

## New modalities in oncology: ribozymes

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For years, one of the assumptions of cell biology was that all enzymatic actions were performed by proteins. Although nucleic acids (DNA and RNA) could serve as blueprints for the transcription and translation that would produce the proper amino acid sequences to make up an enzymatic protein, it was the protein that did the actual work.

However, in 1986, Zaug and Cech found that processing of RNA from the unicellular organism *Tetrahymena thermophila* clearly depended upon an intron from its own RNA (1). This intron exhibited characteristics of an enzyme, and it was soon realized that the RNA was self-splicing. This was the first time a nonprotein enzyme was observed to perform a biochemical reaction. Because the enzyme was ribonucleotide based, it was termed a ribozyme. To some extent, all ribozymes fall under the category of antisense RNAs (reviewed in the previous issue), because they depend upon the binding of their nucleic acid sequence to complementary sequences in the target mRNA. Yet, whereas antisense approaches depend upon the activation of other enzymes (i.e., RNase H) to destroy the target RNA, ribozymes are capable of directly cleaving the target themselves. Ribozymes offer an additional advantage over antisense molecules because one molecule of ribozyme can cleave many mRNA molecules, thus amplifying its effect. As reviewed in the introduction to this series, these new targeted approaches allow therapy to be directed to specific genetic mutations characteristic of the malignant state.

### STRUCTURE

Several different ribozymal structures, including hairpin, maxizymes, minizymes, and hammerhead, have been described. Of these, only the hammerhead ribozyme form is in clinical trials as it offers advantages in manufacturing techniques. Hammerhead ribozymes are made up of 3 base-paired helices composed of approximately 30 nucleotides each that form a wishbone or "Y" configuration (2). The 2 arms of the wishbone form the binding area to the target RNA. Between the binding arms is a catalytic core where cleavage of the bound target RNA occurs (3).

Like protein-based enzymes, ribozyme catalysis obeys Michaelis-Menten principles, with some exceptions. The reaction is composed of 3 steps: binding of the ribozyme to the target, cleavage of the target, and release of the cleavage products (4) (Figure).

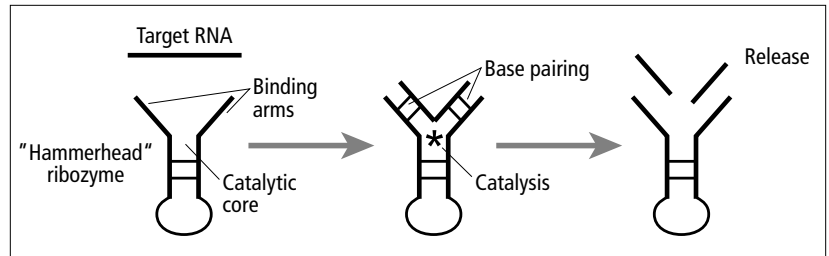


Figure. Scheme detailing the minimum 3-step reaction for hammerhead ribozymes: binding of substrate to ribozyme, cleavage, and release of products.

### RIBOZYMES AS THERAPEUTIC AGENTS

Ribozymes have been proposed for treatment of a variety of diseases, including infectious diseases and cancer. To date, most clinical trials of ribozymes have centered on viral pathogens. Several clinical trials using the hammerhead ribozyme for treatment of HIV-1 have been performed in the USA. These include a protocol for ex vivo transduction and infusion of autologous T lymphocytes from infected individuals via murine vectors, producing a hairpin ribozyme directed against the U5 leader sequence of HIV, and a protocol for transduction and transplantation of CD34 peripheral blood-derived stem cells in HIV-1-infected individuals via murine vectors containing a hammerhead ribozyme targeting HIV Tat RNA (6). In oncology, a phase II clinical trial utilizing a ribozyme against the *flt-2* receptor in combination with cytotoxic chemotherapy has just concluded and is described later in this article.

To use ribozymes as therapeutic agents, several factors must be considered. The first is design. The critical properties of ribozymes are specificity and turnover. Specificity is the ability to cleave at unique sites, whereas turnover is the ability to cleave multiple substrate strands. Both are affected by the lengths of binding arms I and III. If the lengths of the binding arms are too short, the rate of dissociation of the target from the ribozyme will exceed the rate of cleavage, resulting in poor turnover. If the lengths of the substrate-binding arms are too long, the target can be cleaved efficiently, but the ribozyme will turn over slowly because of the rate-limiting release of the cleavage products (4). Thus, the ideal situation is to have arm lengths that aid cleav-

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age yet provide for quick dissociation of the cleaved products. Such considerations come to the fore in designing ribozymes for therapeutic use because long substrates, such as mRNA, are normally very difficult for ribozymes to cleave.

One way to improve hammerhead ribozyme-mediated cleavage is to add oligonucleotide facilitators (7). These facilitators associate with the substrate at ribozyme ends. In long substrates, the facilitators have the potential to preform the substrate for the ribozyme attack to increase both the rate of ribozyme-substrate association and the rate of the cleavage step.

Once designed, the second consideration is delivery. Fell and coworkers found that a large proportion of binding of ribozymes to cells occurred via cell-surface proteins (8). They had been investigating the potential use of chemically stabilized, synthetic ribozymes in down-regulating the *c-erbB1* oncogene, which codes for the epidermal growth factor receptor, which is both amplified and overexpressed in a number of cancers, including brain cancers. In their study, uptake and distribution of exogenously delivered synthetic hammerhead ribozymes were dependent upon temperature, energy, and pH and involved an active endocytic process.

Finally, issues of stability must be considered. Perhaps because RNA is so inherently powerful, nature has evolved myriad different ways to be sure that it doesn't survive long. Powerful degradation enzymes are everywhere, even on our fingertips, so that unprotected RNA has a very short lifespan "in the wild." Thus, for ribozymes to be useful as therapeutic agents, they have to undergo chemical modification to enhance stability. This can assume a variety of forms: chemical modification by methylation of certain bases, substitution of sulfur for oxygen in the phosphate backbone (phosphorothioate linkages), or construction of whole sequences out of DNA instead of RNA. The last approach offers several advantages, including the following: 1) DNA is cheap and easy to synthesize; 2) DNA is more resistant to nucleases than is RNA and can be additionally protected by modification; 3) DNA arms hybridize less easily to the target substrate than do those of RNA, and they may have greater specificity because the hybridizing arms can be longer while still maintaining a competitive substrate dissociation rate; and 4) DNA ribozymes are less prone to self-hybridization than are RNA ribozymes and form inactive structures.

#### TRIALS AT THE MARY CROWLEY MEDICAL RESEARCH CENTER

At the Mary Crowley Medical Research Center, we have just finished participating in a phase II trial of the first ribozyme to be tested as an oncology therapy. Angiozyme is a stabilized ribozyme cleaving *Flt-1* mRNA. This mRNA was chosen as a target based on the following rationale.

Tumor growth is often a multistep process that starts with loss of control of cell proliferation. The cancerous cell then begins to divide rapidly, resulting in a microscopically small spheroid tumor. With further growth, not all the tumor cells are within sufficient proximity of a capillary for exchange of nutrients and oxygen. As this process accelerates, the tumor stops growing and reaches a steady state, in which the number of proliferating cells counterbalances the number of dying cells. In tissues, the oxygen diffusion limit corresponds to a distance of 100  $\mu\text{m}$  between

the capillary and the cells, which is in the range of 3 to 5 lines of cells around a single vessel.

In both normal and pathological cell growth, hypoxia induces expression of vascular endothelial growth factor (VEGF) and its associated receptors. The VEGF family consists of 6 known members: VEGF-A (or VEGF), placenta growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D, and VEGF-E. Each of these is expressed in different tissues—including brain, kidney, liver, and spleen—and by many cell types. *In vitro*, VEGF-A stimulates extracellular matrix degradation, proliferation, and migration, as well as tube formation of endothelial cells (9).

Two high-affinity tyrosine-kinase receptors for VEGF have been identified on vascular endothelium: VEGFR-1 (*Flt-1*) and VEGFR-2 (*Flk-1*). Like VEGF, VEGF receptor gene expression is regulated by hypoxia. An additional member of this family, VEGFR-3 (*Flt-4*), is not a receptor for VEGF but binds VEGF-C and VEGF-D. Decreases in the expression of an important VEGF receptor (in this case *Flt-1*) should then result in the decreased effect of tumor-derived VEGF in promoting new blood vessel formation and subsequent hypoxic injury to the tumor cells. This concept receives support from the results of 2 animal models of cancer in which either continuous or daily bolus subcutaneous injection of Angiozyme inhibited primary tumor growth and metastasis formation. Significantly, Angiozyme administration decreased tumor neovascularization (10).

In preclinical studies, there were few safety concerns with Angiozyme, but animal studies did show dose-dependent effects on the kidneys and, to a slightly lesser extent, the liver in mice and monkeys. Subsequently, 2 separate phase I protocols were performed. One was in healthy volunteers and was limited to single doses of Angiozyme at 10 and 30  $\text{mg}/\text{m}^2$  intravenously and 20  $\text{mg}/\text{m}^2$  subcutaneously. The other phase I trial used single doses of either 100  $\text{mg}/\text{m}^2$  intravenously or 300  $\text{mg}/\text{m}^2$  subcutaneously in patients with advanced cancer. An additional phase I/II trial then investigated subcutaneous doses of 10, 30, 100, and 300  $\text{mg}/\text{m}^2$  daily, with the final recommended dose being 100  $\text{mg}/\text{m}^2$ .

The favorable results of the phase I investigation led to 3 phase II trials in renal cell carcinoma, metastatic melanoma, and metastatic colon cancer. The last trial was performed at Mary Crowley Medical Research Center. The target population was patients with measurable metastatic colon cancer, no previous chemotherapy, and an interval of at least 6 months from previous adjuvant therapy. This was an open-label, nonrandomized trial in which patients received a standard chemotherapy regimen consisting of irinotecan at a dose of 125  $\text{mg}/\text{m}^2$  followed by 20  $\text{mg}/\text{m}^2$  leucovorin and then 500  $\text{mg}/\text{m}^2$  5-fluorouracil given weekly for 4 of every 6 weeks (6 weeks = 1 cycle) (11). Patients also received 100  $\text{mg}/\text{m}^2$  subcutaneous Angiozyme given daily for 24 weeks. A total of 75 patients were treated; the data are being analyzed and will be compared with data for historical controls.

#### CONCLUSION

Ribozyme research is a rapidly expanding area of gene therapy technology. Because of the ability of the ribozyme to recognize even single base-pair differences in sequences, the hammerhead ribozyme lends itself well to gene therapy. Its excellent specificity and turnover are advantageous attributes. The ribozyme can be tailored to the specificity needed to bind a particular substrate

of interest. Not only can it be used in fighting pathogenic viruses, such as HIV-1, but it can be targeted against cancer genes as well. However, in order to use ribozymes in the most efficient and appropriate way in the future, it is imperative that a more comprehensive understanding of their interactions in vivo be attained. Until recently, most experiments involving ribozymes have only been in vitro. With the advent of the clinical trials described here, we are moving into a new age of rapid development of this exciting approach.

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