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Enzymes as Chemotherapeutic Agents

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Introduction

Typical chemotherapeutic agents act by disrupting the flow of biochemical information (Fig. 1). The most common strategy uses small organic molecules to inhibit the function of a protein. Information flow can be disrupted at earlier stages. For example, an antisense oligonucleotide (Vitravene; Isis Pharmaceuticals) acts at the RNA level to treat eye infections caused by cytomegalovirus.

Can enzymes act as chemotherapeutic agents? The answer is clearly "yes," as several enzymes are now in clinical use. Most such comprise regimens to restore catalytic activities in patients with lysosomal storage diseases. Others are hydrolytic enzymes that degrade unwanted extracellular material. For example, deoxyribonuclease I (Pulmozyme from Genentech) is used as an aerosol to cleave DNA in the lungs of cystic fibrosis patients. Tissue plasminogen activator (Activase from Genentech) promotes the degradation of blood clots. Collagenase (Santyl from Healthpoint Biotherapeutics) debrides necrotic tissue.

Certain enzymes have the potential to disrupt the flow of biochemical information by degrading a biopolymer encoding that information (Fig. 1). For example, ribonucleases can do so because cleaving RNA renders indecipherable its encoded information. Yet, no extant enzymic drugs act by blocking the flow of biochemical information. Here, I report on the ability of enzymes to be chemotherapeutic agents, focusing on mammalian ribonucleases for the treatment of cancer.

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Ribonuclease A and Its Homologues

Bovine pancreatic ribonuclease (RNase A; EC 3.1.27.5) is perhaps the best characterized of all enzymes [1]. RNase A was studied extensively during the 1960s and 1970s as a model system, in part because of its prevalence in an accessible source—the cow pancreas—and because of its facile purification, its high conformational stability, and its small size ($C_{575}H_{901}N_{171}O_{193}S_{12}$; 13,682 Da). RNase A was the first enzyme to have its sequence determined and the third for which a structure was revealed. RNase A was also the object of landmark work on the folding and stability of proteins, on enzymology, and on molecular evolution. Recognition of this historic role culminated in 1972 when the Nobel Prize in chemistry was awarded jointly to Stanford Moore, William Stein, and Christian Anfinsen for their collective work on RNase A [2, 3]. In 1984, Bruce Merrifield was awarded the Nobel Prize in chemistry for developing chemical synthesis on a solid matrix; he likewise used RNase A as a model [4].

RNase A catalyzes the depolymerization of RNA by cleaving its P–$O^{5'}$ bonds [1, 5]. A mechanism of catalysis that is consistent with all known data from work on the enzyme is depicted in Fig. 2 [6]. In this mechanism, His12 acts as a base that abstracts a proton from the 2′-oxygen of a substrate molecule and thereby facilitates its attack on phosphorus [7, 8]. The side chain of His119 acts as an acid to protonate the 5″-oxygen to facilitate its displacement. Lys41 stabilizes the negative charge that accumulates on a nonbridging phosphoryl oxygen in the transition state [9, 10]. The attack on phosphorus proceeds in line to displace a nucleoside [11]. Both products are released to solvent. At low salt concentration, the k_{cat}/K_M value for this reaction is the largest known: 3×10^9/M s [12, 13]. The hydrolysis of the 2′,3′-cyclic phosphodiester product occurs in a slow, separate process that resembles the reverse of transphosphorylation [14, 15].

RNase A is but the best known member of a superfamily of secretory enzymes that operate at the crossroads of transcription and translation by catalyzing RNA degradation (Fig. 1) [16]. The physiological function traditionally ascribed to RNase A, RNase 1 (which is its human homologue), and other homologues is to degrade

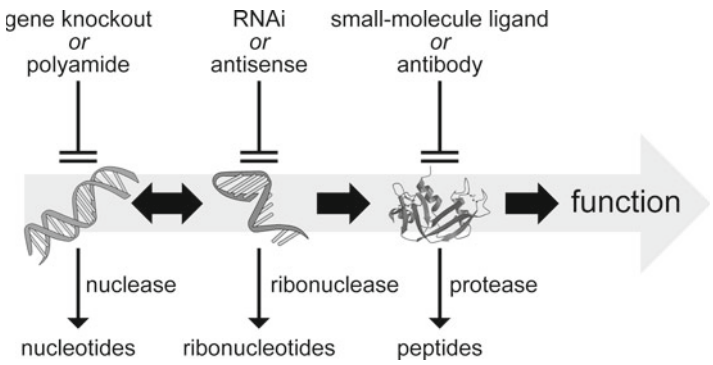

Fig. 1 Flow of biochemical information and strategies to block that flow

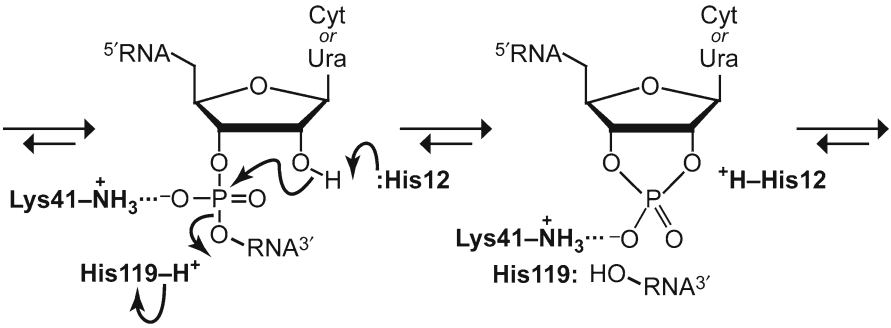

Fig. 2 Putative mechanism for the cleavage of RNA as catalyzed by RNase A [6]

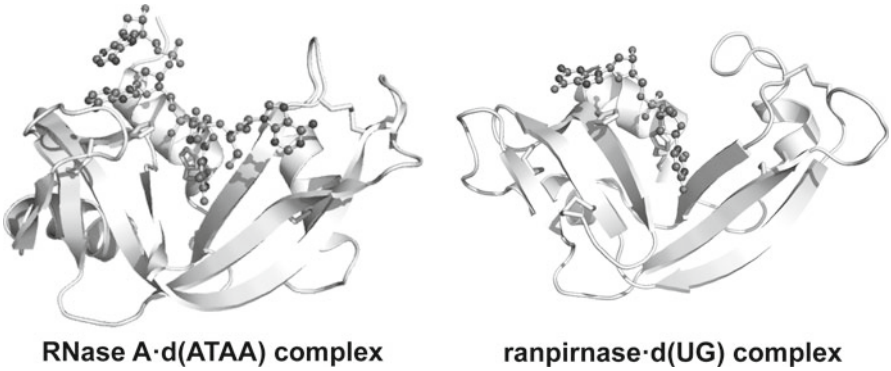

Fig. 3 Three-dimensional structures of RNase A [55] and ranpirnase [56] with bound oligonucleotides. The two active-site histidine residues and the four cystine residues of each enzyme are shown explicitly

dietary RNA [17, 18]. In the 1950s, however, RNase A was shown to be toxic to tumor cells, both in vitro [19] and in vivo [20–22]. These early studies employed extremely large doses of RNase A; effects were observed only after milligrams of enzyme were injected into solid tumors. Thirty years ago, a dimeric homologue of RNase A that is cytotoxic at low levels was discovered in bull seminal fluid [23]. In the past two decades, even more cytotoxic homologues were isolated from the eggs of the bullfrog *Rana catesbeiana*, the Japanese rice paddy frog *Rana japonica*, and the Northern leopard frog *Rana pipiens* [24]. These amphibian ribonucleases are toxic to tumor cells in vitro with IC_{50} values <1 μM.

The *R. pipiens* ribonuclease, ranpirnase (Onconase; Tamir Biotechnology), deserves special attention [25]. Ranpirnase is undergoing a Phase II human clinical trial for the treatment of non-small cell lung cancer and has been granted both orphan-drug and fast-track status in the United States (USA). The enzyme is delivered to patients intravenously, kills cancer cells selectively, and avoids resistance [26]. RNase A and ranpirnase have a similar three-dimensional structure (Fig. 3),

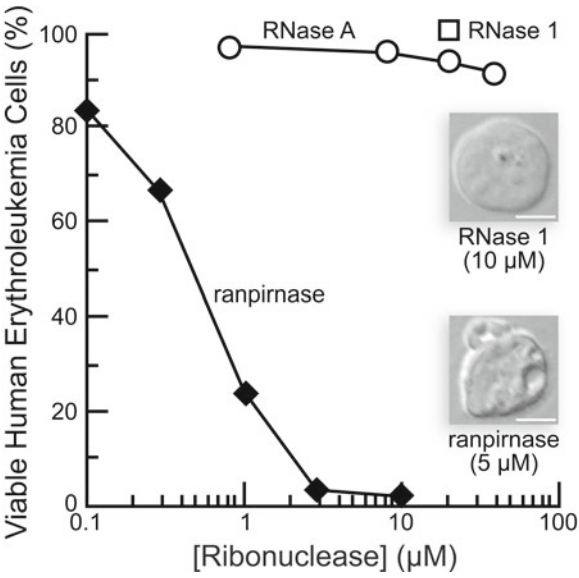

Fig. 4 Effect of wild-type RNase A, RNase 1, and ranpirnase on the viability of human erythroleukemia cells in vitro [36, 57]. *Bars* 10 μm

and both catalyze RNA cleavage after pyrimidine residues. Yet, ranpirnase is toxic to cancer cells at submicromolar (μM) concentrations, whereas high micromolar doses of RNase A exhibit no detectable antitumoral activity (Fig. 4). This discrepancy is made even more puzzling because RNase A cleaves RNA 10^4-fold faster than does ranpirnase [27], and ribonucleolytic activity is essential for the antitumoral activity of ribonucleases [28].

A chemotherapeutic agent based on a mammalian ribonuclease is likely to have many advantages over one derived from an amphibian [29]. Mammalian ribonucleases are markedly less immunogenic than ranpirnase [30]. In humans, the dosing of ranpirnase is limited by renal toxicity. In contrast to ranpirnase, mammalian ribonucleases do not accumulate in the kidney [31]. Finally, mammalian ribonucleases have the intrinsic ability to be much more efficacious chemotherapeutic agents by virtue of their 10^4-fold-greater catalytic activity [27]. The similarity of the structures of RNase A and ranpirnase (Fig. 3) and the difference in their antitumoral activities (Fig. 4) pose an intriguing structure–function problem that is at the core of the work in our laboratory, along with discerning the biochemical basis for the ability of ribonucleases to discriminate between cells from cancerous and noncancerous origins.

Ribonuclease Inhibitor

Ribonuclease inhibitor (RI) is a 50-kDa protein present in the cytosol of mammalian cells [32]. RI contains 15 leucine-rich, β–α repeat units arranged symmetrically in the shape of a horseshoe (Fig. 5). The β-strands form a solvent-exposed β-sheet that

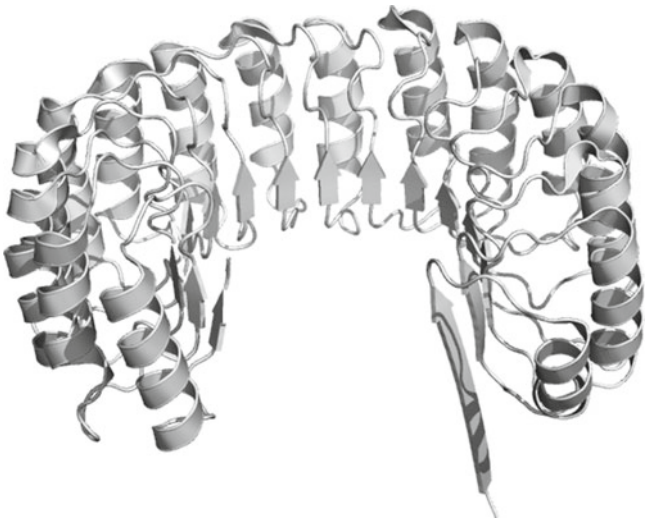

Fig. 5 Three-dimensional structure of human ribonuclease inhibitor (RI) [37]

defines the inner circumference of RI. The α-helices define the outer surface of the inhibitor. RI forms an extremely tight 1:1, noncovalent complex with target ribonucleases. The value of K_d for the human complex is 0.29 fM, making the RI–RNase1 interaction one of the tightest known protein–protein interactions.

Development of a Ribonuclease-Based Drug

RI resides in the cytosol. Yet, all known RI ligands are secreted ribonucleases. Although ranpirnase retains the elements of tertiary structure that characterize pancreatic-type ribonucleases (Fig. 3), ranpirnase does not bind to mammalian RIs [33]. Accordingly, we reasoned that RI evolved to preserve the integrity of cellular RNA should RNase A or a homologue inadvertently reach the cytosol [34, 35].

Starting in 1998 [36], we performed a series of experiments to test the hypothesis that endowing a ribonuclease with RI-evasion makes the ribonuclease into a cytotoxin. Guided by structures of RI–ribonuclease complexes [37, 38], we created variants of RNase A and RNase 1 that evade RI. Most importantly, we found that the ability of a ribonuclease to evade correlates with its cytotoxic activity [37, 39–41]. For reasons that are not yet clear, this toxicity is highly selective for cancer cells, both in vitro and in mice [39, 42, 43]. These findings have led to a Phase I clinical trial of an RI-evasive variant of RNase 1 (QBI-139; Quintessence Biosciences) as a cancer chemotherapeutic agent.

Mechanism of Ribonuclease-Mediated Cytotoxicity

The antitumoral activity of ribonucleases relies on their ribonucleolytic activity [28]. To gain access to cellular RNA, ribonucleases effectively ricochet, following an endocytic pathway that leads eventually to the cytosol (Fig. 6). This process occurs via adsorptive endocytosis rather than receptor-mediated endocytosis [44]. Nonspecific Coulombic forces play a key role in the association of ribonucleases with the cell surface [45]. Analyses in vitro and in cellulo reveal that RNase A interacts tightly with abundant cell-surface proteoglycans containing glycosaminoglycans, such as heparan sulfate and chondroitin sulfate, as well as with sialic acid-containing glycoproteins. The uptake of ribonucleases correlates with both their cationicity [46] and cell anionicity [45] (as quantified by measuring electrophoretic mobility). It is noteworthy that anionic glycans are especially abundant on human tumor cells, perhaps contributing to their favorable therapeutic index.

We have developed a chemical strategy to track the path of ribonucleases (or other molecules) through mammalian cells. Traditional small-molecule fluorophores have been of extraordinary utility to chemical biology [47] but can suffer from incessant fluorescence that can obscure valuable information. We have developed fluorogenic labels that overcome this limitation [48, 49]. At the core of our fluorogenic label is a derivative of rhodamine in which one nitrogen is modified as a urea. That modification enables rhodamine to retain bright fluorescence while facilitating conjugation to a target molecule. The other nitrogen of rhodamine is modified with a "trimethyl lock," which enables fluorescence to be unmasked by a single user-designated chemical reaction. For example, the enzymatic activity of esterases in endocytic vesicles educes the fluorescence of an esterase-reactive fluorogenic label, enabling unprecedented temporal imaging and quantification of the internalization of a pendant analyte into live human cells (Fig. 7). The modular design of this fluorogenic label enables the facile synthesis of an ensemble of small-molecule probes with different enzymatic reactivities and fluorescence wavelengths for the illumination of biochemical and cellular processes [50, 51].

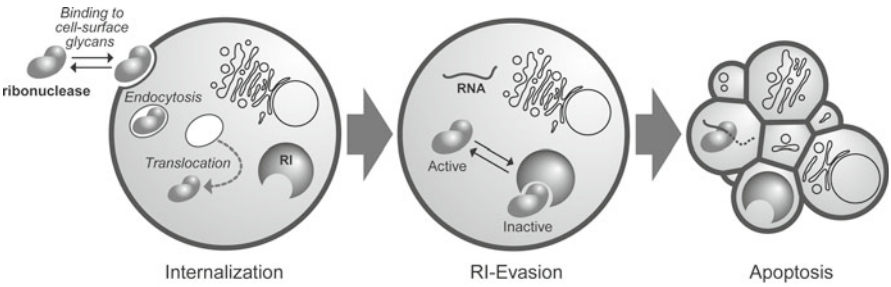

Fig. 6 Mechanism of ribonuclease cytotoxicity. Internalization of ribonucleases involves association with glycans on the cell surface, absorptive endocytosis, and translocation to the cytosol. Upon evasion of RI, a ribonuclease can catalyze the degradation of cellular RNA and induce apoptosis

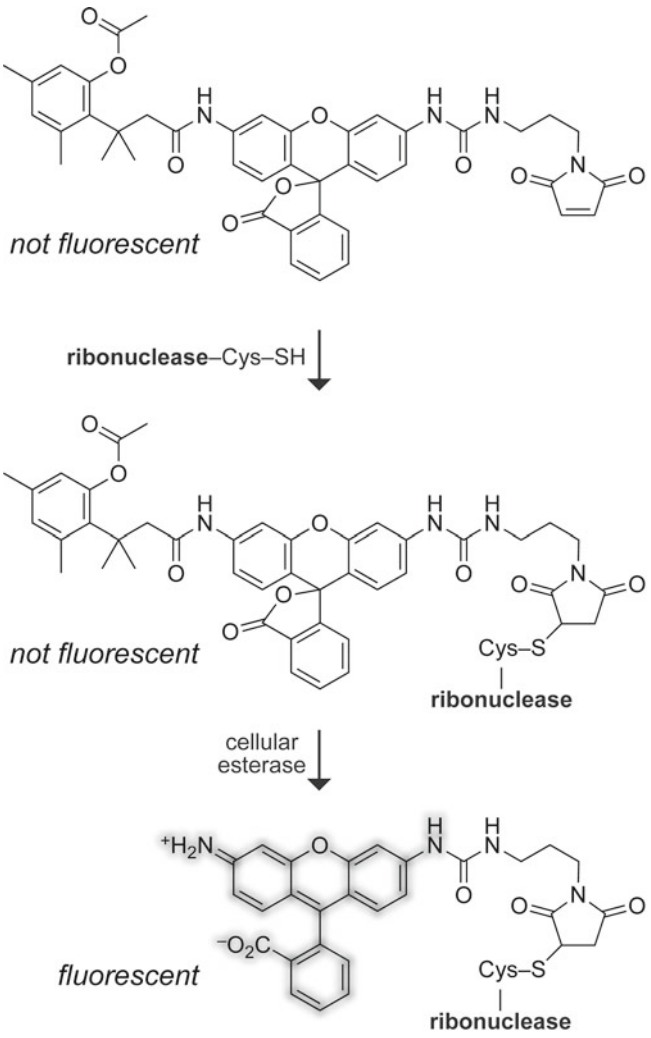

Fig. 7 Mechanism of action of a synthetic esterase-reactive fluorogenic label for spatiotemporal analysis of the cellular internalization of an analyte, here, a ribonuclease [49]

Ribonuclease Zymogens

We are extending the realm of cytotoxic ribonucleases to diseases other than cancer. The biosynthesis of proteases as inactive precursors, or "zymogens," is commonplace. This strategy enables organisms to maintain exquisite control over proteolytic activity. Surprisingly, no zymogens are known to have evolved for other types of enzymes, even those such as ribonucleases that can be cytotoxic. In 2003 [52],

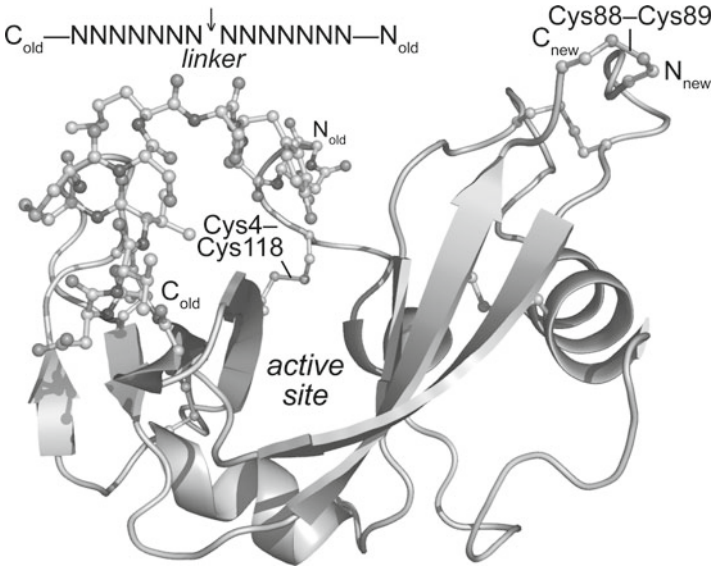

Fig. 8 Structural model of an RNase A zymogen that is activated by a user-designated protease [52–54]. Atoms of the linker (N = any residue) and cysteine residues are shown explicitly; old and new termini, and nonnative cysteines are labeled. Cleavage of the indicated peptide bond in the linker generates ribonucleolytic activity

we created the first artificial zymogen. To do so, we made a circular permutation of the polypeptide chain of RNase A in which the newly installed residues that link the N- and C-termini block access to the active site and contain a sequence recognized by a user-designated protease (Fig. 8). The specificity of zymogen activation can be altered simply by changing the identity of the 14 amino-acid residues in the linker. To date, we have reported on three ribonuclease zymogens. The original one is activated by a protease that is necessary for the propagation of the parasite that causes malaria [52], a second zymogen is made active by a protease that is essential for the replication of the hepatitis C virus [53], and the most recent zymogen is activated by the renowned HIV-1 protease [54]. Our "Trojan horse" strategy, which relies on the *function* of a viral enzyme to elicit toxicity rather than its inhibition, could elude the resistance mechanisms that frustrate existing anti-pathogen therapies.

Prospectus

Enzymes are the most potent of known catalysts, and thus have an intrinsic potential to be superb chemotherapeutic agents. The appearance of ribonucleases in the clinic is heraldic, both by its leveraging the storied role of RNase A in the history of biological chemistry and by its bringing cytosolic targets within the reach of enzymic

drugs. The future for ribonucleases and other enzymes as chemotherapeutic agents is bright, and its manifestation is likely to inspire new chemistry and to reveal new biology.

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