Bioorganic & Medicinal Chemistry Letters 20 (2010) 4367–4370

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

10-23 DNAzyme modified with (2′*R*)- and (2′*S*)-2′-deoxy-2′-*C*-methyluridine in the catalytic core

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ARTICLE INFO

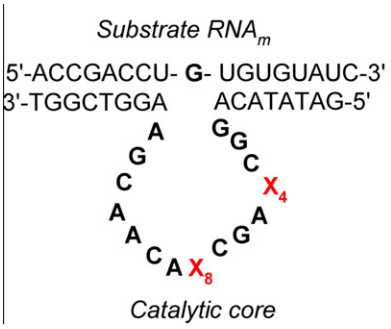
Article history:
Received 11 May 2010
Revised 9 June 2010
Accepted 11 June 2010
Available online 16 June 2010

Keywords:
10-23 DNAzyme
2′-Deoxy-2′-*C*-methyl nucleosides

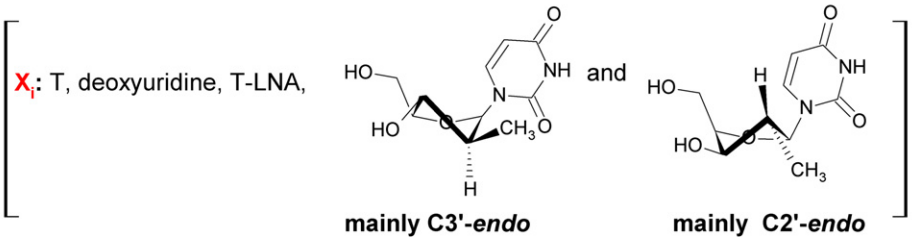
ABSTRACT

The catalytic core of a 10-23 DNAzyme was modified using (2′*R*), (2′*S*)-2′-deoxy-2′-*C*-methyluridine and LNA-T. Catalytic activities under *pseudo* first order conditions were compared at different Mg^{2+} concentrations, indicating that certain 2′-*C*-methyl modified DNAzymes have significant activities. Resistance against MCF-7 cell lysate and endonuclease RQ1 was also measured, showing that the introduction of 2′-*C*-methyl-2′-deoxynucleosides increased the stability.

RNA or DNA sequences can perform non conventional activities, like ribozymes,[1] DNAzymes,[2] aptamers[3] and more recently siRNA.[4] DNAzymes (or deoxyribozymes) are single stranded DNA molecules, selected by in vitro evolution, which are able to catalyze



Modification	DNAzyme	Catalytic core sequence
No modified	DZ-nm	5'-GGCTAGCTACAACGAT-3'
2'-*C*-methyl	DZ-4S	5'-GGCXAGCTACAACGAT-3'
	DZ-4R	5'-GGCXAGCTACAACGAT-3'
	DZ-8S	5'-GGCTAGCXACAACGAT-3'
	DZ-8R	5'-GGCTAGCXACAACGAT-3'
	DZ-4,8S	5'-GGCXAGCXACAACGAT-3'
	DZ-4,8R	5'-GGCXAGCXACAACGAT-3'
	DZ-4S,8R	5'-GGCXAGCXACAACGAT-3'
	DZ-4R,8S	5'-GGCXAGCXACAACGAT-3'
LNA	DZ-4LNA	5'-GGCXAGCTACAACGAT-3'
	DZ-8LNA	5'-GGCTAGCXACAACGAT-3'
	DZ-48LNA	5'-GGCXAGCXACAACGAT-3'



Scheme 1. Modified 10-23 DNAzymes prepared.

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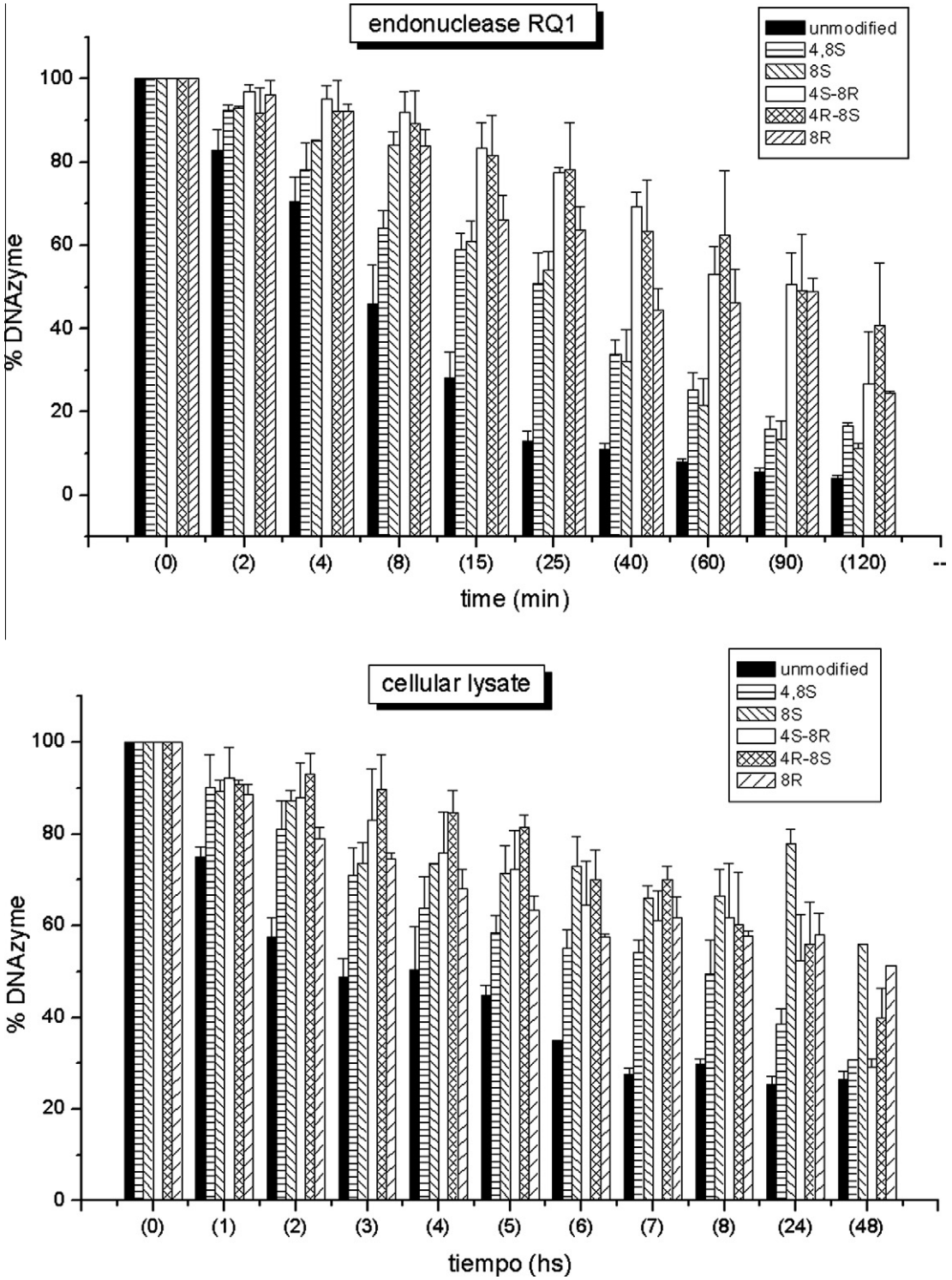
doi:10.1016/j.bmcl.2010.06.071

chemical transformations. In particular, the 10-23 DNAzyme possesses RNA hydrolytic activity and have been used to inhibit protein expression in cell cultures and in vivo applications.[5] The 10-23 DNAzyme consists in a cation-dependent catalytic core of 15 nucleotides and two recognition arms that bind to the target mRNA. In this molecule the conservation of the catalytic core sequence is important in order to keep the hydrolytic activity.[6] The active conformation, cleavage mechanism and three dimensional structure of the substrate:10-23 DNAzyme complex, remains unknown.[7] Spite these limitations, there are several modified deoxyribozymes that have been designed to increase the stability against degradation by nucleases. Most of them are placed in the recognition arms using inverted nucleotides,[8] phosphorothioates,[9] LNA analogs[10] or 2′-*O*-methyl nucleosides.[11]

On the other hand, there are only a few examples of chemical modifications in the 10-23 DNAzyme catalytic core, using for



Scheme 2. 10-23 DNAzyme stability in endonuclease and cellular lysate solutions.

example 2′-*O*-methyl[11] or phosphorothioate nucleotides.[12] Although numerous efforts have been performed to establish the role of each nucleotide in the catalytic core,[13] the conformational requirements of the sugar moiety has not been yet investigated. In this sense, the 2′-*C*-methyl-2′-deoxynucleosides[14] constitutes an interesting modified nucleoside class, because they show differential preferred sugar conformations depending on the absolute configuration at the 2′-carbon. The (2′*S*)-2′-*C*-methyl-2′-deoxynucleosides mainly adopt the C3′-*endo* conformation while those with (2′*R*) configuration prefer the C2′-*endo* conformation (Scheme 1).[15] In addition, oligonucleotides containing these nucleosides have shown enhanced nuclease resistance.[16]

We present in this work the evaluation of the kinetic activity, the nuclease resistance and the magnesium dependence of punctual and double mutated 10-23 DNAzymes with either (2′*S*)- or (2′*R*)-2′-*C*-methyl-2′-deoxyuridine. Finally, we also compare the activity of modified sequences carrying 2′-*C*-methyl-2′-deoxy or LNA nucleosides at the same positions.

In first place, (2′*R*)- and (2′*S*)-2′-*C*-methyl-2′-deoxyuridine phosphoramidites were prepared as previously reported.[14] A set of 10-23 DNAzymes directed against the +964 site of the estrogen receptor α mRNA (Scheme 1), was synthesized using conventional solid phase chemistry. This collection included DNAzymes carrying: unmodified nucleosides (DZ-nm, Scheme 1), 2′-deoxyuridine (data not shown) and 2′-*C*-methyl uridines (2′-*C*-methyl, Scheme 1) and LNA-T (LNA, Scheme 1) at the same positions.

The basic motivation was to establish if the conformational restriction at positions 4 and/or 8 of the catalytic core would have a positive effect on activity, by slightly modifying the three-dimensional structure to a nearer transition state form. With this idea in mind, the cleavage activity of unmodified and modified DNAzymes was determined under single turnover conditions and the kinetic parameters were calculated at different magnesium concentrations: 0.5 and 1 mM to emulate physiological Mg^{2+} concentrations and 5 and 10 mM for in vitro testing.

DNAzymes activity was assayed using the ^{32}P labelled short target RNA (17 nt, Scheme 1). The cleavage reactions were analyzed by PAGE and the results are presented in Table 1.

In preliminary experiments it was established that DNAzymes carrying 2′-deoxyuridine at 4- and/or 8-positions of the catalytic core have the same activity than the unmodified DNAzyme (data not shown). This result indicated that the 5-methyl groups of 4- and 8- thymidines have no relevant effect on activity. Then, 2′-*C*-methyl DNAzymes activity was tested at different Mg^{2+} concentrations, finding that the DZ-4S and DZ-4R were inactive. When these modifications were introduced at 8-position a differential effect was observed depending on 2′-*C*-configuration. At physiological Mg^{2+} concentrations DZ-8R was five to ten times less active than the control, while DZ-8S was almost as efficient as DZ-nm. The low activity showed by DZ-8R is unexpected, since it is known that 8-position of the catalytic core supports nucleoside modifications, even deletion.[6] At the same Mg^{2+} concentrations, double mutated DNAzymes DZ-4,8*S* and DZ-4*S*,8*R* kept 22–54% of DZ-nm activity, respectively (Table 1).

In order to assess the effect of another modified nucleoside sharing similar conformational preferences, LNA-T was introduced at 4- and/or 8-positions, finding no activity up to 1 mM Mg^{2+} at least. Preliminary molecular modelling studies carried out in our lab suggest that the sugar moiety of (2′*S*) 2′-*C*-methyl-2′-deoxynucleosides show certain degree of flexibility when it is present in a double stranded structure, On the contrary, LNA nucleosides contain a covalent linkage that locks their conformations. The results here obtained may be attributed to the requirement of a partially flexible sugar ring to allow the catalytic core to adopt an active conformation. In addition, the presence of the hydrophobic methyl group at 2′-position could also contribute to obtain a catalytically active core conformation.

To evaluate the stability against nucleolytic degradation, 2′-*C*-methyl-2′-deoxy modified DNAzymes were incubated in MCF-7 cellular lysate and with endonuclease RQ1 (Scheme 2). It can be observed that the introduction of 2′-*C*-methyl-2′-deoxynucleosides increased the stability under the aforementioned conditions in all cases.

To conclude, we have presented a group of modified 10-23 DNAzymes showing enhanced biological stability, being the most active DZ-8*S* and DZ-4*S*,8*R*. We have also shown that the 2′-*C*-methyl-2′-deoxyuridine modifications have different effects depending on the 2′-*C*-configuration. On the other hand, the mutations with the 3′-*endo* rigid LNA analogues have produced inactive DNAzymes under physiological magnesium concentrations.

Table 1
K_{obs} for modified DNAzymes at different Mg^{2+} concentrations

DZ-	K_{obs}/min^{-1}			
	0.5 mM Mg^{2+}	1.0 mM Mg^{2+}	5.0 mM Mg^{2+}	10.0 mM Mg^{2+}
nm	0.45 ± 0.03	0.74 ± 0.003	1.49 ± 0.16	2.38 ± 0.20
4*S*	NM[a]	ND[b]	ND	ND
4*R*	NM	ND	0.021 ± 0.003	0.071 ± 0.008
8*S*	0.50 ± 0.05	0.52 ± 0.03	0.84 ± 0.06	1.39 ± 0.14
8*R*	0.041 ± 0.003	0.15 ± 0.02	0.20 ± 0.02	0.21 ± 0.02
4,8*S*	0.101 ± 0.008	0.27 ± 0.03	0.34 ± 0.04	0.50 ± 0.05
4,8*R*	NM	ND	0.021 ± 0.004	0.051 ± 0.002
4*S*,8*R*	0.18 ± 0.01	0.40 ± 0.02	1.22 ± 0.06	1.75 ± 0.09
4*R*,8*S*	0.017 ± 0.004	0.06 ± 0.01	0.35 ± 0.01	0.44 ± 0.01
4LNA	NM	ND	NM	1.86 ± 0.14
8LNA	NM	ND	NM	ND
4,8LNA	NM	ND	NM	0.015 ± 0.003

[a] NM: not measured.
[b] ND: not detected.

Acknowledgments

This work was financially supported by ANPCyT and CONICET. J.M.M. and A.M.I. are CONICET members.

Supplementary data

Supplementary data (detailed description of the experimental procedures and NMR assignments) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.06.071.

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