been identified to date. Kim *et al.* (99) employed the *Pichia pastoris* expression system to produce recombinant glycosylated Onconase® (gONC) protein. Approximately 10 mg of gONC protein was secreted per liter of culture media, of which about 80% was glycosylated at N69. Importantly, glycosylation of Onconase® at N69 greatly increased its toxicity against K-562 cancer cells. Specifically, the IC_{50} value of gONC was 50-fold lower than that of Onconase®. Glycosylation increased both the Tm of Onconase® and its resistance to proteinase K, suggesting that the elevated cytotoxicity of gONC is related to higher conformational stability.

R. catesbeiana RNase has also been modified to increase its cytotoxicity. For example, the antitumor activity of sialic acid-binding lectin from *R. catesbeiana* was increased through chemical modification with a water-soluble carbodiimide in the presence of nucleophiles such as ethylenediamine and glycine methylester (100). The effect of replacing the aspartic acid/glutamic acid residues of this RNase sialic acid-binding lectin with asparagine/glutamine and arginine was to enhance its antitumor activity in murine leukemia P388 cells (101).

4. Potential chemotherapeutic application of amphibian RNases

Onconase®, also known as P-30 protein or ranpirnase, is a promising antitumor therapeutic that has reached phase III clinical trials (67).

Two phase I studies were conducted in patients with a variety of solid tumors. Based on phase I study data, the maximum tolerated dose (MTD) was established at 960 $\mu g/m^2$, with the dose-limiting toxicity (DLT) consisting of proteinuria with or without azotemia, peripheral edema and fatigue (77, 102). The first of the studies included 32 patients treated with daily bolus i.v. Onconase® for 30 days (6 and 105 $\mu g/m^2$). Three (9.3%) of the 32 patients demonstrated stabilization of previously progressive disease (77). The second phase I study included 71 patients treated with a weekly Onconase® schedule administered at eight different dose levels (60, 120, 240, 360, 480, 720, 960 and 1200 $\mu g/m^2$). DLT of proteinuria with or without azotemia, peripheral edema and fatigue was encountered at 1200 $\mu g/m^2$. Objective responses were seen in patients with non-small cell lung cancer (NSCLC; 1 partial response), colorectal carcinoma (1 minor response) and esophageal carcinoma (1 partial response). One patient each with malignant thymoma, colorectal carcinoma and NSCLC exhibited disease stabilization (77, 102).

Phase II studies involved 207 patients with malignant mesothelioma (103), pancreatic cancer (77), breast cancer (104) and renal cell cancer (105). The largest phase II trial was conducted in malignant mesothelioma (105 patients). Onconase® was administered i.v. weekly at a dose of 480 $\mu g/m^2$. The overall median survival was 6 months (95% confidence interval [CI] = 4.7-10 months). Evidence of clinical activity (*i.e.,* complete response, partial response, minor response or stable disease for a minimum of 3 months) was seen in 41 (51%) patients. Partial responses were obtained in 4 (5%) patients, minor responses in 2 (3%) patients and stable disease in 35 (43%) patients. Whether disease stabilization reflects benefit from Onconase® or is an intrinsic difference in tumor biology can not be determined by this type of phase II trial (77). The overall median survival time was 6 months (95% CI = 4.7-10.0 months), with 1- and 2-year survival rates of 34.3% and 21.6%, respectively. Sixteen of 105 (15.2%) patients discontinued the study prematurely because of adverse events, including renal insufficiency in 4 patients, allergic reaction in 4 patients, arthralgia in 2 patients and proteinuria in 2 patients. Aanaphylactoid reaction, hypotension, peripheral edema and asthenia were reported in 1 patient each (103).

A phase I/II study of combination therapy with Onconase® and tamoxifen was also conducted in 71 patients with advanced pancreatic adenocarcinoma.

Onconase® was given as a weekly i.v. infusion at six different dose levels, ranging from 60 to 720 μg/m^2, with oral tamoxifen 20 mg/day. The MTD was 720 μg/m^2. For all patients, the median survival was 3.1 months (range: 2.8-3.8 months) and the overall response rate, including disease stabilization of previously progressive disease, was 15% (11/71) (77). This trial was discontinued in 1998 because tolerated levels of Onconase® did not offer a significant therapeutic advantage over gemcitabine (Gemzar®) (2).

The combination of Onconase® (480 μg/m^2 weekly) and tamoxifen (20 mg/day) was also evaluated in patients with early prostate cancer recurrence, as evidenced by rising prostate-specific antigen (PSA) levels. Thirteen patients with rising PSA after initial radiation therapy or prostatectomy were enrolled in the trial and 11 patients were evaluated for safety and efficacy. Side effects included fatigue (1/11) and arthralgia (1/11); however, 6 patients discontinued therapy due to toxicity or patient choice prior to completing 3 cycles of therapy (1 patient each due to deep venous thrombosis, fatigue/depression, proteinuria, arthralgia and nausea). One of the 5 patients who completed 3 months of therapy demonstrated stable PSA (77).

The activity of Onconase® was further tested in 17 patients with breast cancer. The trial used a dose of 240 μg/m^2. Of the 17 patients entered, 1 patient achieved a partial response, another had a minor response and 2 patients achieved stable disease for at least 3 months. Mean survival time was 350 days (range: 48-1,258 days) and median survival was 230 days. Adverse effects observed included decreased creatinine clearance (2/17) and arthralgia, dyspnea, peripheral edema, asthenia and nausea (each 1/17) (104).

Another phase II trial was conducted in 30 patients with unresectable or previously resected NSCLC (106). A weekly dose of 240 μg/m^2 was also used in this trial. Treatment with Onconase® produced disease stabilization in 6 (20%) patients, with an overall median survival of 7.7 months. The 6 patients with stable disease had a median survival of 10 months, compared to 7.0 months for patients with progressive disease. Side effects included decreased creatinine clearance (13%), peripheral edema (10%) and proteinuria (7%).

A phase II trial was also conducted in 14 patients with refractory advanced renal cell cancer. The patients were treated weekly at a dose of 480 μg/m^2 (105). No responses were seen in any of the 14 patients. The median survival was 16 months (range: 2-28 months). At this dose and schedule, Onconase® demonstrated minimal activity in metastatic renal cell cancer.

Phase III clinical trials have been completed or are in progress in patients with unresectable malignant mesothelioma. An exploratory phase III trial of single-agent Onconase® in patients with unresectable malignant mesothelioma was completed in April 1999. This study enrolled a total of 154 patients who were randomized (3:2) to receive either 480 μg/m^2 Onconase® (n=84) administered weekly or 60 mg/m^2 doxorubicin (n=70) every 3 weeks for 6 cycles. The primary endpoint was survival. The intent-to-treat (ITT) analysis included all patients with a confirmed diagnosis of malignant mesothelioma randomized during the study for which follow-up information (and alternative treatment information for refusal patients) was available —a total of 144 patients (Onconase®, n=75; doxorubicin, n=69). The median survival time (MST), 1- and 2-year survival rates for the ITT populations were similar for both treatments; MSTs were 8.4 and 8.2 months, 1-year survival rates were 33.3% and 34.8%, and 2-year survival rates were 13.7% and 10.9%, respectively, for Onconase® and doxorubicin. Alfacell is presently conducting a confirmatory phase IIIb registration trial of Onconase® plus doxorubicin *versus* doxorubicin alone in more than 360 patients with unresectable malignant mesothelioma, with survival as the primary endpoint. The trial is being conducted in the U.S., Canada, Poland, Italy, Germany, Australia, New Zealand, Russia, Romania, Mexico and Brazil (25).

Concluding remarks and future directions

As our knowledge of the structure and function of cytotoxic RNases advances, a new potential class of chemotherapeutic agents has begun to emerge. The RNases addressed in this review are particularly interesting, since they are highly selective for malignant cells. Onconase®, a prototype example of this drug class, is the first cytotoxic RNase to enter cancer clinical trials. Although an amphibian protein, Onconase® is immunologically tolerated by humans (98). This is evident from phase I clinical trials indicating that it is well tolerated, with an MTD of 960 μg/m^2. A few side effects have been reported, including renal toxicity, but this effect is reversible (97) and probably related to the RNase's high stability (54, 77). Although advanced clinical trials have shown only moderate efficacy for this drug in cancer patients, there is enormous potential for improved results. Some of the modifications and variants of these drugs mentioned in this review have not yet been tested in clinical trials. Furthermore, with more research, new rational drug combinations will surely emerge that can improve these results.

In summary, cytotoxic RNases have great potential as therapeutic drugs for cancer treatment. Very few examples of drugs with such specificity for cancer cells are found in the current cancer drug arsenal. Future research to explore this potential is highly warranted.

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