De novo synthesis of artificial ribonucleases with benign metal catalysts

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A zinc(II)–neocuproine based ribozyme mimic has been constructed for the sequence-selective transesterification of RNA using a begin metal catalyst.

We report the *de novo* synthesis of a Zn-based ribozyme mimic that can cleave HIV mRNA in a sequence-specific manner, with excellent activity. The design of artificial ribonucleases that are both biocompatible and sequence-specific has attracted much attention for their potential use as catalytic, gene-specific therapeutics.[1–8] Many of the artificial ribonucleases prepared thus far are constructs that contain chelates of copper or chelates of heavy metals. Two of our goals in the construction of artificial ribonucleases are: (i) the use of bioavailable, innocuous metals such as zinc, and (ii) the optimization of activity. The most specific artificial ribonucleases are referred to as 'ribozyme mimics' owing to the fact they mimic the Watson–Crick derived sequence-specific activity of some ribozymes. With ribozyme mimics, the large catalytic domain of a natural ribozyme is replaced by a small-molecule catalyst.

The RNA cleavage ability of free mononuclear and dinuclear zinc complexes and conjugates has been demonstrated in previous work from our lab and from other groups, but the cleavage activity of Zn reagents has generally been low. In 1995, sequence-specific cleavage of RNA was demonstrated by Lönnberg and coworkers[9] when they used a 5′-imidazole DNA conjugate in concert with free zinc ions to form an active artificial ribonuclease that cleaved 2–5% of the target RNA in 19 h at room temperature. It was followed by a 1998 communication by Matsuda *et al.* where a novel dinuclear Zn(II)–DNA conjugate was used to effect sequence-specific cleavage of RNA.[10] The dinuclear construct cleaved 5% of the RNA after 3 h at 37 °C ([RNA] = 0.2 μM; [cleavage agent] = 5 μM). Here we report the *de novo* construction of an active, sequence-specific artificial ribonuclease containing an internal serinol-neocuproine residue embedded in a DNA 17-mer (Fig. 1).

We have previously reported the synthesis and activity of a ribozyme mimic derived from serinol and copper(II)–terpyridine (Cu–terpy) (Probe 1, Fig. 1).[1,11] The serinol residue introduced an abasic site into the ribozyme mimic–RNA duplex, and was shown to be three times more efficient at RNA cleavage than a fully duplexed Cu(II)–terpy derived ribozyme mimic. The phosphoramidite **1**, a serinol-neocuproine conjugate, was synthesized according to Scheme 1 and incorporated into a DNA 17-mer (Probe 2) using standard automated DNA synthesis.§ This 17-mer is complimentary to a region of the mRNA from the HIV *gag*-gene. Probe 3 (Fig. 1), which contains a three-carbon spacer produced from a commercially available (Glen Research) phosphoramidite, was used to test for cleavage associated with the presence of an abasic site in the RNA/DNA duplex.

The modified DNA conjugates that were synthesized are shown here where X indicates the modified position. RNA cleavage reactions were conducted with two RNA substrates: a 159-mer from a relatively well conserved sequence from the *gag* mRNA of HIV (775-933), and a 28-mer that contains the same 17-mer recognition sequence (Fig. 2). The 28-mer substrate allowed identification of cleavage products and the 159-mer allowed assessment of the sequence-specificity of the ribozyme mimic in the presence of numerous binding sites. Reactions were conducted at both 37 and 45 °C, at pH 7.4 (HEPES Buffer).

High-resolution polyacrylamide gel-electrophoresis was used to analyse the RNA cleavage, and a typical image of the 28-mer reaction mixture is shown in Fig. 1. No sequence-specific cleavage was observed with probe 2 in the absence of added metal. In the presence of EDTA, probe 1 gave no

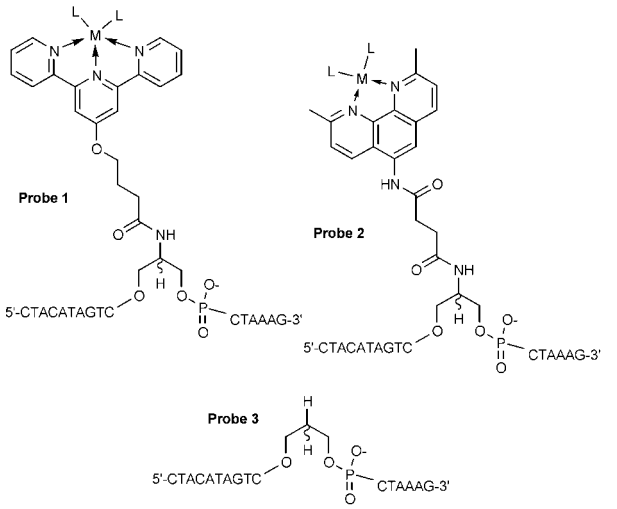
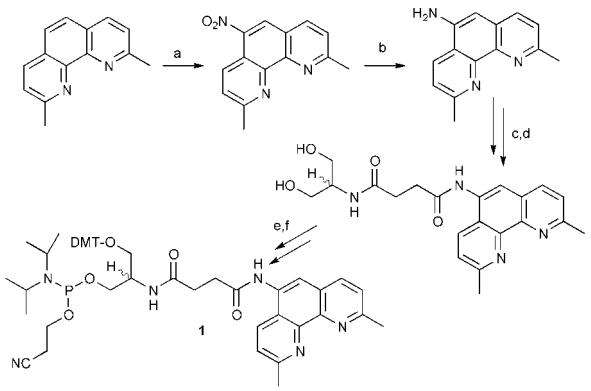


Fig. 1 Ribozyme mimic probes.



Scheme 1 Synthesis of phosphoramidite **1**. *Reagents and conditions*: (a) H_2SO_4, fuming HNO_3, reflux, 5 h, 23%; (b) NH_2NH_2, Pd/C, EtOH, reflux, 5 h, 81%; (c) succinic anhydride, pyridine, room temp., 12 h, 59%; (d) EDC–HCl, serinol, DMF, room temp., 12 h, 63%; (e) DMTCl, pyridine, room temp., 12 h, 51%; (f) 2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite, room temp., 1 h, 82%.

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(a)
5′-^{1}(775)GGAGAA^{6} AUUUAUAAAA^{16} GAUGGAUAAU^{26} CCUGGGAUUA^{36} AAUAAAAUAG^{46}
UAAGAAUGUA^{56} UAGCCCUACC^{66} CAGCAUUCUG^{76} GACAUAAGAC^{86} AAGGACCAAA^{96}
GGAACCUUUA^{106} GAGACUAUGU^{116} AGACCGGUUC^{126} UAUAAAACUC^{136} UAAGAGCCGA^{146}
GCAAGCUUCA^{156} CAG^{159} (933)-3′

(b)
5′-A^{1}A^{2}A^{3}C^{4}C^{5}A^{6}A^{7}C^{8}C^{9}C^{10}U^{11}U^{12}U^{13}A^{14}G^{15}A^{16}G^{17}A^{18}C^{19}U^{20}A^{21}U^{22}G^{23}U^{24}A^{25}G^{26}A^{27}C^{28}-3′

Fig. 2 RNA Substrates. (a) The 159-mer target RNA strand, a relatively well-conserved sequence from the *gag* mRNA of HIV (775-933 HIVHXB2R), is shown, with the 17-mer target region underlined. (b) The 28-mer target RNA strand containing the same 17-mer recognition sequence from the *gag*-mRNA of HIV (HIVHXB2R), is shown, with the 17-mer target region underlined.

Table 1 Sequence-specific cleavage of 28-mer and 159-mer RNA targets by ribozyme mimics derived from Cu(II) and Zn(II) with probes 1–3 (exact reaction conditions given in experimental)

Probe no. (metal)	Cleavage (%) (28-mer, 15 h 37 °C)	Cleavage site (28-mer)	Cleavage (%) (159-mer, 10 h, 37 °C	Cleavage site (159-mer)
1 (Cu)	14	A^{16}	8	A^{108}
2 (Cu)	65	G^{15}, A^{16}	80	G^{109}, A^{108}
2 (Zn)	25	G^{15}, A^{16}	18	G^{109}, A^{108}
3	0	n/a	0	n/a

sequence-specific RNA cleavage. In accordance with previous reports, EDTA treatment is necessary to suppress cleavage in the case of probe 1 because of the high binding constant of terpy for copper. This extremely high affinity allows ribozyme mimics that contain pendant terpy ligands to scavenge trace amounts of Cu(II) from commercial buffer salts.[11] In the presence of copper, both probe 1 and 2 form active ribozyme mimics, and in the presence of zinc, probe 2 forms an active ribozyme mimic. The products of all of the cleavage reactions co-migrate with alkaline hydrolysis and Ribonuclease T_1 (G specific) digestion products, which lends strong evidence that, in the presence of either metal, these ribozyme mimics react *via* a biomimetic pathway of transesterification and hydrolysis.

The extent of cleavage is shown in Table 1. The major site of RNA scission by probes 1 and 2 varies by one base. Probe 1 induces nucleophilic cleavage of the RNA at the 3′ side of A^{16} while probe 2 cleaves on the 5′ side of this nucleotide. No sequence-specific cleavage is observed in the presence of Cu(II) or Zn(II) alone (with no DNA probe). Probe 3, the control oligonucleotide containing the abasic site and no pendant ligand, is completely inactive for RNA cleavage in the presence of Cu(II) and Zn(II) (data not shown for Zn). This control tested for any site-specific cleavage derived solely from the presence of metal ions and an abasic site.

The Zn(II) conjugate of probe 2 is only the third example of a zinc based artificial ribonuclease derived from Zn(II), and the second mononuclear example. Direct comparisons of sequence-specific RNA cleavage efficiencies are very difficult because of differences in reaction time and temperature, but the zinc–neocuproine-mediated cleavage reported here is higher than the sequence-specific cleavage previously reported. We have demonstrated that high cleavage efficiencies that can be attained using benign inorganic chemistry. The extent of cleavage by ribozyme mimics observed in these studies is as follows:

Cu(II)–neocuproine > Zn(II)–neocuproine > Cu(II)–terpy.

The observed trends result predominately from two phenomena: (i) The speciation between metal-bound water and metal bound hydroxide (the most probable active species) *i.e.* the trends in the pK_a of the coordinated waters and (ii) the suppression of bis(μ-hydroxide) dimers by the methyl groups of neocuproine.[12]

The *in vivo* concentration of zinc in human serum is 17.2 μM,[13] three times greater than the concentration of zinc used in these *in vitro* experiments. There remains controversy over the amount of free zinc in the cell. Note also that the free copper concentration *in vivo* has recently been determined to be approximately zero.[14] The large cleavage efficiency of the zinc

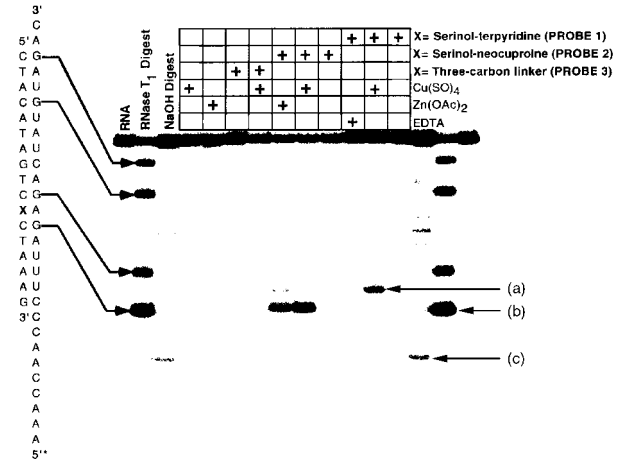

Fig. 3 Audioradiogram of the sequence-specific cleavage of the 5′-end labeled 28-mer RNA substrate 5′-CTA CAT AGT CXC TAA AG-3′ by ribozyme mimics; (a) sequence-specific cleavage by probe 1 (minor site probe 2), (b) sequence-specific cleavage by probes 2, (c) hot spot for RNA cleavage.

based ribozyme mimic bodes well for the production of biocompatible sequence-specific artificial ribonucleases.

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Notes and references

§ The compounds synthesized in Scheme 1 were fully characterized by ^{1}H, ^{13}C and ^{31}P(phosphoramidite **1**) high-resolution NMR spectroscopy and high-resolution fast-atom-bombardment (HR-FAB) mass spectrometry (MS). All probes were characterized by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry and electrospray mass spectrometry.(Mass spectrometry was provided by the Washington University Mass Spectrometry Resource, an NIH Research Resource (Grant No. P41RR0954)).

The 159-mer RNA was synthesized by runoff transcription and 5′ end labeled with ^{32}P (We thank Dr Lee Ratner for the plasmid containing the HIV *gag*-gene fragment). The 28-mer RNA was purchased (Oligo's *etc.*) and ^{32}P 5-end labeled in a similar fashion. RNA cleavage reactions were carried out in 10 mM *N*-(2-Hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) buffer pH 7.4, with 0.1 M $NaClO_4$, [0.1 μM RNA (28-mer) and 0.01 μM RNA (159-mer), 5 μM probe, and 5 μM metal (total reaction volume = 10 μL. The reactions were heated to the desired temperature and time. The reactions were quenched by the addition of 5 μL loading buffer, and loaded on a denaturing polyacrylamide gel (20% 28-mer and 6% 159-mer). The gel image was quantified using a Molecular Dynamics PhosphorImager™ and the ImageQuant™ software package.

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