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Introduction of guanidinium-modified deoxyuridine into the substrate binding regions of DNAzyme 10–23 to enhance target affinity: Implications for DNAzyme design

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ABSTRACT

Deoxyribozymes (DNAzymes) are important catalysts for potential therapeutic RNA destruction and no DNAzyme has received as much notoriety in terms of therapeutic use as the Mg^{2+}-dependent RNA-cleaving DNAzyme 10–23 (Dz10–23). As such, we have investigated the synthetic modification of Dz10–23 with a guanidinium group, a functionality that reduces the anionic nature and can potentially enhance the membrane permeability of oligonucleotides. To accomplish this, we synthesized a heretofore unknown phosphoramidite, 5-(*N*,*N*′-biscyanoethoxycarbonyl)-guanidinoallyl-2′-deoxyuridine and then incorporated it into oligonucleotides via solid phase synthesis to study duplex stability and its effect on Dz10–23. This particular modification was chosen as it had been used in the selection of Mg^{2+}-free self-cleaving DNAzymes; as such this will enable the eventual comparison of modified DNAzymes that do or do not depend on Mg^{2+} for catalysis. Consistent with antecedent studies that have incorporated guanidinium groups into DNA oligonucleotides, this guanidinium-modified deoxyuridine enhanced the thermal stability of resulting duplexes. Surprisingly however, Dz10–23, when synthesized with modified residues in the substrate binding regions, was found to be somewhat less active than its non-modified counterpart. This work suggests that this particular system exhibits uniform binding with respect to ground state and transition state and provides insight into the challenge of re-engineering a Mg^{2+}-dependent DNAzyme with enhanced catalytic activity.

Oligonucleotides represent a group of therapeutic agents that are being explored (1) as aptamers that target many different proteins, (2) as antisense, siRNA, ribozyme, and DNAzyme motifs that can regulate specific mRNA levels, and (3) as triplex forming oligonucleotides that inhibit mRNA transcription.[1–5] In addition, numerous chemical approaches have been used to (a) increase target affinity, (b) enhance stability against circulating nucleases, (c) improve cellular uptake, and in some cases, (d) improve catalytic activity through the addition of extra functionalities.[6–8] A privileged functionality that has the potential to promote all of these goals is the guanidinium cation. Indeed, this functionality enhances the membrane permeability of oligonucleotides by reducing the overall anionic nature of them[9] and is thought to confer the same functions as arginine residues that are found in many arginine-rich cell penetrating peptides.[10] Hence, guanidinium groups have been incorporated into oligonucleotides at select positions on the sugar,[11] base,[9,12] and phosphate regions[13] of oligonucleotides. Further highlighting the utility of the guanidinium functionality, oligonucleotides with guanidinium groups attached to the nucleobase have been successfully used in the in vitro selection of (1) aptamers that recognize glutamate[14] and (2) Mg^{2+}-*independent* DNAzymes with high catalytic activity.[15,16] In the former case, aptamers exhibit enhanced affinity for the ground state while in the latter case, guanidinium-modified dUs, which are essential for activity, likely provide for both ground state and transition state stabilization in the total absence of Mg^{2+}.

DNAzymes offer potential therapeutic action for anti-mRNA targeting,[17–19] and towards these ends, no DNAzyme has received as much notoriety in terms of potential therapeutic use as has the Mg^{2+}-dependent RNA-cleaving DNAzyme 10–23 (Dz10–23).[20] Dz10–23 is a typical DNAzyme that is composed of a catalytic motif, which is flanked by two substrate binding regions. With the exception of the dinucleotide that undergoes transphosphorylative scission, the catalytic loop does not appear to interact with the extended substrate sequence. Hence, the composition of the guide arms can be varied to specify targeted cleavage of any given sequence of RNA. Indeed, this noteworthy versatility accounts for broad academic and applied interests directed toward using Dz10–23 to target *any* mRNA sequence.[17,21] When the flanking guide arms are increased to lengths ranging from 9 to 11 bases, higher target affinity and specificity are achieved. However, further

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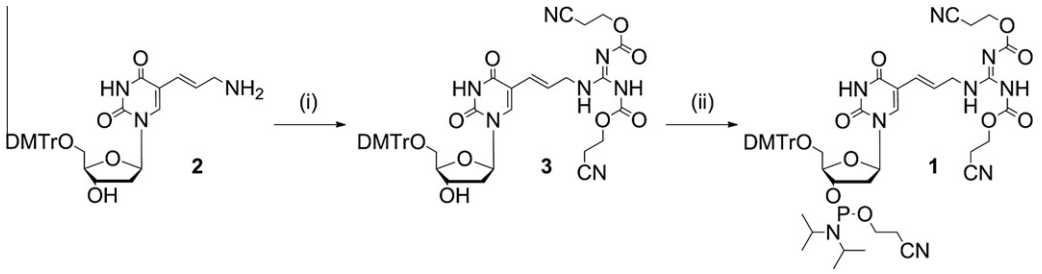

Scheme 1. Synthesis of **1**. Reagents and conditions: (i) *N*,*N*′-bis-(2-cyanoethoxycarbonyloxy)-2-methyl-2-thiopseudourea, Et_3N, DMF, 89%; (ii) 2-cyanoethyl *N*,*N*′-diisopropylchlorophosphoramidite, diisopropylethylamine, CH_2Cl_2, 67%.

increases in guide arm length do not appear to enhance k_{cat}/K_m values, and increased length may accommodate mismatches as well as result in undesired product inhibition.

Because of the alleged potential of Dz10–23 for targeting many different genes, chemical modification in the guide arms has been investigated with regards to enhancing target recognition. For example, Vester et al.[22] have incorporated locked nucleic acids (LNA), residues that also increase helical thermostability, into the binding arms of Dz10–23 and found that resulting catalysts became more active compared to unmodified counterparts. Other examples have employed 2′-OMe sugars, phosphorothioates[23] as well as appended intercalators.[24] Although none of these modifications address the need for overcoming the high levels of Mg^{2+} needed for optimal activity, a limitation that now appears to undermine DNAzyme utility in cells, they reflect an ongoing medicinal interest in improving DNAzyme action through synthetic modification. Towards this end, our interest in guanidinium-modified DNAzymes led us to test the effect of this important functionality in the context of the Mg^{2+}-dependent Dz10–23. Herein, we address two properties of guanidinium groups: their effect on target affinity and on catalysis by Dz10–23. We (1) synthesized and characterized a modified DNA phosphoramidite (**1**) (Scheme 1) containing an arginine group that is tethered from the 5-position of 2′-deoxyuridine, (2) studied its effect on oligonucleotide duplex structures, and (3) performed kinetic experiments on the Dz10–23 that was constructed with substrate recognition domains utilizing this new phosphoramidite and targeting hepatitis C virus (HCV) RNA.

Our target molecule is the phosphoramidite of a modified nucleoside triphosphate that we successfully used in two separate in vitro selection experiments that led to fully Mg^{2+}-independent self-cleaving DNAzymes. The modification is attached to the 5-position such that the sugar–phosphate backbone and the standard Watson–Crick base pairing remain unaffected in order to facilitate the enzymatic incorporation of modified nucleoside triphosphates to construct modified nucleic acid libraries. Previous studies of guanidinium-modified oligonucleotides have demonstrated that the guanidinium group adds stability to duplex structures,[11,12] and hence we hypothesized that our modification would enhance substrate affinity and thereby possibly facilitate catalysis by promoting tighter substrate association, particularly at low substrate concentrations and at physiological concentrations of Mg^{2+} (~0.5 mM)[25–28] where Dz10–23 is not especially active.[29]

As shown in Scheme 1, the phosphoramidite **1** was synthesized following the protocols that have been used by Prakash et al. to guanidinylate a 2′-aminoethyl- and 2′-aminohexylribose. The nucleoside 5′-dimethoxytrityl-5-(3-amino-1-propenyl)-2′-deoxyuridine (**2**) was coupled to *N*,*N*′-bis-(2-cyanoethoxycarbonyloxy)-2-methyl-2-thiopseudourea[30,31] in the presence of triethylamine and DMF. The resulting product (**3**) was then phosphitylated in diisopropylamine and CH_2Cl_2. This phosphoramidite was successfully incorporated into oligonucleotides using standard solid phase coupling with a slightly modified deprotection method that called for a 24 h treatment of the protected product with piperidine to first remove the β-cyanoethoxycarbonyl groups.[31]

The duplex stabilities of the oligonucleotides shown in Table 1 are summarized in Table 2. The modified DNA oligonucleotides **O2** and **O3** were hybridized to either complementary RNA **O4** or DNA **O5** and analyzed by monitoring their temperature-dependent absorbance at 260 nm. In all cases, the presence of modified residues resulted in more stabilized duplexes. The melting temperatures were increased by 2.3 and 2.5 °C per modified nucleotide for DNA/RNA and DNA/DNA duplexes, respectively. These results were consistent with studies that have been done with similarly modified oligonucleotides.[12]

In order to test the base pairing specificity of our modified residue, two DNA oligonucleotides **O6** and **O7**, which contain a C and G, respectively, in place of the A that base pairs with the guanidinium containing deoxyuridine, were hybridized with **O2** as well. As expected, both mismatches significantly lowered the melting temperatures. Comparing the results for the mismatched duplexes containing the unmodified and modified residues, it was found that the destabilization of a mismatch was more pronounced ($\Delta T_m \sim 1$ °C) in the case of modified residues. Based on these observations, it would seem that our modified nucleotide retains proper Watson and Crick base pairing interactions because once these base pairing interactions are removed, the stabilizing effect of the modification is also reduced. Hence, the modification on d*T* effectively increases the base pairing specificity with d*A*, at least for the specific site tested.

Kinetic studies on DNAzymes containing 2, 4 or 6 guanidinium modifications were performed using the sequences shown in Figure 1. Although the catalytic motif of Dz10–23 can be inserted between guide arms that allow targeting of any sequence, **O9** represents a variant of Dz10–23 that was previously shown to cleave HCV RNA spanning the 5′-untranslated region-core genomic position 1–976 in vitro, and a similar but more active variant was found to reduce the amount of HCV RNA in vivo as well.[32] Thus, we sought to modify this catalyst based on previous reports of its in vitro activity against a sequence contained within the HCV genome that results in cleavage of the phosphodiester bond at position

Table 1
Oligonucleotides used for melting temperature studies[a]

O1	d-TTC TTT TTC TTC TCT TT
O2	d-TTC TTT TTC TTC TCT MT
O3	d-TTC TTM TTC TTC TCT MT
O4	r-CUC AAA GAG AAG AAA AAG AAC U
O5	d-CTC AAA GAG AAG AAA AAG AAC T
O6	d-CTC ACA GAG AAG AAA AAG AAC T
O7	d-CTC AGA GAG AAG AAA AAG AAC T

[a] Guanidinium containing nucleotides are indicated with M.