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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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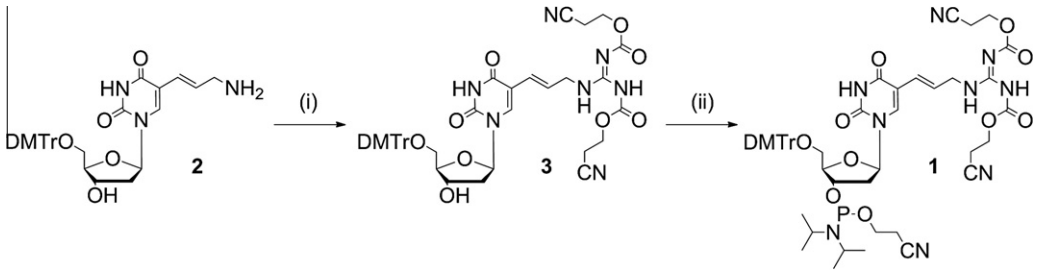

Scheme 1. Synthesis of **1**. Reagents and conditions: (i) *N*,*N*′-bis-(2-cyanoethoxycarbonyloxy)-2-methyl-2-thiopseudourea, Et_3N, DMF, 89%; (ii) 2-cyanoethyl *N*,*N*′-diisopropylchlorophosphoramidite, diisopropylethylamine, CH_2Cl_2, 67%.

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.

Table 2
Melting temperatures for duplexes[a]

	O4		O5		O6		O7	
	T_m (°C)	ΔT_m (°C)	T_m (°C)	ΔT_m (°C)	T_m (°C)	ΔT_m (°C)	T_m (°C)	ΔT_m (°C)
O1	49.3		40.8		36.3		37.0	
O2	51.5	+2.2	43.3	+2.5	37.7	+1.4	38.4	+1.4
O3	53.9	+4.6	45.8	+5.0	N/A		N/A	

[a] Data measured in 100 mM NaCl, 10 mM sodium phosphate (pH 7.0) and 1 μM of each strand. Absorbance at 260 nm measured. Numerical data from four separate experiments of forward and reverse melting temperatures were averaged. N/A, T_m not measured.

699 as indicated by the arrow in the figure. Indeed, with this in mind, we constructed duly modified catalytic sequences of the same length with modified residues in either the 5′ substrate binding region, the 3′ substrate binding region, or both substrate binding regions.

Multiple turnover kinetic experiments, as shown in Figure 2, were carried out to study the effect of the modifications on catalysis. Under physiological conditions (e.g., 0.5 mM Mg^{2+}, 150 mM NaCl, pH 7.4, 37 °C), reactions were carried out at two different concentrations of both DNAzyme and excess 19 ribonucleotide substrate: 5 nM and 50 nM or 250 nM and 5 μM, respectively. A

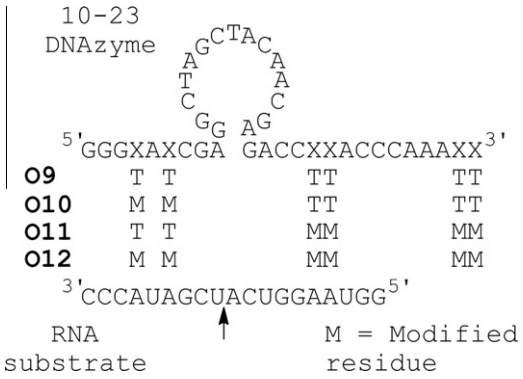

Figure 1. Sequences of Dz10–23 and its RNA substrate. The site of cleavage is noted with an arrow.

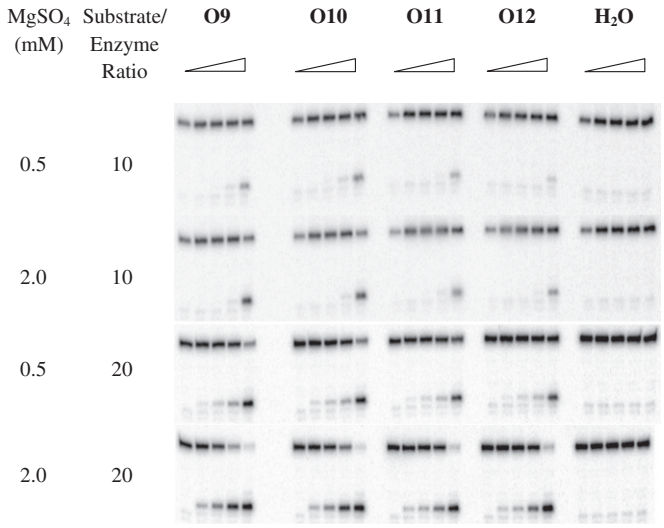

Figure 2. Denaturing PAGE (20%) of Dz10–23 cleavage reactions. Four DNAzymes and a H_2O control were incubated with RNA substrate under two concentrations of $MgSO_4$, 0.5 mM and 2 mM. Final concentrations of the catalyst and substrate were either 5 nM and 50 nM or 0.25 μM and 5 μM, respectively. Kinetic reactions were performed in 150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 37 °C. Aliquots were removed and quenched at 0, 0.5, 1.5, 4.5, 25 h with increasing time indicated by the triangle above each set of lanes.

substrate concentration of 5 μM reflects saturating conditions, while a substrate concentration of 50 nM represents conditions under which substrate binding may limit catalysis, and whereby the effect of modifications in the modified catalysts that lower the dissociation rate could afford apparent catalytic enhancement. The observed rate constants were calculated and are reported in Table 3. Compared to the unmodified sequence **O9**, all three modified sequences were found to be slightly less active. Nevertheless the guanidinium groups are well-tolerated in the 5′ substrate binding region as the rates observed for **O10** were only slightly lowered. However, modifications in the 3′ binding arm are tolerated to a lesser extent. The rates of cleavage were further reduced when both substrate binding regions contained modified residues.

Several other kinetic experiments were also conducted (see Supplementary data). These included two sets of kinetic studies involving the use of substrates of different lengths. A shorter, 17 nucleotide RNA substrate was cleaved much less effectively by both unmodified and modified catalysts, while a longer 25 nucleotide RNA substrate, designed to hybridize to the entire substrate binding region of the catalysts was also cleaved at somewhat reduced rates. The shorter substrate was not long enough to form catalyst–substrate complexes of sufficient stability whereas the cleavage of the longer substrate was limited by product release inhibition as seen in previous reports.[29] These results confirmed that the 19 nucleotide RNA substrate used in this study was not too long or too short. Finally, the analysis of catalysis under low substrate conditions was taken one step further. The substrate concentration was halved to 25 nM and the concentration of Mg^{2+} was varied from a physiologically relevant value of 0.5 mM[25–28] to a non-physiological value of 10.0 mM. Under these conditions, none of the modified catalysts was superior to the unmodified control.

In addition to the aforementioned kinetic studies, melting temperature measurements of the dissociation of various Dz10–23 species with the 19 nucleotide RNA substrate were also performed (see Supplementary data). These were done in the absence and presence of 0.5 mM Mg^{2+} (substrate cleavage is negligible over the course of the T_m assay). DNAzyme **O10**/substrate complexes were slightly less stable (~0.5 °C) than the unmodified control; however, as expected, substrate complexes with the more heavily

Table 3
DNAzyme observed rate constants (min^{-1}) from multiple turnover experiments[a,b]

Mg^{2+} (mM)	0.5		2.0	
Substrate/enzyme ratio	10	20	10	20
O9	1.5×10^{-3}	1.1×10^{-2}	5.5×10^{-3}	5.2×10^{-2}
O10	1.5×10^{-3}	9.4×10^{-3}	4.0×10^{-3}	3.9×10^{-2}
O11	6.7×10^{-4}	7.5×10^{-3}	2.0×10^{-3}	2.1×10^{-2}
O12	2.6×10^{-4c}	3.6×10^{-3}	1.1×10^{-3}	1.6×10^{-2}

[a] Reaction conditions: 150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 37 °C. Final concentrations of the catalyst and substrate were either 5 nM and 50 nM or 0.25 μM and 5 μM, respectively.

[b] Rate constants were obtained by first dividing the ratio of cleaved product at certain time points of the reaction by time and then multiplying by the substrate to catalyst ratio.

[c] This value was obtained as an average of two experiments, and all other values were averages of three experiments performed on different days.

modified DNAzymes **O11** and **O12** were substantially more stable (5–8 °C). This suggests that the guanidinium ion greatly enhances DNAzyme target affinity at low Mg^{2+} concentrations.

Nevertheless, the same interactions did not provide catalytic enhancement. Such may be explained by the following logic: although this modification minimizes the need for Mg^{2+} in terms of substrate recognition, as evidenced by the higher T_m values, it did not alleviate the need for Mg^{2+} in terms of catalysis. The guanidinium-modified DNAzymes herein were less active at all concentrations of Mg^{2+} and substrate tested (saturating and subsaturating); observed rate constants fell by approximately a factor of two in accord with increasing modification. This small change suggests generally uniform binding between the ground state and transition state such that $\Delta\Delta G^{\ddagger}$ remains largely unchanged. To the extent that observed k_{cat} values were modestly reduced by a factor of two or less, is consistent with a near-negligible energetic preference for the binding of ground state over the transition state (<1 kcal/mol). While it is also possible that the modified Dz10–23 recognizes the ground state in a different conformation and then undergoes a conformational change en-route to the transition state, the T_m values obtained from the substrates suggest this is not the case.

In conclusion, a new phosphoramidite of a guanidinium containing 2′-deoxyuridine was synthesized and characterized. It was successfully incorporated into oligonucleotides by standard solid phase techniques and was found to enhance the stability of DNA/DNA and DNA/RNA duplexes. Although the presence of these modifications in the substrate binding regions of Dz10–23 did not enhance catalytic activity, all modified species were found to be catalytically active and such results now demonstrate that Dz10–23 variants can be selectively modified with groups that are known to enhance cellular uptake and thereby manifest enhanced effects in cells. Inasmuch as the catalytic properties of Dz10–23 are shared by other Mg^{2+}-dependent DNAzymes, this work underscores the difficulty of incorporating modified nucleotides into the guide arms to improve Mg^{2+}-dependent activity; clearly, Mg^{2+} must bind the catalytic loop to activate catalysis,[33] a critical requirement, that is, apparently not satisfied by the cellular milieu.[34] Because the catalytic loop of Dz10–23 is somewhat refractory to post-synthetic modification,[35] we did not investigate the introduction of guanidinium-dU into this region, and thus cannot know for certain if its presence in the catalytic loop could have alleviated the need for Mg^{2+} for catalysis. While incorporating modified nucleosides into DNAzymes post-selection may prove promising, a more effective approach for alleviating the need of Mg^{2+} in catalysis is to select modified catalysts de novo,[36–39] especially in the case of DNAzymes that contain guanidinium ions, cationic amines, and imidazoles in the catalytic loop.[15,16]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.07.027.

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