Biodrugs 2008; 22 (1): 53-58
1173-8804/08/0001-0053/$48.00/0

Ribonucleases as Novel Chemotherapeutics

The Ranpirnase Example

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Contents

Abstract

Ranpirnase, a cytotoxic ribonuclease from the frog *Rana pipiens*, is the archetype of a novel class of cancer chemotherapeutic agents based on homologs and variants of bovine pancreatic ribonuclease (RNase A). Ranpirnase in combination with doxorubicin is in clinical trials for the treatment of unresectable malignant mesothelioma and other cancers. The putative mechanism for ranpirnase-mediated cytotoxicity involves binding to anionic components of the extracellular membrane, cytosolic internalization, and degradation of transfer RNA leading to apoptosis. The maintenance of ribonucleolytic activity in the presence of the cytosolic ribonuclease inhibitor protein is a key aspect of the cytotoxic activity of ranpirnase. The basis for its specific toxicity for cancer cells is not known. This review describes the development of ranpirnase as a cancer chemotherapeutic agent.

RNA is the intermediate in the flow of biochemical information from genes to proteins (figure 1). Accordingly, intervention in the metabolism of RNA presents an opportunity for the development of chemotherapeutic agents.[1] Since the 1980s, antisense oligonucleotides and ribozymes have been pursued as the basis for treatments of viral infections, inflammatory disorders, hematological diseases, and cancer.[2-4] In 1998, the phosphorothioate antisense oligonucleotide fomivirsen was approved by the US FDA for the treatment of cytomegalovirus retinitis in immunocompromised patients.[5] More recently, manipulation of the RNA interference machinery has garnered much interest as a basis for drug development,[6-8] though the safety of this approach is a concern.[9,10]

Ribonucleases also have potential therapeutic utility.[11-15] These proteins are efficient catalysts of RNA cleavage, acting in effect as RNA depolymerases.[16] Much interest has focused on homologs and variants of bovine pancreatic ribonuclease (RNase A), which is renowned as a model system in protein biochemistry.[17] RNase A itself is not cytotoxic. In contrast, bovine seminal ribonuclease, which is a homodimer, is endowed with antitumoral, immunosuppressive, and antiviral activities.[18] Ranpirnase (which is also known as P-30 protein) is an amphibian homolog that has marked toxicity for tumor cells,[19] and is the

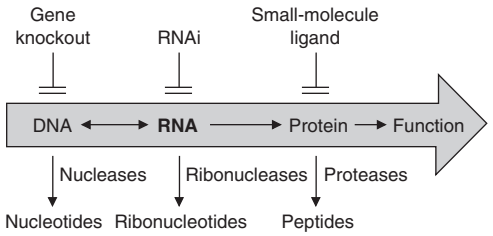

Fig. 1. Flow of chemical information in biology. Ribonucleases can be cytotoxic because their degradation of RNA renders genetic information indecipherable. **RNAi** = RNA interference.

only ribonuclease to have been studied in a human clinical trial.[20,21] Here we review the structure and function of ranpirnase, which has become the archetype of a new class of cancer chemotherapeutic agent.[22]

1. History of Ranpirnase

In the early 1970s, Shogen and Yoan[23] discovered that extracts from embryos of the Northern Leopard frog (*Rana pipiens*) have antitumoral activity. Nearly two decades later, Ardelt et al.[24] attributed that activity to a basic protein, ranpirnase (meaning, *Ran*a *pi*piens *r*ibo*n*ucl*ease*), which belongs to the RNase A superfamily.[25] In oocytes, ranpirnase localizes with yolk proteins.[26] It has been postulated that ranpirnase is synthesized in the liver of female frogs in a seasonal manner, and then secreted into the blood and deposited in oocytes as they mature.[27] There and in embryos, ranpirnase has been speculated to play a role in host defense.[26]

Ranpirnase is both cytotoxic and cytostatic toward cultured tumor cells and inhibits the growth of xenograft tumors in mice.[28,29] Currently, ranpirnase in combination with doxorubicin is in a confirmatory phase IIIb clinical trial for the treatment of unresectable malignant mesothelioma, a cancer associated with exposure to asbestos.[20,21] Moreover, ranpirnase has been granted both orphan-drug and fast-track status by the FDA.

2. Biochemical Attributes of Ranpirnase

The amino acid sequence of ranpirnase was determined in 1991,[24] and its 3-dimensional structure was reported 3 years later.[30] Ranpirnase is a relatively small enzyme, with a molecular formula of $C_{520}H_{810}N_{142}O_{155}S_9$ and a molecular mass of 11 820 Da. The active site of ranpirnase contains the catalytic triad (His10, Lys31, and His97) that is characteristic of the RNase A superfamily.[31] Ranpirnase possesses two additional active-site residues: Lys9 and an N-terminal pyroglutamate residue, which is formed by the co-translational cyclization of the encoded glutamine residue in the endoplasmic reticulum.[32] Like other members of the RNase A superfamily, ranpirnase catalyzes the cleavage of the P–$O^{5'}$ bond on the 3′ side of a pyrimidine nucleobase in an RNA strand.

The ribonucleolytic activity of ranpirnase is necessary for its cytotoxicity. A decrease in ribonucleolytic activity leads to a corresponding reduction in cytotoxicity.[24] Although ranpirnase assumes the kidney-shaped tertiary structure that is typical of the RNase A superfamily (figure 2)[30,33] and has the key catalytic residues, its value of k_{cat}/K_M (which is the second-order rate constant and thus a measure of catalytic efficiency) is 10^4-fold less than that of RNase A for cleavage of their best known substrates under similar conditions.[34] A low affinity for its substrate contributes to its low k_{cat}/K_M value. In addition, nuclear magnetic resonance spectroscopy and molecular dynamics simulations have revealed that ranpirnase has an extremely rigid β-sheet,[35,36] which could deter an 'induced fit'[37] necessary for substrate binding and turnover.

The substrate specificity of ranpirnase can be considered on two levels. On the nucleobase level, ranpirnase prefers to cleave the phosphodiester bond on the 5′ side of a guanine. This preference is in marked contrast to that of RNase A, which has little preference for guanine versus adenine at this position. In the cell, transfer RNA (tRNA) has been reported to be the main target for ranpirnase.[39] The cleavage of tRNA occurs at the guanosine-guanosine bond in the variable loop or D-arm.[40] The revelation of the atomic structure of a ranpirnase-nucleic acid complex has provided insight into the structural basis for this substrate specificity.[38]

A notable feature of ranpirnase is its extraordinary conformational stability. Ranpirnase has a T_m value of 87°C (which is the temperature at the midpoint of the thermal transition between folded and unfolded states and thus a measure of conformational

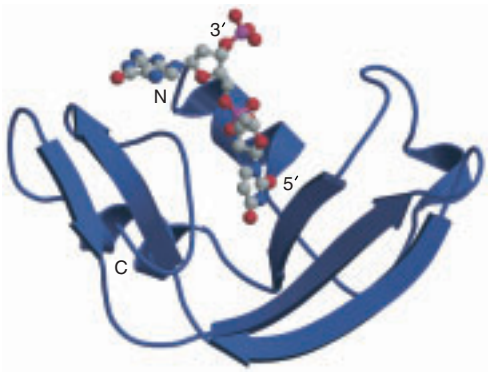

Fig. 2. Ribbon diagram of the 3-dimensional structure of a crystalline ranpirnase-nucleic acid (dAdUdGdA) complex (PDB entry 2I5S).[38] The N- and C-termini of the protein, and 3′- and 5′-termini of the nucleic acid, are noted explicitly. The image was created with the programs MOLSCRIPT (Avatar Software AB, Stockholm) and RASTER3D (D.J. Bacon and W.F. Anderson, http://skuld.bmsc.washington.edu/raster3d/raster3d.html).[33]

stability) and resists degradation by various proteases.[41] The exceptional conformational stability of ranpirnase is largely because of its tethered C-terminus, created by a C-terminal half-cystine residue.[42] This synapomorphic C-terminal disulfide bond is conserved in amphibian ribonucleases but is absent from mammalian homologs.[31] The hydrogen-bond network at the N-terminus[41] and the absence of a *cis*-proline residue[43] also contribute to the conformational stability of the enzyme. This exceptional stability is critical for cytotoxicity. Variants of ranpirnase with reduced conformational stability have less cytotoxic activity.[41,42] On the other hand, glycosylation of ranpirnase at its consensus N-linked glycosylation site (Asn69-Val70-Thr71) increases both conformational stability and cytotoxic activity.[44]

3. Mechanism of Ranpirnase-Mediated Cytotoxicity

Ranpirnase is an atypical biodrug in that it is administered extracellularly but acts intracellularly. To exert its antitumoral effect, ranpirnase must reach the cytosol and there cleave RNA substrates. The generally accepted mechanism of ranpirnase-mediated cytotoxicity is divided into two major stages (as depicted in figure 3): (i) cytosolic internalization; and (ii) catalytic degradation of RNA.[45]

3.1 Cytosolic Internalization

The first step for the cytosolic internalization of ranpirnase is its binding to the cell surface. The existence of low- and high-affinity ranpirnase receptors on the cell surface has been reported,[46] but other findings contradict their existence.[47] The cell surface is highly anionic due to the abundance of sulfate, phosphate, and carboxylate groups of its carbohydrates and lipids. It is probable that ranpirnase, which is a highly cationic protein with a calculated isoelectric point of >9.5,[24] binds to the cell surface through favorable Coulombic interactions.

After binding to the cell surface, ranpirnase is internalized through energy-dependent endocytosis. The role of the GTPase dynamin in this process is under investigation.[47,48] Internalized ranpirnase is routed to endosomes. Drugs that disrupt retrograde transport from the *trans*-Golgi network to the endoplasmic reticulum potentiate the cytotoxicity of ranpirnase.[47-49] These and other results suggest that the *trans*-Golgi network is an inefficient site for the translocation of ranpirnase, and that endosomes are a key compartment for cytotoxic delivery.

The means by which ranpirnase, which is extremely hydrophilic, ultimately crosses a lipid bilayer is not understood. To facilitate successful entry into the cell, the diphtheria toxin and ricin proteins utilize a distinct translocation domain, which dissociates from a catalytic domain upon cytosolic entry. In contrast, ranpirnase is a hyperstable, single-domain protein, which remains intact during its endocytosis. The mechanism of ranpirnase translocation could be related to that used by cationic peptides, such as residues 47–57 of the HIV-1 TAT protein and nonaarginine.[50]

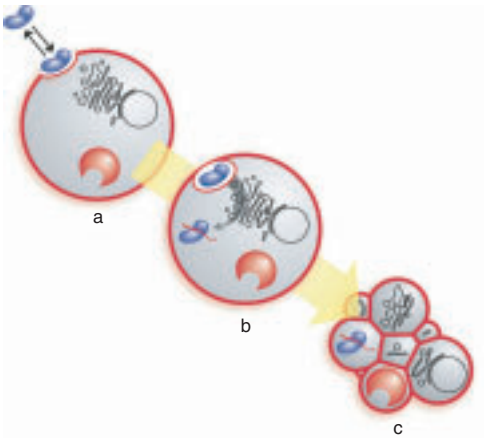

Fig. 3. Putative mechanism of ranpirnase-mediated cytotoxicity.[45] Cationic and anionic biomolecules are depicted in blue and red, respectively. (**a**) Ranpirnase (blue) forms an extracellular equilibrium complex with cell-surface heparan sulfate (red); (**b**) ranpirnase is internalized by endocytosis, translocates to the cytosol, evades the ribonuclease inhibitor protein (red horseshoe), and degrades transfer RNA (tRNA; red line); and (**c**) tRNA degradation leads to apoptosis.

3.2 Degradation of Cellular RNA and Induction of Apoptosis

Once in the cytosol, ranpirnase degrades cellular RNA. Ranpirnase is an unusual homolog of RNase A in that it seems to evade completely the cytosolic ribonuclease inhibitor protein (RI).[51,52] RI is a 50-kD protein present in every surveyed mammalian cell. RI is composed of 15 leucine-rich repeats, a motif that often participates in protein-protein interactions.[53] RI binds to certain members of the RNase A superfamily with femtomolar affinity, and renders them inactive. The complex formed by human RI and human pancreatic ribonuclease is among the tightest known in biology ($K_d = 2.9 \times 10^{-16}$ mol/L).[54] The ability of ranpirnase to evade RI is likely to be necessary for its cytotoxic activity, as non-cytotoxic mammalian ribonucleases become cytotoxic by incorporating residues that enable RI evasion.[54,55] Moreover, the cytotoxicity of variants correlates with their RI-evading ability.[56]

In the cell, the ribonucleolytic activity of ranpirnase is directed predominantly towards tRNA, leaving ribosomal RNA (rRNA) and messenger RNA (mRNA) largely intact.[39] The basis for this specificity is not understood, though bound proteins could protect rRNA and mRNA from ranpirnase cleavage. The susceptibility of

non-coding RNA, such as microRNA or small-interfering RNA (siRNA), to ranpirnase cleavage is unknown.

Degradation of tRNA by ranpirnase inhibits protein synthesis in the cell and leads to apoptosis.[57,58] The cytotoxic effect of ranpirnase becomes noticeable after a longer incubation (≈48 h, *in vitro*) than required for drugs that block translation, such as cycloheximide (≈2 h). In addition, ranpirnase-induced apoptosis does not require the high level of translation inhibition observed with cycloheximide, suggesting that the inhibition of protein synthesis is not the sole cause of ranpirnase-induced apoptosis.[59] In HeLa cells, ranpirnase-induced cytotoxicity is initiated with the activation of the stress-activated c-Jun N-terminal kinase (JNK), followed by the activation of caspase-9, which activates the executioner caspase-3 and -7. Caspase-8 or the tumor-suppressor protein p53 are not required in this pathway.[60] Other studies with the HL-60 leukemic cell line implicate the activation of serine proteases along with these caspases.[61] The induced apoptosis is enhanced by mild hyperthermia.[62]

3.3 Basis for Therapeutic Index

Ranpirnase is more toxic to tumor cells than to normal cells *in vitro* and *in vivo*. The mechanism for this selectivity is unknown, but a promising hypothesis is that ranpirnase is selectively internalized by tumor cells. In general, tumor cells are more negatively charged than are homologous normal cells.[63,64] Moreover, the level of sialic acid-rich gangliosides is greater and the phospholipid content is altered in certain tumor cells.[65,66] The elevated anionic character of tumor cells could promote their interaction with the highly cationic ranpirnase. Other viable hypotheses include a different and more efficient intracellular routing of ranpirnase to the cytosol in tumor cells, and a greater susceptibility of rapidly growing tumor cells to RNA degradation.

4. Therapeutic Applications

In vitro, ranpirnase has been shown to be cytotoxic/cytostatic to a range of cell lines, including 9L rat glioma,[46] K-562 human leukemia,[34,42] Colo 320 CM human colon adenocarcinoma,[28] HL-60 human leukemia,[67] LNCaP and JCA-1 human prostate cancer,[67] HT-29 human colorectal cancer,[68] and U937 human lymphoma cell lines.[69] Typical 50%-inhibitory concentrations (IC_{50}) for the proliferation of 9L rat glioma[28,46] and K-562 human leukemia cells[34] are near 10^{-7} mol/L. Concomitant administration of ranpirnase with tamoxifen,[70,71] cisplatin,[71] or vincristine[68] results in increased toxicity. In combination with vincristine, ranpirnase has shown toxicity against multidrug-resistant tumors.[68] *In vivo*, ranpirnase treatment has prolonged the survival of mice transplanted with human[68,72] and murine tumors.[73,74]

Ranpirnase has been administered as a single agent in two phase I clinical studies to determine the optimal dose and schedule in patients with various solid tumors.[20,75] These phase I studies indicated that ranpirnase is well tolerated. The maximum tolerated dose was 960 μg/m^2, and the recommended dose for phase II studies was 480 μg/m^2/week. Ranpirnase has been evaluated in phase II clinical trials as a single agent in patients with non-small-cell lung cancer,[76] breast cancer,[77] renal cell cancer,[78] and malignant mesothelioma.[79] The largest phase II trial for ranpirnase was in patients with malignant mesothelioma. Among 81 patients who were evaluated for tumor response, 41 patients showed a decrease in tumor progression, which justified subsequent phase III studies on this tumor type. In the initial phase III studies, 154 patients were treated with either ranpirnase (84 patients) or doxorubicin (70 patients). In these studies, ranpirnase treatment provided markedly increased survival compared with doxorubicin treatment.[20,21] Reversible renal toxicity was the major adverse effect. The current confirmatory phase IIIb study is an open-label, multicenter, and international study, with the goal of comparing the efficacy of ranpirnase plus doxorubicin versus doxorubicin alone.[20,21]

5. Engineering Ranpirnase and Future Directions

There have been attempts to endow ranpirnase with increased toxicity toward tumor cells. Nearly all non-Hodgkin lymphoma cells display a specific cell-surface receptor, CD-22. A human monoclonal antibody against CD-22 has been covalently linked to ranpirnase.[80] This fusion protein was 10^4-fold more toxic to non-Hodgkin lymphoma cells than was wild-type ranpirnase because of increased binding to the tumor cells. In addition, this protein showed enhanced potency and specificity along with decreased systemic toxicity in mice.

The ribonucleolytic activity of ranpirnase is 10^4-fold lower than that of other mammalian homologs.[34] Accordingly, it could be both possible and advantageous to engender ranpirnase with greater ribonucleolytic activity without compromising other attributes required for its cytotoxicity, such as cationicity, RI evasion, and conformational stability. Either enhancing substrate binding or alleviating β-sheet rigidity could yield variants with increased ribonucleolytic activity and, hence, greater chemotherapeutic efficacy.

6. Conclusions

Ranpirnase is a cytotoxic ribonuclease that affords a novel strategy for cancer chemotherapy. Ranpirnase is internalized by tumor cells and degrades tRNA, which leads to the inhibition of protein synthesis and apoptosis. The cationicity and maintenance

of ribonucleolytic activity in the presence of RI are critical for its cytotoxicity. The efficacy of ranpirnase can be augmented by other cytotoxic agents such as doxorubicin, and a confirmatory phase IIIb clinical trial for the treatment of malignant mesothelioma is ongoing. Emerging knowledge on the mechanism of action of ranpirnase could aid in the development of other ribonucleases, including those from mammals, as cancer chemotherapeutic agents.

Acknowledgments

Work in the Raines laboratory on ribonucleases is supported by grant CA073808 (NIH). We are grateful to R.F. Turcotte and T.-Y. Chao for comments on the manuscript. The authors have no conflicts of interest that are directly relevant to the content of this review.

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