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Hydrolysis of Bulged Nucleotides in Hybrids Formed by RNA and Imidazole-Derivatized Oligo-2'-O-methylribonucleotides

Anthony D. Saleh[†] and Paul S. Miller^{*}

Department of Biochemistry and Molecular Biology, Bloomberg School of Public Health, Johns Hopkins University, 615 North Wolfe Street, Baltimore, MD 21205

Abstract

In order to enhance the efficacy of small antisense molecules, we examined a series of antisense oligonucleotides derivatized with functional groups designed to enable them to hydrolyze their RNA target. Solid phase synthetic methods were used to prepare imidazole-derivatized antisense oligo-2'-O-methylribonucleotides. Upon binding, these oligonucleotides create internal bulged bases in the target RNA that serve as sites for hydrolysis. We observed that an oligonucleotide derivatized with a side chain containing two imidazole groups was capable of hydrolyzing 58% of its RNA target when incubated with the target for 48 hours at 37°C and physiological pH.

1. Introduction

Antisense oligonucleotides provide a means to control gene expression at the RNA level. These oligonucleotides have been used as research tools to knockdown gene expression in cells in culture and as tools for drug target validation. Efforts have also been made to develop antisense-based therapeutics [1,2].

Antisense oligonucleotides can function by essentially two different mechanisms. By binding to its RNA target, the oligonucleotide can physically block proteins or other nucleic acids from interacting with the target and thus prevent target expression. This mode of action is termed steric blockage and requires at a minimum one antisense oligonucleotide for each target RNA. Under certain circumstances, the RNA strand of the hybrid formed by the antisense oligonucleotide and its RNA target can be hydrolyzed by RNase H, thus rendering the target inactive. RNase H is found in most cells and antisense oligonucleotides that use this endonuclease are able to target multiple RNAs for destruction. Although RNase H dependent antisense oligonucleotides represent an attractive and potentially efficient way to knockdown intracellular RNAs [3,4], the types of oligonucleotides that function by this mechanism are somewhat limited structurally. Oligodeoxyribonucleotides, which support RNase H mediated hydrolysis, are themselves readily degraded by cellular nucleases. Attempts to increase nuclease resistance through the use of modified nucleosides or sugar-phosphate backbones often results in reduced or lost cleaving activity. In addition, formation of as few as 4–5 base pairs between the antisense oligonucleotide and RNA is sufficient to initiate RNase H mediated cleavage [5]. This minimal complementarity requirement can lead to degradation of non-targeted RNAs [6–8].

An alternative to the use of RNase H dependent antisense oligonucleotides are those whose structures include a functional group that is capable of hydrolyzing the RNA when the

^{*}Correspondence should be addressed to Paul S. Miller, pmiller@jhsp.h.edu., Phone: (410)-955-3489, Fax: (410)-955-2926.

[†]Current Address: Radiation Oncology Branch, National Cancer Institute, National Institute of Health, 10 Center Drive, Bethesda, MD 20892

oligonucleotide binds to its target. A number of these RNA-cleaving antisense oligonucleotide have been described [9]. For example, RNA-cleaving oligonucleotides have been designed that contain imidazole and primary amine functional groups conjugated to the end of the oligonucleotide [10–17]. The imidazole groups in these constructs target and cleave RNA outside the binding site of their oligonucleotide, while the oligonucleotide remains bound to the target after hydrolysis occurs. A potentially more effective strategy that has been developed that involves targeting metal based cleaving moieties to internal unpaired or bulged RNA bases formed when the oligonucleotide binds to its RNA target [18–20]. Hydrolysis at the site of these bases disrupts the oligonucleotide binding site thus freeing the oligonucleotide to bind another copy of the target.

We have explored the possibility of targeting imidazole groups to cleave the nucleotides of an internal bulge that is created when the oligonucleotide hybridizes to its RNA target. In this report we demonstrate that bulged nucleotides are susceptible to hydrolysis by imidazole buffers. We describe the solid-phase preparation of antisense oligo-2'-O-methylribonucleotides that contain imidazole groups conjugated to nucleotides located in the middle of the oligonucleotide. These oligonucleotides create a one or two base bulge when hybridized to their complementary RNA target and effect hydrolysis of the target at physiological pH.

2. Results and Discussion

2.1 Imidazole Catalyzed Hydrolysis of RNA Bulges

Base catalyzed hydrolysis of RNA resulting from in-line attack by the 2'-hydroxyl group of an RNA nucleotide on an adjacent phosphodiester bond is sterically impossible in A-type double-stranded molecules, the conformation that is adopted when an antisense oligonucleotide binds to its RNA target. However, single-stranded RNA is more susceptible to cleavage because rotation around the phosphodiester linkage can allow proper positioning of the 2'-hydroxyl for in-line attack [21,22]. Structural studies suggest that internal bulged or unpaired nucleotides in an RNA duplex can assume an orientation around the phosphate allowing in-line attack by the 2'-hydroxyl group [23–28]. This idea is supported by the observations that bulged bases in RNA duplexes have greatly increased susceptibility to hydrolysis catalyzed by metal complexes [29] or by imidazole [30].

We examined the susceptibility to imidazole-mediated hydrolysis of RNA bulged nucleotides that are created when an antisense oligo-2'-O-methylribonucleotide binds to an RNA target. The target, **RNA-1** whose sequence is shown in Table 1, corresponds to the nucleotides comprising the 5' side of stem-loop II B of the HIV-1 rev response element (RRE). When complexed with the oligo-2'-O-methylribonucleotide 13-mer **oligo-1**, a single bulged adenosine is created in the hybrid duplex as shown schematically in Figure 1A. Additional targets, **RNA-2**, **RNA-3**, and **RNA-4**, were synthesized which give duplexes that have 2, 3, or 4 bulged adenosines when hybridized to **oligo-1**. We also synthesized **oligo-2**, which upon binding to **RNA-1**, produces a duplex with a -CA- two nucleotide bulge.

The stabilities of the hybrid duplexes formed by **oligo-1** or **oligo-2** and the RNA targets were determined by thermal denaturation experiments. A typical melting curve and the melting temperatures for the various hybrid duplexes are shown in Figure 1B. The melting temperatures of the duplexes range from 65.2°C to 73.0°C. The duplex with a single bulged adenosine, **RNA-1/oligo-1**, has the highest T_m. The presence of two bulged adenosines in duplex **RNA-2/oligo-1** reduces the T_m by 3.5°C. However no further decrease in T_m was observed for duplexes having 3 or 4 bulged adenosines, duplexes **RNA-3/oligo-1** and **RNA-4/oligo-1**, respectively. The melting temperatures and the shapes of the melting curves show that all of the hybrids are in duplex form at 37°C.

The susceptibility of hybrid duplex **RNA-1/oligo-1** to imidazole-mediated hydrolysis was determined. A solution of 100 nM 5'-³²P-labeled-**RNA-1** and 10 μ M **oligo-1** in buffer containing 1.0 M imidazole and 0.5 mM EDTA pH 7.0 was incubated at 37 °C, aliquots were removed at various times, and the reaction mixtures were analyzed by denaturing polyacrylamide gel electrophoresis. As shown in Figure 2A, hydrolysis was observed at position 7, the site of the bulge, and at position 8, the nucleotide immediately adjacent on the 3'-side of the bulge. No significant hydrolysis was observed at other positions in the duplex. This result is consistent with the idea that the orientations of the sugar phosphate backbones of the ribonucleotides in an A-form duplex adjacent to the site of the bulge restrict inline attack on the phosphate group by the 2'-hydroxyl group.

Incubation of duplex **RNA-1/oligo-2**, which contains a -CA- two base bulge, in imidazole buffer resulted in hydrolysis at the two bulged nucleotides and the nucleotide directly 3' of the bulge as shown by the gel and schematically in Figure 2B. Similar results were seen with duplex **RNA-2/oligo-1**, which contains an -AA- two base bulge (see Figure S1A). The extent of hydrolysis for both of these hybrid duplexes appeared to be greater than that observed for the duplex with a single adenosine bulge. This difference may reflect the ability of the single adenosine to adopt both extrahelical and helical conformations, with the latter conformation stabilized by stacking interactions [28]. In contrast, the nucleotides involved in the 2 base bulges would exist primarily in an extrahelical conformation and would thus be more susceptible to hydrolysis.

Hydrolysis experiments were also carried out on duplexes formed between **oligo-1** and **RNA-3**, or **RNA-4** which have 3 and 4 adenosine nucleotide bulges, respectively. As was the case with duplexes having two bulged nucleotides, hydrolysis was observed at the positions of the bulged nucleotides and the 3'-nucleotide immediately adjacent to the bulge (see Figures S1B and S1C). No hydrolysis was observed in a control experiment where **oligo-1** and **RNA-2** were incubated in buffer without imidazole (Figure S1D).

Taken together, the results described above demonstrate that bulged RNA nucleotides in an otherwise intact hybrid 2'-O-methyl-RNA/RNA duplex are susceptible to imidazole-catalyzed hydrolysis at neutral pH. These results are similar to those reported in previous studies where hydrolysis of bulges in hybrid duplexes formed between oligodeoxyribonucleotides and RNA were probed with metal complexes [18,28] or imidazole [29]. One difference is that in the studies by Husken et al., which were carried out at 60°C, additional bases surrounding the bulge were also hydrolyzed. The reduced relative stability of DNA/RNA hybrids, which can lead to increased breathing around the bulged bases, and the higher reaction temperature may account for the additional hydrolysis products observed in those reactions.

2.2 Synthesis and Cleaving Activities of Imidazole Conjugated Oligonucleotides

As shown in Figure 3A, molecular models, generated using Hyperchem 8.0 software, of the duplexes formed between **oligo-1** and **RNA-1** or **RNA-2** suggested that an imidazole tethered to **oligo-1** in the minor groove of the duplex near the site of the bulge should be able to interact with and deprotonate the 2'-hydroxyl of the RNA bulged nucleotide to initiate hydrolysis. This might be achieved by conjugating histamine to the 2' position of a nucleotide of **oligo-1** located one or two bases 3' from the bulge. In addition the models suggested that conjugation of histamine or an aminobutyl group to the 5'-end of **oligo-4** could position these groups near the 5'-oxygen or phosphate, respectively, of the bulged nucleotide where they could further facilitate hydrolysis of the phosphodiester bond.

To test this possibility, we synthesized the imidazole-conjugated oligo-2'-O-methylribonucleotides whose sequences and structures are shown in Table 1 and Figure 5A.

Oligo-3 and **oligo-4** have histamines conjugated to both the 2'-hydroxyl of an internal ribonucleotide (rC in Table 1) and to the 5'-hydroxyl of the oligonucleotide. **Oligo-5** and **oligo-6** each have histamine conjugated to an internal ribonucleotide (rC in Table 1) and an aminobutyl group conjugated to the 5'-hydroxyl of the oligonucleotide. When hybridized to **RNA-1**, the duplexes formed by these oligonucleotides have a single A bulge in the middle of the duplex. In the case of **oligo-3** and **oligo-5**, the internal histamine is located one base removed from the bulged nucleotide. In the case of **oligo-4** and **oligo-6**, the internal histamine is directly adjacent to the site of the bulge as illustrated in Figure 5A.

The conjugates were synthesized as outlined in Figure 4. Derivatization reactions were carried out on the protected, support-bound oligonucleotide. This procedure allows the use of excess reagents to drive the derivatization reactions to completion and simple washing steps to remove unreacted reagents and solvents, thus avoiding potential losses of material associated with precipitation or column purifications. The conjugates were purified by C-18 reversed phase HPLC and their compositions were confirmed by MALDI-TOF mass spectrometry.

The abilities of the imidazole-conjugated oligonucleotides to hydrolyze **RNA-1** were examined by incubating 10 μ M conjugate and 100 nM 5'-³²P-labeled **RNA-1** in a solution containing 50mM MOPS pH 7.5, 100 mM sodium chloride, and 0.5 mM EDTA at 37°C for 24 hrs. A large excess of conjugated oligonucleotide was used in order to ensure that the RNA target was completely bound and consequently maximum cleavage of the target occurred. Electrophoretic mobility shift assays (EMSA) with 1 nM **RNA-2** showed that the RNA targets were completely bound in the presence of 50-fold oligonucleotide (data not shown).

The reaction mixtures were analyzed by electrophoresis on a 20% denaturing polyacrylamide gel. As shown in Figure 5B, **oligo-3** and **oligo-5** each hydrolyzed **RNA-1** to an extent of 1% - 2%. A fragment 8 nucleotides in length was observed that corresponds to hydrolysis at the site of the single A bulge. An additional RNA fragment, 14 nucleotides in length was observed, which most likely results from hydrolysis of the target at the site of the 5'-end of the oligonucleotide. These bands are not observed when RNA is incubated alone or with unconjugated oligonucleotide (Figure S6). Increasing the size of the bulge did not increase the amount of hydrolysis. The reaction of **oligo-3** or **oligo-5** with **RNA-2**, **RNA-3** or **RNA-4**, which created duplexes having a 2, 3, or 4 nucleotide bulge respectively, gave essentially no hydrolysis, even after incubation for 96 hrs at 37°C (data not shown). As mentioned previously, the single base bulge formed when **RNA-1** interacts with either **oligo-3** or **oligo-5** may reside in both extrahelical and helical conformations, an arrangement that may allow the bulged residue to remain in relatively close proximity to the functional groups of the conjugate. In contrast, residues in the larger bulges formed between **oligo-3** or **oligo-5** with **RNA-2**, **RNA-3** or **RNA-4** would most likely be exclusively extrahelical and thus not accessible to the functional groups of the conjugates.

The internal histamine is located one base pair removed from the bulge, in the duplexes formed by oligonucleotides **oligo-3** and **oligo-5**. To determine the effect of moving the internal histamine closer to the site of the bulge, oligonucleotides **oligo-4** and **oligo-6** (see Figure 5A) were incubated with each of the RNA targets, **RNA-1** through **RNA-4**, at 37°C for up to 96 hrs. The reaction products were analyzed by denaturing polyacrylamide gel electrophoresis. No hydrolysis products were detected (see Figure S5).

In order to function effectively, it is likely that both imidazole groups of **oligo-3** or **oligo-4** or the imidazole and the aminobutyl groups of **oligo-5** or **oligo-6** must encounter the phosphodiester of the bulged nucleotide at the same time. Although the molecular models

suggested that internally tethered histamine and 5'-tethered imidazole or aminobutyl groups could reside in the vicinity of this phosphodiester group, the lack of significant hydrolysis by any of the imidazole or aminobutyl/imidazole conjugated oligonucleotides suggests that a hydrolytically productive simultaneous encounter by the two functional groups of these oligonucleotides is a very rare event. In theory, this problem could be ameliorated by attaching both imidazole groups to the same tether. In this way both imidazole groups would at least reside simultaneously at the targeted phosphodiester group, where, if in the correct orientation, they could facilitate in-line attack by the conformationally pre-organized 2'-hydroxyl of the bulged nucleotide. Such an arrangement is modeled in Figure 3B.

To test this idea we prepared oligonucleotide **oligo-7** whose structure is shown in Figure 6. This oligonucleotide contains two histamine groups conjugated through carbamate linkages to a tris(2-aminoethyl) tether that is attached to the 2'-position of an internal ribonucleotide. This functional group, when attached to the 5'-end of antisense oligonucleotide has been shown to effectively catalyze hydrolysis of tRNA [11]. Hybridization of **oligo-7** to **RNA-1** or **RNA-2** creates duplexes that contain a 1 or 2 nucleotide bulge respectively in the RNA target. Molecular models show that the tether is sufficiently long to reach across the minor groove of the duplex and allow the imidazole groups of the histidine residues to reside in the vicinity of the bulged nucleotides of the RNA target.

The conjugated oligonucleotide was synthesized as shown in Figure 6. The deprotected conjugate was first purified by SAX and then C18 HPLC and the composition of the conjugate was confirmed by MALDI-TOF mass spectrometry. As shown in Figure 7A, essentially no hydrolysis was observed when 100 nM **RNA-1** was incubated with 10 μ M **oligo-7** at 37°C in buffer containing 50 mM MOPs, pH 7.5, 100 mM sodium chloride and 0.5 mM EDTA. The duplex created by **oligo-7** and **RNA-1** contains a single adenosine bulge. Increasing the size of the bulge to two nucleotides, however, resulted in modest hydrolysis. Thus when **RNA-2** was incubated with **oligo-7** (15 % of the total RNA was cleaved after incubation for 96 hrs (Figure 7B). In addition to the expected hydrolysis at the bulged nucleotides, nucleotides 7, 8 and 9, hydrolysis was also observed near the 3' end of the RNA at positions 17 and 18, which is outside the oligonucleotide binding site. These additional cleavage fragments do not result from non-specific hydrolysis of the target, because no hydrolysis was observed when either **RNA-1** or **RNA-2** was incubated with buffer alone (see Figures 7A and 7B, lanes C₁ and C₃, respectively) or with unconjugated oligo (see Figure 7A and 7B, lanes C₂ and C₄, respectively). Furthermore, it seems unlikely that these products arise from reaction of the RNA with the large excess of free conjugate present in the assay, because incubation of 100 nM non-complementary **RNA-5** with 10 μ M **oligo-7** resulted in no hydrolysis, even after 96 hrs (see Figure S4). Molecular models suggest that the tethered imidazoles cannot reach the 3' end of the single stranded RNA when **oligo-7** is bound as shown in Figure 7. Possibly the RNA/oligonucleotide conjugate hybrid adopts an alternate conformation that allows these groups to be positioned near the 3' end of the duplex where it can carry out hydrolysis at positions 17 and 18.

All of the reactions described above were carried out in the absence of magnesium. However, previous studies have shown that in some cases, magnesium can enhance the rates of hydrolysis of RNA targets by ribozymes [31] and antisense cleavers [11]. Therefore we decided to test if magnesium could enhance the rate of hydrolysis by **oligo-7**, the oligonucleotide conjugate that has the highest hydrolysis rate in the absence of magnesium. As seen in Figure 8, addition of magnesium to the reaction mixture greatly increased the rate of RNA hydrolysis by **oligo-7**. Incubation of **RNA-1** with **oligo-7** in the presence of 20 mM Mg²⁺ resulted in hydrolysis of 78 % of the RNA after 96 hrs (see Figure 8A). Hydrolysis was observed at position 7 and 8, the sites of the single A bulge and directly 3' of the bulge, respectively. Hydrolysis was also observed near the 3' end of the RNA at positions 15, 16

and 17. Incubation of **RNA-2** with **oligo-7** and 20 mM Mg^{2+} resulted in 58% hydrolysis of **RNA-2** after 48 hrs and complete hydrolysis after 96 hrs (see Figure 8B). After 48 hrs several RNA fragments were observed, including those 7, 8, and 9 nucleotides in length that correspond to cleavage at the site of the bulge. Fragments 16, 17, and 18 nucleotides in length were also observed and these represent hydrolysis near the 3' end of the RNA target. The hydrolysis pattern seen after 48 hrs is similar to that observed in the reactions without Mg^{2+} (Figure 7B). However, after 96 hours of incubation only products arising from hydrolysis at the bulge are observed. This suggests that the RNA target undergoes multiple rounds of hydrolysis during the course of the experiment. Essentially no hydrolysis was observed when **RNA-1** or **RNA-2** was incubated in buffer with magnesium (Figure 8A and 8B, lanes C₁ and C₂, respectively) or with unconjugated oligo (figure S7 C₂).

There are several possible reasons why the rates of hydrolysis of **RNA-1** and **RNA-2** by **oligo-7** are enhanced in the presence of magnesium. Interaction of Mg^{2+} with the oligonucleotide conjugate/RNA duplex may alter the structure of the bulged bases in such a way as to provide a more favorable conformation for hydrolysis. It is known that Mg^{2+} induced changes in ribozyme structure can lead to as much as a tenfold enhancement of the hydrolysis rate^[31]. Alternatively, the imidazole groups of **oligo-7** may chelate magnesium which could then facilitate hydrolysis of the target. However under the conditions of our reactions (pH 7.5), the imidazole groups of oligo-7 are most likely protonated or partially protonated thus making Mg^{2+} chelation less likely. Finally, association of Mg^{2+} with the negatively charged phosphate groups of the RNA target may stabilize the formation of the pentacoordinate intermediate thus facilitating hydrolysis of the phosphodiester bond.

3. Conclusion

The majority of imidazole-based antisense cleavers that have been reported in the literature are conjugated at the 5' or 3' end of the oligonucleotide^[9]. The studies presented here show that internally conjugated imidazole-based cleavers can effect hydrolysis when targeted to bulged nucleotides in RNA. It appears that hydrolysis is most effective when both imidazoles are conjugated to a single tether as in the case of **oligo-7**. Cleaving the RNA at positions internal to the binding site of the antisense oligonucleotide should enable the oligonucleotide to dissociate from the cleaved target and be available to cleave additional targets.

Our results may be compared to those of Reynolds et al.^[21], who described constructs in which primary amine and imidazole functional groups were conjugated to a non-nucleoside linker at an internal position of a methylphosphonate antisense oligonucleotide. The non-nucleoside linker is similar to an abasic site. In this configuration, the unpaired base across from the linker is still accommodated in the helix but is free to rotate about its phosphates. This is different than our system where the bulged nucleotides are forced out of the helix. In Reynolds' studies, 10% hydrolysis was observed at the unpaired base after a five-day incubation period. This activity was significantly less than that observed for **oligo-7** targeting a two nucleotide bulge, which cleaves 58% of its target within 48 hrs.

Antisense oligonucleotides that are internally conjugated with a chelating moiety have also been used to catalyze hydrolysis of bulged RNA bases. These molecules have been shown to cleave target RNAs at substoichiometric concentrations, at considerably faster rates than reported in this study^[19,20], a result that suggests the possibility of catalytic turnover. Although the antisense molecules reported in this study possess a rather modest rate of hydrolysis, further modifications of the tether or functional groups may result in greater hydrolytic efficiency and consequently provide antisense oligonucleotides that could be used to attack cellular targets.

4. Experimental

Materials

Protected ribo- and 2'-O-methylribonucleoside-3'-O- (β -cyanoethyl-N,N-diisopropylamino) phosphoramidites, nucleoside-derivatized controlled pore glass supports, 4,5-dicyanoimidazole, 1-*H*-tetrazole, 5-ethylthiotetrazole, Cap A solution, Cap B solution, and oxidizing solution were purchased from Glen Research Inc., Sterling, VA. The exocyclic amino groups of the 2'-O-methylribonucleoside phosphoramidites mr-C and mr-G were protected with acetyl and isobutryl protecting groups, respectively. Phosphoramidite solutions were prepared using synthesis grade acetonitrile (Fisher) that was dried and stored over calcium hydride. MALDI-TOF mass spectra analyses were performed at the AB Mass Spectrometry/Proteomics Facility at the Johns Hopkins School of Medicine with support from a National Center for Research Resources Shared Instrumentation Grant 1S10-RR14702. Gamma-³²P-adenosine triphosphate was purchased from Amersham/GE HealthCare Products, Inc. T4 Polynucleotide kinase was purchased from New England Biolabs. 1,4-diaminobutane, carbonyl diimidazole, tris(2-aminoethyl)-amine, and dimethylsulfoxide (DMSO) were purchased from SIGMA/Aldrich. DMSO was dried and stored over molecular sieves.

General Methods

Strong anion exchange (SAX) HPLC was carried out using a Dionex DNAPac PA-100 analytical (4 × 250 mm) column purchased from Dionex Corp., Sunnyvale, CA. The column was eluted with a 30 min linear gradient of 0.0 M to 0.5 M sodium chloride in a buffer containing 100 mM Tris-hydrochloride, pH 7.8, and 10% acetonitrile at a flow rate of 1.0 mL/min. The UV absorbance of the eluant was monitored at 260 nm for analytical runs, and 290 nm for preparative runs. C18 reversed phase HPLC was carried out using a Dynamax Microsorb C18 column purchased from Rainin Instrument Inc. The column was eluted with a 20 min linear gradient of 2–20% acetonitrile in buffer containing 50 mM sodium phosphate, pH 5.2. The UV absorbance of the eluant was monitored at 260 nm for analytical runs, and 290 nm for preparative runs. Polyacrylamide gel electrophoresis (PAGE) was carried out on 20 × 20 × 0.75 cm gels (unless otherwise noted), containing 17% acrylamide, 7 M urea (denaturing gels only), and 90 mM Tris, 90 mM boric acid, and 0.2 mM ethylenediaminetetraacetate buffered at pH 8 (1X TBE). The gel running buffer was also 1x TBE. The denaturing gel loading buffer contained 90% formamide, 0.05% xylene cyanol (XC), and 0.05% bromophenol blue (BPB), and 10 mM EDTA. Oligonucleotide extinction coefficients were calculated based on nearest neighbor approximations [32].

Chemical Synthesis and Purification of Target RNAs

The short RNA targets used for thermal melt and hydrolysis analysis, **RNA-1**, **RNA-2**, **RNA-3**, and **RNA-4**, whose sequences are shown in Table 1 and Figure 1, were synthesized using protected ribonucleoside-3'-O-(β -cyanoethyl)phosphoramidites and standard automated procedures on an ABI DNA/RNA synthesizer model 392 or 3400. The coupling time was 450 sec and 5-ethylthiotetrazole was used as the activating agent. The oligoribonucleotides were removed from their supports by incubating the supports with 0.4 mL of a solution containing 95% ethanol/concentrated ammonium hydroxide (1:3 v/v) for 24 h at room temperature, and then for 3 h at 55°C. The 2'-O-t-butyldimethylsilyl (TBS) protecting groups were removed by treating the partially deprotected oligonucleotides with 150 μ L of triethylamine trihydrofluoride for 24 h at room temperature. The RNA was precipitated by the addition of 400 μ L dry methanol followed by incubation on ice for 15 min. The precipitate was recovered by centrifugation, washed twice with 200 μ L of dry methanol, and dried under vacuum. Deprotected RNA was then redissolved in 50% formamide, 10 mM EDTA gel loading buffer and purified by electrophoresis on a 20%

denaturing polyacrylamide gel. The full-length RNA was located by UV-shadowing and extracted from the gel by incubation with 1 mL of 0.1 M ammonium acetate, pH 6.5, 20% aqueous acetonitrile, and 10 mM EDTA overnight at 37°C. The RNAs were then desalted on C-18 SEP PAK cartridges. RNA concentrations were determined based on their UV absorbance at 260 nm. The purified RNAs were analyzed by MALDI-TOF mass spectrometry: **RNA-1**, m/z calcd: 5807.75, obsvd: 5810.40; **RNA-2**, m/z calcd: 6136.79, obsvd: 6137.32. **RNA-3**, m/z calcd: 6465.84 obsvd: 6468.60; **RNA-4**, m/z calcd: 6794.89, obsvd: 6795.75.

Syntheses and Purification of Oligo-2'-O-methylribonucleotides

The oligo-2'-O-methylribonucleotides **oligo-1** and **oligo-2**, whose sequences are shown in Table 1 and Figure 1A, were synthesized on an ABI model 392 or 3400 DNA/RNA synthesizer. The coupling time was 120 sec. and 5-ethylthiotetrazole was used as the activating agent. The oligonucleotides were deprotected by incubating the supports with 0.4 mL of a solution containing 95% ethanol/concentrated ammonium hydroxide (1:3 v/v) for 3.5 h at 55 °C. The supernatant was recovered and after evaporation, the residue was redissolved in 50% acetonitrile/water. The oligonucleotides were then purified by SAX HPLC using linear gradients of 0–0.5 M sodium chloride and desalted on C18 SEP PAK cartridges (Waters Corporation). The purified oligonucleotides were analyzed by MALDI-TOF mass spectrometry: **oligo-1**, m/z calcd: 4294.95, obsvd: 4294.37; **oligo-2** m/z calcd: 4255.54 obsvd: 4257.53.

Syntheses of Imidazole Conjugated Oligonucleotides **oligo-3**, **oligo-4**, **oligo-5** and **oligo-6**

The sequences of the imidazole conjugated oligonucleotides are shown in Table 1 and Figure 5A, and the synthetic scheme is shown in Figure 4. Syntheses were carried out on controlled pore glass supports using 2'-O-methylribonucleoside phosphoramidites up to the position of the internally derivatized nucleotide, at which point a ribonucleoside phosphoramidite with 5'-dimethoxytrityl (DMT) and 2'-*t*-butyldimethylsilyl (TBS) protecting groups was then coupled to this oligonucleotide to give **4.1**. Several mg of support were deprotected and the product, **4.1**, was examined by C-18 reversed phase HPLC (Figure S2A). The synthesis was paused, leaving the 5' dimethoxytrityl protecting group on the oligonucleotide. The synthesis column was washed with 2 × 10 mL portions of dry 100% acetonitrile and dried under vacuum. The cyanoethyl phosphate protecting groups were removed by treating the support with 1 mL of dry triethylamine overnight at room temperature. The support was then washed with 2 × 10 mL portions of dry acetonitrile. In order to remove the 2'-TBS protecting group from the ribonucleotide, the support was first washed with 2 mL of dry tetrahydrofuran. The support was then washed with 2 mL of a 1 M solution of tetra-*n*-butylammonium fluoride in tetrahydrofuran over a period of 15 minutes to give oligonucleotide **4.2**. The support was then washed with 2 mL of tetrahydrofuran THF, followed by 2 × 10 mL portions of dry acetonitrile and then dried under vacuum. Several mg of support were removed from the synthesis column and deprotected in order to ensure removal of the TBS group which was monitored by C-18 reversed phase HPLC (Figure S2 B).

The remaining support was treated with 1 mL of a solution containing 0.5 M carbonyldiimidazole dissolved in dry acetonitrile for 3.5 hours at room temperature with periodic mixing to yield the imidazole carbamate derivative **4.3**. Excess CDI was removed by washing the support with 2 × 10 mL portions of dry acetonitrile. The support was dried under vacuum and then transferred to a 4 mL glass autosampler vial. The support was treated with 300 µL of 0.33 M triethylamine and 0.2 M histamine in DMSO for 18 hrs with slow shaking to yield the desired 2'-carbamate, **4.4**. After which it was washed with 2 × 10 mL portions of acetonitrile, followed by 2 × 10 mL portions of 50% aqueous acetonitrile and

dried under vacuum. A 2 mg portion of the support was removed; the protecting groups were removed from the oligonucleotide; and the sample subjected to C-18 reversed phase HPLC to verify that the conjugation reactions had occurred. The remaining support was returned to the synthesis column. The imidazole group was first protected by acetylation on the DNA synthesizer after which oligonucleotide synthesis was continued with removal of the 5'-terminal DMT group to give oligonucleotide **4.5**.

Upon completing the synthesis of the oligonucleotide, the support was washed with 2×10 mL portions of acetonitrile, followed by 2×10 mL portions of 50% aqueous acetonitrile and then dried under vacuum. A 2 mg portion of the support was removed; the oligonucleotide deprotected; and the product analyzed by SAX HPLC to verify that synthesis was complete (Figure S2C). The remaining support was treated with 1 mL of a solution containing 0.5 M carbonyldiimidazole dissolved in dry acetonitrile for 3.5 hours at room temperature with periodic mixing to give imidazole carbamate derivative **4.6**. Excess CDI was removed by washing with 2×10 mL portions of dry acetonitrile. The remaining 20 mg of support was divided into two 10 mg portions. The 5'-end of one portion was derivatized with histamine as described above to give 2', 5'-bis-histamine conjugate **4.7**. The other portion was treated with 1 mL of a solution of 0.3 M 1,4-diaminobutane in acetonitrile for 30 min with periodic mixing to give the 2'-histamine-5'-aminobutyl conjugate **4.8**. Both supports were then washed with 2×10 mL portions of acetonitrile, followed by 2×10 mL portions of 50% aqueous acetonitrile and then dried under vacuum.

The oligonucleotide conjugates were deprotected by incubating the supports with 0.4 mL of a solution containing 95% ethanol/concentrated ammonium hydroxide (1:3 v/v) for 3.5 h at 55 °C. HPLC analyses of the crude oligonucleotides showed that derivatization of their 5' ends were essentially quantitative as shown, for example, by the chromatogram of **oligo-6** in Figure S2D. The oligonucleotides were purified by SAX HPLC using linear gradients of 0–0.5 M sodium chloride and desalted on C18 SEP PAK cartridges (Waters Corporation). The purified oligonucleotides were analyzed by MALDI-TOF mass spectrometry: **oligo-3**, m/z calcd: 4555.0 obsvd: 4554.73; **oligo-5**, m/z calcd: 4532.12 obsvd: 4531.62; **oligo-4**, m/z calcd: 4555.0 obsvd: 4556.80; **oligo-6**, m/z calcd: 4532.12 obsvd: 4531.90. The yields of the purified conjugates were (calculated based on yield of underivatized oligonucleotide): **oligo-3**, 18%; **oligo-4**, 19%; **oligo-5**, 14%; **oligo-6** 19%.

Synthesis of Imidazole-Conjugated Oligonucleotide **oligo-7**

The sequence of the oligonucleotide is shown in Table 1 and Figure 7, and the synthetic scheme is shown in Figure 6. The fully protected oligo-2'-O-methylribonucleotide containing a single 2'-O-TBS protected cytidine at position 8 was synthesized on a polystyrene support. Following synthesis, the 5'-terminal DMT protecting group was removed and the 5'-hydroxyl acetylated by reaction with acetic anhydride to give **7.1**. The support was washed with 2×10 mL of acetonitrile and dried under vacuum. A 2 mg sample of the support was deprotected and the product analyzed by SAX HPLC to ensure that the synthesis had gone to completion. The cyanoethyl phosphate protecting groups were removed from the oligonucleotide by treating the support with 1 mL of dry triethylamine overnight at room temperature. The support was washed with 2×10 mL portions of dry acetonitrile and dried under vacuum. The 2'-O-TBS protecting group was then removed to yield **7.2** by treating the support with 2×1.0 mL portions of triethylamine trihydrofluoride (TEA:3HF) for 90 min each at room temperature with periodic mixing. The support was again washed with acetonitrile and dried under vacuum. TEA:3HF reagent is more reactive than TBAF^[33], the reagent used to deprotect the imidazole conjugated oligonucleotides described above. A 2 mg sample of support was deprotected and the product analyzed by C-18 reversed phase HPLC which showed that approximately 70% of the TBS group was removed by this procedure (Figure S3A). The remaining support was then treated with 1 mL

of a solution containing 0.5 M carbonyldiimidazole in dry acetonitrile for 3.5 hours at room temperature with periodic mixing to give imidazole carbamate derivative **7.3**. Excess CDI was removed by washing with 2×10 mL portions of dry acetonitrile. The support was treated with 1.0 mL of a solution containing 0.3 M tris(2-aminoethyl)-amine in acetonitrile for 30 min with constant mixing to give **7.4**. Excess tris(2-aminoethyl)-amine was removed by washing the support with 2×10 mL portions of acetonitrile. A 2 mg sample of the support was deprotected and the product analyzed by SAX HPLC (Figure S3B) and MALDI-TOF mass spectrometry to confirm that conjugation occurred. The remaining support was treated with a solution containing 1 mL of 0.5 M carbonyldiimidazole in dry acetonitrile for 3.5 hours at room temperature with periodic mixing to yield the bis-imidazole carbamate derivative **7.5**. The support was washed with acetonitrile, dried under vacuum and transferred to a glass autosampler vial. The support was then treated with 300 μ L of a solution containing 0.33 M triethylamine and 0.4 M histamine in DMSO for 18 hrs on a bench top shaker to give the desired bis-histamine conjugate **7.6**. Following this treatment the support was transferred to the synthesis column, washed sequentially with 2×10 mL portions of acetonitrile and by 2×10 mL portions of 50% aqueous acetonitrile, and dried under vacuum. The conjugated oligonucleotide was deprotected by treating the support with 0.4 mL of a solution containing of 95% ethanol/concentrated ammonium hydroxide (1:3 v/v) for 3.5 h at 55 °C. The supernatant was recovered and after evaporation, the residue was redissolved in 50% aqueous acetonitrile. The conjugate is then purified by SAX HPLC (Figure S3C) using a linear gradient of 0–0.5 M sodium chloride and further purified by C18 reversed phase HPLC using a non-linear gradient of 2–10% acetonitrile for 5 min followed by a gradient of 10–15% acetonitrile from 5–20 min. The resulting pure oligonucleotide was desalted using a C18 SEP PAK cartridge and analyzed by MALDI-TOF mass spectrometry: **oligo-7**, m/z calcd: 4354.03, obsvd: 4355.03. The yield of purified conjugate was 25 %.

Thermal Denaturation Experiments

Oligonucleotides were annealed to their complementary RNA targets by heating a 1 mL solution containing 1 μ M of each strand in 50 mM MOPS [3-(N-morpholino) propane sulfonic acid], pH 7.0, 100 mM sodium chloride and 0.5 mM EDTA at 65°C for 15 min followed by slow cooling to room temperature. Thermal denaturation experiments were carried out using a thermostat-controlled Cary 3 E UV-visible spectrophotometer (Varian). The absorbance was monitored at 260 nm as the sample was heated at a rate of 0.4°C per min over the temperature range 20 °C to 90 °C. Data were collected at 0.25°C intervals and analyzed with Sigma Plot. Melting temperatures (T_m) were determined from smoothed first derivative data sets obtained using Sigma Plot's cubespline #1 and #2 transformations.

Assay for Measuring the Susceptibility of Bulged Bases to Imidazole-Mediated Hydrolysis

The RNA target strands were 5'-[32 P]-labeled enzymatically using T4 polynucleotide kinase and 32 P- γ -ATP (100 Ci/mmol). Excess 32 P- γ -ATP was removed using a Sephadex G25 spin column (Amersham Biosciences). RNA and oligonucleotide stock solutions were each prepared at 2 X concentration in buffer containing 1.0 M imidazole and 0.5 mM EDTA at pH 7.0. Equal volumes of each stock solution were then mixed to give solutions containing 10 μ M oligonucleotide and 100 nM RNA. The solutions were incubated at 37 °C and 5 μ L aliquots were removed at specified times. The reactions were stopped by adding 5 μ L of gel loading buffer (90 % formamide, 0.3% xylene cyanol and bromophenol blue, 10 mM EDTA) and the solutions were placed on ice. The reaction products were analyzed by gel electrophoresis on a 17% denaturing polyacrylamide sequencing gel. The gels were dried and the dried gels exposed to a phosphorimaging plate.

Assay for Oligonucleotide Conjugate Mediated Hydrolysis of RNA

The RNA target strands were 5'-[³²P]-labeled enzymatically using T4 polynucleotide kinase and γ -³²P-ATP (100 Ci/mmol). Excess γ -³²P-ATP was removed using a Sephadex G25 spin column. RNA and oligonucleotide conjugate stock solutions were each prepared at 2X concentration in buffer containing 50 mM MOPS, 100 mM sodium chloride, and 0.5 mM EDTA. Equal volumes of the RNA and conjugate solutions were mixed to give 5–10 μ L of reaction mixture that contained 100 nM 5'-[³²P]-labeled RNA and 10 μ M conjugate unless otherwise specified. The reaction mixtures were incubated at 37 °C and the reactions stopped by addition of 10 μ L of formamide gel loading buffer. The reaction products were then analyzed by gel electrophoresis on a 17% denaturing polyacrylamide sequencing gel. Sodium bicarbonate and RNase T₁ partial digests of the target RNA were prepared and run on the gel as markers to identify hydrolysis products. The gels were dried and the dried gels were exposed to a phosphorimaging plate. Hydrolysis products were quantitated using Multigauge version 3.01 imaging software.

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Figure 1.

RNA-N/oligo-2'-O-methylribonucleotide hybrid duplexes used to test susceptibility of bulged RNA bases to imidazole hydrolysis (Panel A). UV thermal denaturation profile of **RNA-2/Oligo-1** (Panel B). The melting temperatures of the hybrid duplexes are shown in the table.

Figure 2.

Susceptibility of bulged nucleotides to hydrolysis in imidazole buffer. Duplexes formed between 100 nM 5'-[32 P]-labeled **RNA-1** and 10 μ M **oligo-1** (Panel A) or **oligo-2** (Panel B) were incubated at 37°C in buffer containing 1.0 M imidazole, pH 7.0 and 0.5 mM EDTA for the times indicated. The reactions were analyzed on a 20% denaturing polyacrylamide gel.

Figure 3.

Molecular models showing the position of the cleaving moieties relative to the bulged nucleotides when these groups are attached to the 5'-end and an internal position in the oligonucleotide (Panel A) or when both groups are attached at internal position in the oligonucleotide (Panel B). The models were generated using Hyperchem 8.0 modeling software.

Figure 4.

Synthesis of imidazole-conjugated oligo-2'-O-methylribose nucleotides. Abbreviations: control pore glass (CPG); 2'-*t*-butyldimethylsilyl (TBS); tetra-*n*-butylammonium fluoride (TBAF); dimethoxytrityl (DMT); carbonyldiimidazole (CDI); 1,4-diaminobutane (DAB).

Figure 5.

Hydrolysis of **RNA-1** by imidazole-conjugated oligonucleotides. The structures of the hybrids formed between **RNA-1** and the imidazole-conjugated oligonucleotides are shown in Panel A. A phosphorimage of the gel used to analyze reactions between 10 μ M **oligo-3**, or **oligo-5** and 100 nM **RNA-1** incubated in buffer containing 50mM MOPS pH 7.5, 100 mM sodium chloride, 0.5 mM EDTA for 24 hrs is shown in Panel B. Lane B: **RNA-1** target partially digested with sodium bicarbonate at 90 °C. Lane C: **RNA-1** incubated in pH 7.5 buffer for 24 hrs. Note: All lanes were run on the same gel. Irrelevant lanes were deleted for clarity.

Figure 6.

Synthesis of **oligo-7**. Abbreviations: polystyrene (PS); 2'-*t*-butyldimethylsilyl (TBS); dimethoxytrityl (DMT); triethylamine trihydrofluoride (TEA:THF); carbonyldiimidazole (CDI); tris(2-aminoethyl)-amine (TAEA).

Figure 7.

Hydrolysis of RNA by **oligo-7**. Panel A shows the structure of the **oligo-7** and the duplexes formed with **RNA-1** or **RNA-2**. 10 μ M **Oligo-7** and 100 nM 5'-[32 P]-labeled **RNA-1** (Panel B) or **RNA-2** (Panel C) were incubated at 37 °C in buffer containing 50mM MOPS pH 7.5, 100 mM sodium chloride, 0.5 mM EDTA. Aliquots were withdrawn at the times indicated and subjected to PAGE on a 17% sequencing gel. Lane B: RNA target partially digested with sodium bicarbonate at 90 °C; Lane T₁: **RNA-2** target partially digested with RNase T₁; Lane C₁: **RNA-1** incubated in buffer for 96 hrs; Lane C₂: 10 μ M unconjugated **oligo-7** and 100 nM **RNA-1** incubated in buffer for 96 hrs; Lane C₃: **RNA-2** incubated in buffer for 96 hrs; Lane C₄: 10 μ M unconjugated **oligo-7** and 100 nM **RNA-2** incubated in buffer for 96 hrs.

Table 1

Oligonucleotide and conjugate sequence and mass spec validation

Oligonucleotide	Sequence	Calculated Mass (m/z)	Observed Mass (m/z)
RNA-1-4	5' UGGGCGC(A) _n GCGUCAAUGA 3'	--	See experimental
Oligo-1	5' UGACGGCGGCCCA 3'	4294.95	4294.37
Oligo-2	5' UUGACGGCGGCCCA 3'	4255.54	4257.53
Conjugates			
Oligo-3	5' UGACGGCG(rC)GCCCA 3'	4555.0	4554.73
Oligo-4	5' UGACGC(rG)CGGCCCA 3'	4555.0	4556.80
Oligo-5	5' UGACGGCG(rC)GCCCA 3'	4532.12	4531.62
Oligo-6	5' UGACGC(rG)CGGCCCA 3'	4532.12	4531.90
Oligo-7	5' UGACGGCG(rC)GCCCC 3'	4354.03	4355.03