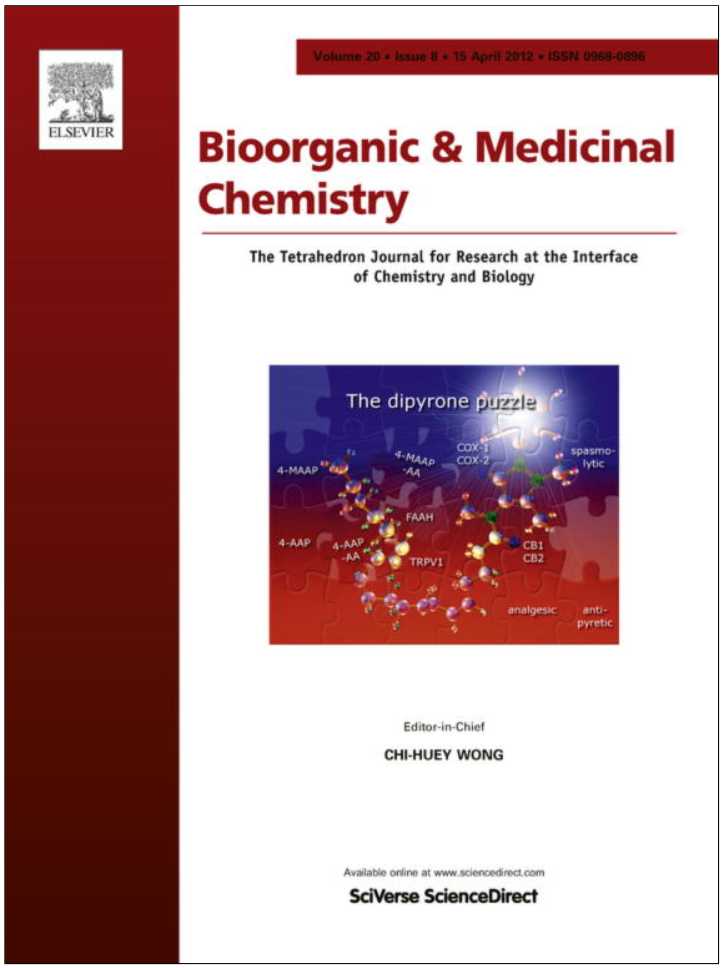

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# Influence of conformationally restricted pyrimidines on the activity of 10–23 DNAzymes

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ABSTRACT

The catalytic core of a 10–23 DNAzyme was modified introducing conformationally restricted nucleosides such as (2′*R*)-, (2′*S*)-2′-deoxy-2′-*C*-methyluridine, (2′*R*)-, (2′*S*)-2′-deoxy-2′-*C*-methylcytidine, 2,2′-anhydrouridine and LNA-C, in one, two or three positions. Catalytic activities under *pseudo* first order conditions were compared at different $Mg^{2+}$ concentrations using a short RNA substrate. At low $Mg^{2+}$ concentrations, triple modified DNAzymes with similar kinetic performance to that displayed by the non-modified control were identified. In the search for a partial explanation of the obtained results, in silico studies were carried out in order to explore the conformational behavior of 2′-deoxy-2′-*C*-methylpyrimidines in the context of a loop structure, suggesting that at least partial flexibility is needed for the maintenance of activity. Finally, the modified 2′-*C*-methyl DNAzyme activity was tested assessing the inhibition of Stat3 expression and the decrease in cell proliferation using the human breast cancer cell line T47D.



## 1. Introduction

Last decades research has shown that oligonucleotides can perform very different activities from the transmission and storage of genetic information, like in the case of RNA (ribozymes[1]) or DNA (DNAzymes or deoxyribozymes[2]) sequences with catalytic properties. In particular, DNAzymes obtained using SELEX (Systematic Enhancement of Ligands by Exponential Enrichment) techniques, are able to catalyze a wide reaction repertoire,[3] which includes transformation of nucleic acids such as cleavage,[4] ligation,[5,6] and phosphorylation[7] and also other reactions like nucleopeptide linkage formation,[8] Diels–Alder addition[9] and porphyrin metalation.[10]

The RNA-cleaving 10–23 DNAzyme has been employed to inhibit protein expression in cell cultures and in vivo experiments.[11] This molecule consists of a 15-nucleotides catalytic core whose conserved sequence is important in order to keep the hydrolytic activity, and two recognition arms that bind to the target mRNA.[12] Its active conformation, cleavage mechanism and three dimensional structure remain unknown.[13] Despite these limitations, several modified deoxyribozymes have been designed to increase the stability against degradation by nucleases, which is a major drawback in cellular media. Most of the modifications, such as inverted nucleotides,[14] phosphorothioates,[15] LNA analogs[16] or 2′-*O*-methylnucleosides, have been placed in the recognition arms.[17] On the other hand, due to the sensitive structure/activity relationship the catalytic core presents, there are only a few examples of chemical modifications in the 10–23 DNAzyme catalytic core, using for example 2′-*O*-methylnucleosides,[17] phosphorothioates,[18] amino-acid-like bases[19] or abasic phosphoramidites.[20] In this sense, the 2′-*C*-methyl-2′-deoxynucleosides[21] constitute an interesting nucleoside class, because they show differential preferred sugar conformations depending on the absolute configuration at the 2′-carbon. The (2′*S*)-2′-deoxy-2′-*C*-methylnucleosides mainly adopt the C3′-*endo* puckering while the 2′*R* isomers prefer the C2′-*endo* state (Fig. 1).[22]

The results recently reported by us regarding the activity of DNAzymes carrying 2′-deoxy-2′-*C*-methyluridine, encouraged us to further explore the influence of these and other analogs.[23] Therefore, we present in this work the evaluation of the kinetic activity and the magnesium dependence of modified 10–23 DNAzymes with (2′*R*)-, (2′*S*)-2′-deoxy-2′-*C*-methylpyrimidines, 2,2′-anhydrouridine and LNA-C. These results confirm previous studies that showed that the incorporation of 2′-deoxy-2′-*C*-methylnucleosides into oligonucleotides increases their nuclease stability.[24] For specific examples of 2′-*C*-methyl modified DNAzymes, an improvement in the activity/resistance relationship respect to the non-modified sequence is also shown. In the search

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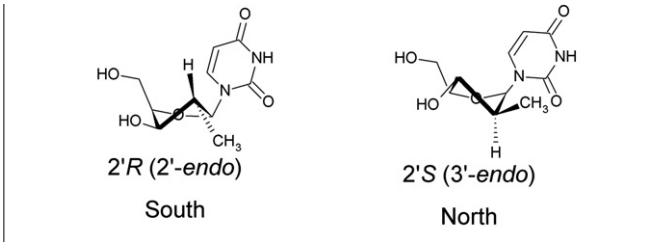


| | | 2'R | | 2'S | |
|---|---|---|---|---|---|
| | | NMR[21] | MP2 6-31G(d) | MP2 6-31G(d) | NMR[21] |
| vo | C4'-O4'-C1'-C2' | -19.3 | -18.1 | 1.7 | 6.6 |
| v1 | O4'-C1'-C2'-C3' | 32.8 | 33.5 | -24.8 | -26.7 |
| v2 | C1'-C2'-C3'-C4' | -33.1 | -35.3 | 36.7 | 35.6 |
| v3 | C2'-O3'-C4'-O4' | 22.6 | 25.9 | -36.8 | -32.3 |
| v4 | C3'-C4'-O4'-C1' | -2.3 | -5.1 | 22.2 | 16.4 |
| v5 | C5'-C4'-C3'-C2' | -98 | -95.8 | -157.5 | -153 |
| v6 | C5'-O4'-C3'-O3' | 145.2 | 142.5 | 84.6 | 84.8 |
| v7 | O4'-C4'-C3'-O3' | -94.2 | -95.8 | -154.7 | -154.6 |
| v8 | C4'-O4'-C1'-N1 | -142.3 | -141.3 | -121.1 | -114.6 |
| | P | 179.74 | 179.8 | 0.28 | 0.14 |
| | Φm | 33.1 | 35.3 | 36.7 | 35.6 |

**Figure 1.** Experimental and calculated dihedral angles of 2′-*C*-methylnucleosides.

for a partial explanation of the obtained results, in silico studies were carried out in order to explore the conformational behavior of 2′-deoxy-2′-*C*-methylpyrimidines in a single stranded nucleic acid environment. Finally, we assessed the modified DNAzyme activity in signal transducer and activator of transcription 3 (Stat3) expression, in the human breast cancer cell line T47D.

Stat3 is a member of the Stat family of transcription factors implicated in growth factor and cytokine signaling. Its activation is due to transient phosphorylation of cytoplasmic monomers that dimerize, translocate to the nucleus, and bind to specific promoter sequences, thereby regulating gene expression.[25] Persistently phosphorylated Stat3 is observed in nearly 70% of human cancers. This protein is implicated in a vast range of normal and pathogenic processes including cellular proliferation, differentiation, survival, angiogenesis, metastasis, inflammation, and immune response, and acts as an oncogene to induce tumorigenesis while contributing to tumor-immune escape mechanisms.[26–28] Stat3′s pivotal position at the convergence of many oncogenic pathways and dependence of some cancers on persistent Stat3 signaling[29,30] makes it a prime target for cancer therapy.[28,31] Indeed, direct inhibition of Stat3 activity by overexpression of dominant-negative isoforms,[31,32] antisense oligos,[33] RNAi,[34,35] peptides[36] and small drug inhibitors[37,38] blocks growth and induces apoptosis in various model systems.[28]

## 2. Experimental

### 2.1. General

For oligonucleotide synthesis the following solvents and reagents were used: triethylamine (Sintorgan), glacial acetic acid (Sintorgan), acetonitrile (HPLC grade, J.T. Baker, <13 ppm water), THF (J.T. Baker), Pyridine (J.T. Baker), dichloromethane (J.T. Baker), 2,6-lutidine (Aldrich), acetic anhydride (Aldrich) and iodine (Aldrich). Commercial amidites were from ChemGenes (USA). Short RNA substrate was purchased from Sigma-Genosys.

### 2.2. Modified phosphoramidite synthesis

The synthesis of (2′*S*)- and (2′*R*)-2′-deoxy-2′-*C*-methyluridine and cytidine phosphoramidites was carried out according to the previously reported procedures.[23,24,38–44]

### 2.3. DNAzyme synthesis

Modified and unmodified DNAzymes were synthesized using an ABI392-DNA synthesizer (Applied Biosystem, USA) or a MARMADE 6 synthesizer (BioAutomation USA), at 40 or 50 nmol scale respectively. All reagents and solvents were high purity grade and the syntheses were performed according to the manufacturer recommendations. The coupling time for modified positions was increased in order to improve reaction efficiency. DMTr-ON oligonucelotides were purified on C18 chromatography column. The purity of DNAzymes was confirmed by RP-HPLC (Gilson).

### 2.4. Oligonucelotide labeling

Short RNA substrate (17nt) or DNAzymes (1 pmol) were radiolabeled with [$\gamma$-$^{32}$P]-ATP (20 μCi), using T4 polynucleotide kinase (2U) (Invitrogen). The reaction was carried out for 1 h at 37 °C, extracted with chloroform–isoamilic alcohol (96:4) and then purified on Sephadex G-25 columns (*GE* Healthcare Life Sciences).[39]

### 2.5. Kinetic analysis

DNAzymes kinetic was evaluated under single turnover conditions. The DNAzyme (100 pmol) and labeled substrate (10 pmol, 10,000 cpm) were combined in Tris–HCl, pH 7.5 (20 μL of 50 mM), heated at 95 °C for 1 min, and then equilibrated at 37 °C for 5 min. Reaction was initiated by the addition of a solution of $MgCl_2$ in the same buffer to give a ranging $Mg^{2+}$ concentration from 0.5 to 10 mM. Aliquots were removed at different times and the reactions were stopped by the transfer of 1 μL of reaction aliquots to 10 μL of ice-cold stop buffer (50% formamide 50% buffer TBE 100 mM EDTA 100 mM). The cleavage fractions were resolved on denaturing PAGE 20%, and the percentage of cleavage was analyzed using a Molecular Dynamics Storm 840 Phosphoimager. The time dependent increase of the cleaved fraction was fitted with $\%P = \%P_{max} \times (1-\exp(-k_{obs} \times x))$. All time measurements were performed as triplicates.

### 2.6. Stability assay

To assess DNAzyme stability against nucleolytic degradation, unlabeled DNAzyme (100 pmol) and labeled DNAzymes (20,000 cpm) were incubated with nuclease RQ1 (Invitrogen). Aliquots were removed at different time points and the reaction was stopped by transferring 1 μL aliquots of reaction to 10 μL of ice-cold stop buffer (formamide 50%, buffer TBE 100 mM, EDTA 100 mM). The degradations products were resolved on denaturing PAGE 20% and then analyzed using Molecular Dynamics Storm 840 Phosphoimager.

### 2.7. Western blots

To study Stat3 expression at protein level, whole-cell extracts were prepared. In brief, T47D cells were lysed by scraping at 4 °C in buffer 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM ethylene glycolbis(β-aminoethyl ether)-*N,N,N*9,*N*9-tetraacetic acid (EGTA), 10% glycerol, 0.5% Nonidet P-40, 1 mM $MgCl_2$, 0.1% sodium dodecyl sulfate [SDS], 1 mM PMSF, 5 μg of leupeptin/mL, 5 μg of pepstatin/mL, 5 μg of aprotinin/mL, 5 mM NaF, 0.15 mM spermine, 0.5 Mm spermidine, 10 mM β-glycerophosphate and 1 mM sodium orthovanadate. Lysates were centrifuged at 12,000 rpm for 30 min. Soluble proteins in clarified lysates were quantified by using a Bio-Rad (Richmond, Calif.) kit. Proteins were resolved by SDS–PAGE on 7.5% gels, electroblotted onto nitrocellulose, and immunoblotted with an anti-Stat3 rabbit polyclonal antibody (C-20) from Santa Cruz Biotechnology (Santa Cruz, CA). Filters were reprobed with an anti-actin antibody (clone ACTN05) from Neomarkers

(Freemont, CA). Enhanced chemiluminescence was performed according to the manufacturer's instructions (Amersham). Signal intensities of Stat3 bands were analyzed by densitometry and normalized to actin bands.

### 2.8. Cell cultures, treatments, and proliferation assays

T47D cells were obtained from the American Type Culture Collection. Cell proliferation was evaluated by [$^3$H]thymidine incorporation assays. In earlier experiments we had demonstrated that thymidine uptake correlates with the number of cells/well.[45] Cells were plated in culture flasks with DMEM–F12 (Dulbecco modified Eagle medium–Ham F12 [1:1]) plus 10% heat-inactivated fetal calf serum (FCS; Gen S.A., Buenos Aires, Argentina) and allowed to attach for 24 h. Then, they were transfected for 24 h and starved in serum free medium for another 24 h, treated with the synthetic progestin medroxyprogesterone acetate (MPA) or left untreated in the presence of 0.8 μCi of [$^3$H]thymidine (specific activity, 70–90 Ci/mmol; NEN/Dupont, Boston, Mass). Cells were then trypsinized and harvested. Assays were performed in octuplicate. The differences between control and experimental groups were analyzed by analysis of variance and the Tukey *t* test between groups. Fugene HD transfection reagent (Roche Biochemicals) was used as recommended by the manufacturer. Transfection efficiencies were evaluated by using the pEGFP-N1 vector and determined by the percentage of cells that exhibited GFP 4 days after transfection, observing variations between 60% and 70%.

## 3. Results and discussion

### 3.1. 10–23 DNAzymes carrying 2′-deoxy-2′-*C*-methylcytidine at single positions of the catalytic core

A set of modified DNAzymes with 2′-deoxy-2′-*C*-methylcytidine at single positions of the catalytic core were synthesized. These modified DNAzymes were directed against the +964 position of the Estrogen Receptor α (ERα) mRNA since we have previously shown the feasibility of using core-modified hammerhead ribozymes targeted to the same sequence in MCF-7 cells.[39] A non-modified DNAzyme (Dz, positive control, Table 1) and sequences carrying (2′*R*)- or (2′*S*)-2′-deoxy-2′-*C*-methylcytidine at positions 3, 7, 10 or 13 of the catalytic core (Dz-3R, Dz-3S, Dz-7R, Dz-7S, Dz-10R, Dz10S, Dz-13R, Dz-13S, Table 1) were synthesized and the catalytic activity was determined by measuring the $k_{obs}$ under pseudo first order conditions (DNAzyme excess) at 0.5, 1.0, 5.0 and 10.0 mM $Mg^{2+}$.[39] Only the introduction of the methyl modified cytidine at position 7 (Dz-7S, Dz-7R, Table 1), gave active DNAzymes, even at 0.5 mM $Mg^{2+}$. This is in agreement with previous results that indicated that this position could be replaced by thymidine or adenosine,[40] or even deleted,[12] without losing significant hydrolytic activity.

### 3.2. 10–23 DNAzymes with 2′-deoxy-2′-*C*-methylcytidine and 2′-deoxy-2′-*C*-methyluridine at multiple positions of the catalytic core

Having in mind that the nucleolytic resistance of the DNAzymes will depend on the modification degree, and taking into account our previous results,[23] simultaneous modification of two and three positions of the catalytic core was explored.

In first place (2′*S*)- and (2′*R*)-2′-deoxy-2′-*C*-methylcytidine were introduced at position 7 of the catalytic core keeping (2′*S*)-2′-deoxy-2′-*C*-methyluridine at position 8 (Dz-7S8S; Dz-7R8S, Table 2) affording sequences with similar activities than the non-modified control. These results encouraged us to study the effect of three modified nucleosides at the catalytic core. A set of DNAzymes carrying 2′-deoxy-2′-*C*-nucleosides at positions 4, 7 and 8 were prepared and their respective $k_{obs}$ were measured (Dz-4S7R8S; Dz-4S7S8S; Dz-4S7R8R; Dz-4S7S 8R; Table 2). Dz-4S7R8S and Dz-4S7S8R emerged as the most promising, showing kinetic constants (0.50 ± 0.05 and 0.53 ± 0.05 $min^{-1}$ at 1 mM $Mg^{2+}$, respectively) similar to the value found for the non-modified control (0.74 ± 0.03 $min^{-1}$).

### 3.3. 10–23 DNAzymes with conformationally locked nucleosides at the catalytic core

In order to compare the results here obtained with 2′-deoxy-2′-*C*-methylcytidine, LNA-C nucleotides (fixed in C3′-*endo*) were also introduced at positions 3, 7, 10 and 13 (Table 3). All the synthesized DNAzymes were inactive at 1 mM $Mg^{2+}$ (Table 3). When the activity was explored at 10 mM $Mg^{2+}$, Dz-3LNA and Dz-13LNA were completely inactive while Dz-7LNA and Dz-10LNA were respectively 43 and 23 times less active than the non modified sequence (Table 3).

These results are in line with those previously obtained by us using 2′-deoxy-2′-*C*-methyluridine and LNA-T.[23] With the intention to explore the effect of a conformationally locked analogue with an inverted conformation than the LNA-nucleotides, 2,2′-anhydrouridine (fixed in C2′-*endo*) was introduced at 4- and 8- and 4,8-positions of the catalytic core, finding that Dz-8Anh (Table 3) was the only with partial activity: it was inactive at 1 mM $Mg^{2+}$ and 14 times less active than the control at 10 mM $Mg^{2+}$.

### 3.4. In silico study of 2′-*C*-methylnucleosides in a single stranded loop structure

In order to understand the different properties observed when using well known conformationally locked nucleosides and the 2′-

**Table 1**
$K_{obs}$ for DNAzymes mutated at single positions with 2′-deoxy-2′-*C*-methylcytidine

| Name | DNAzyme sequence | $k_{obs}$ ($min^{-1}$) | | | |
|---|---|---|---|---|---|
| | | 0.5 mM $Mg^{2+}$ | 1 mM $Mg^{2+}$ | 5 mM $Mg^{2+}$ | 10 mM $Mg^{2+}$ |
| Dz | 5′ TGGCTGGA $G_1$ $G_2$ $C_3$ $T_4$ $A_5$ $G_6$ $C_7$ $T_8$ $A_9$ $C_{10}$ $A_{11}$ $A_{12}$ $C_{13}$ $G_{14}$ $A_{15}$ ACATATAG 3′ | 0.45 ± 0.03 | 0. 74 ± 0.03 | 1.5 ± 0.2 | 2.4 ± 0.3 |
| Dz-3S | 5′ TGGCTGGA $G_1$ $G_2$ $C_{3S}$ $T_4$ $A_5$ $G_6$ $C_7$ $T_8$ $A_9$ $C_{10}$ $A_{11}$ $A_{12}$ $C_{13}$ $G_{14}$ $A_{15}$ ACATATAG 3′ | 0.024 ± 0.003 | NM[b] | 0.059 ± 0.001 | NM |
| Dz-3R | 5′ TGGCTGGA $G_1$ $G_2$ $C_{3R}$ $T_4$ $A_5$ $G_6$ $C_7$ $T_8$ $A_9$ $C_{10}$ $A_{11}$ $A_{12}$ $C_{13}$ $G_{14}$ $A_{15}$ ACATATAG 3′ | ND | NM | 0.014 ± 0.003 | NM |
| Dz-7S | 5′ TGGCTGGA $G_1$ $G_2$ $C_3$ $T_4$ $A_5$ $G_6$ $C_{7S}$ $T_8$ $A_9$ $C_{10}$ $A_{11}$ $A_{12}$ $C_{13}$ $G_{14}$ $A_{15}$ ACATATAG 3′ | 0.51 ± 0.04 | 0.69 ± 0.09 | 1.38 ± 0.09 | 1.8 ± 0.2 |
| Dz-7R | 5′ TGGCTGGA $G_1$ $G_2$ $C_3$ $T_4$ $A_5$ $G_6$ $C_{7R}$ $T_8$ $A_9$ $C_{10}$ $A_{11}$ $A_{12}$ $C_{13}$ $G_{14}$ $A_{15}$ ACATATAG 3′ | 0.76 ± 0.04 | 0.8 ± 0.1 | 1.25 ± 0.09 | 1.80 ± 0.09 |
| Dz-10S | 5′ TGGCTGGA $G_1$ $G_2$ $C_3$ $T_4$ $A_5$ $G_6$ $C_7$ $T_8$ $A_9$ $C_{10S}$ $A_{11}$ $A_{12}$ $C_{13}$ $G_{14}$ $A_{15}$ ACATATAG 3′ | 0.11 ± 0.01 | NM | 0.27 ± 0.01 | NM |
| Dz-10R | 5′ TGGCTGGA $G_1$ $G_2$ $C_3$ $T_4$ $A_5$ $G_6$ $C_7$ $T_8$ $A_9$ $C_{10R}$ $A_{11}$ $A_{12}$ $C_{13}$ $G_{14}$ $A_{15}$ ACATATAG 3′ | 0.058 ± 0.004 | NM | 0.22 ± 0.01 | NM |
| Dz-13S | 5′ TGGCTGGA $G_1$ $G_2$ $C_3$ $T_4$ $A_5$ $G_6$ $C_7$ $T_8$ $A_9$ $C_{10}$ $A_{11}$ $A_{12}$ $C_{13S}$ $G_{14}$ $A_{15}$ ACATATAG 3′ | ND[a] | NM | 0.033 ± 0.005 | NM |
| Dz-13R | 5′ TGGCTGGA $G_1$ $G_2$ $C_3$ $T_4$ $A_5$ $G_6$ $C_7$ $T_8$ $A_9$ $C_{10}$ $A_{11}$ $A_{12}$ $C_{13R}$ $G_{14}$ $A_{15}$ ACATATAG 3′ | ND | NM | 0.08 ± 0.01 | NM |

The core sequence is indicated in italic and the modified position is marked with bold fonts.
[a] ND: not detected.
[b] NM: not measured.

**Table 2**
$k_{obs}$ for DNAzymes mutated at double and triple positions with 2′-deoxy-2′-*C*-methylcytidine and 2′-deoxy-2′-*C*-methyluridine

| Name | DNAzyme sequence | $k_{obs}$ ($min^{-1}$) | |
|---|---|---|---|
| | | 1 mM $Mg^{2+}$ | 10 mM $Mg^{2+}$ |
| Dz | 5′ TGGCTGGA $G_1$ $G_2$ $C_3$ $T_4$ $A_5$ $G_6$ $C_7$ $T_8$ $A_9$ $C_{10}$ $A_{11}$ $A_{12}$ $C_{13}$ $G_{14}$ $A_{15}$ ACATATAG 3′[a] | 0. 74 ± 0.03 | 2.4 ± 0.3 |
| Dz-7S8S | 5′ TGGCTGGA $G_1$ $G_2$ $C_3$ $T_4$ $A_5$ $G_6$ **$C_{7S}$** **$U_{8S}$** $A_9$ $C_{10}$ $A_{11}$ $A_{12}$ $C_{13S}$ $G_{14}$ $A_{15}$ ACATATAG 3′ | 0.66 ± 0.04 | 2.4 ± 0.2 |
| Dz-7R8S | 5′ TGGCTGGA $G_1$ $G_2$ $C_3$ $T_4$ $A_5$ $G_6$ **$C_{7R}$** **$U_{8S}$** $A_9$ $C_{10}$ $A_{11}$ $A_{12}$ $C_{13S}$ $G_{14}$ $A_{15}$ ACATATAG 3′ | 0.77 ± 0.05 | 3.0 ± 0.3 |
| Dz-4S7R8S | 5′ TGGCTGGA $G_1$ $G_2$ $C_3$ **$U_{4S}$** $A_5$ $G_6$ **$C_{7R}$** **$U_{8S}$** $A_9$ $C_{10}$ $A_{11}$ $A_{12}$ $C_{13}$ $G_{14}$ $A_{15}$ ACATATAG 3′ | 0.50 ± 0.05 | 1.8 ± 0.2 |
| Dz-4S7S8S | 5′ TGGCTGGA $G_1$ $G_2$ $C_3$ **$U_{4S}$** $A_5$ $G_6$ **$C_{7S}$** **$U_{8S}$** $A_9$ $C_{10}$ $A_{11}$ $A_{12}$ $C_{13}$ $G_{14}$ $A_{15}$ ACATATAG 3′ | 0.24 ± 0.02 | 0.69 ± 0.05 |
| Dz-4S7S8R | 5′ TGGCTGGA $G_1$ $G_2$ $C_3$ **$U_{4S}$** $A_5$ $G_6$ **$C_{7S}$** **$U_{8R}$** $A_9$ $C_{10}$ $A_{11}$ $A_{12}$ $C_{13}$ $G_{14}$ $A_{15}$ ACATATAG 3′ | 0.20 ± 0.01 | 0.90 ± 0.07 |
| Dz-4S7R8R | 5′ TGGCTGGA $G_1$ $G_2$ $C_3$ **$U_{4S}$** $A_5$ $G_6$ **$C_{7R}$** **$U_{8R}$** $A_9$ $C_{10}$ $A_{11}$ $A_{12}$ $C_{13}$ $G_{14}$ $A_{15}$ ACATATAG 3′ | 0.53 ± 0.03 | 1.1 ± 0.1 |

[a] The core sequence is indicated in italic and the modified position is marked with bold fonts.

**Table 3**
$k_{obs}$ for DNAzymes mutated at single positions with 2,2′-anhydrouridine and LNA-C

| Name | DNAzyme sequence | $k_{obs}$ ($min^{-1}$) | |
|---|---|---|---|
| | | 1 mM $Mg^{2+}$ | 10 mM $Mg^{2+}$ |
| Dz | 5′ TGGCTGGA $G_1$ $G_2$ $C_3$ $T_4$ $A_5$ $G_6$ $C_7$ $T_8$ $A_9$ $C_{10}$ $A_{11}$ $A_{12}$ $C_{13}$ $G_{14}$ $A_{15}$ ACATATAG 3″[a] | 0.74 ± 0.03 | 2.4 ± 0.3 |
| Dz-4Anh | 5′ TGGCTGGA $G_1$ $G_2$ $C_3$ **$U_{4A}$** $A_5$ $G_6$ $C_7$ $T_8$ $A_9$ $C_{10}$ $A_{11}$ $A_{12}$ $C_{13}$ $G_{14}$ $A_{15}$ ACATATAG 3′ | ND | ND |
| Dz-8Anh | 5′ TGGCTGGA $G_1$ $G_2$ $C_3$ **$U_{4A}$** $A_5$ $G_6$ $C_7$ **$U_{8A}$** $A_9$ $C_{10}$ $A_{11}$ $A_{12}$ $C_{13}$ $G_{14}$ $A_{15}$ ACATATAG 3′ | ND | 0.17 ± 0.02 |
| Dz-4,8Anh | 5′ TGGCTGGA $G_1$ $G_2$ $C_3$ $T_4$ $A_5$ $G_6$ $C_7$ **$U_{8A}$** $A_9$ $C_{10}$ $A_{11}$ $A_{12}$ $C_{13}$ $G_{14}$ $A_{15}$ ACATATAG 3′ | ND | ND |
| Dz-3LNA | 5′ TGGCTGGA $G_1$ $G_2$ **$C_{3LNA}$** $T_4$ $A_5$ $G_6$ $C_7$ $T_8$ $A_9$ $C_{10}$ $A_{11}$ $A_{12}$ $C_{13}$ $G_{14}$ $A_{15}$ ACATATAG 3′ | ND | ND |
| Dz-7LNA | 5′ TGGCTGGA $G_1$ $G_2$ $C_3$ $T_4$ $A_5$ $G_6$ **$C_{7LNA}$** $T_8$ $A_9$ $C_{10}$ $A_{11}$ $A_{12}$ $C_{13}$ $G_{14}$ $A_{15}$ ACATATAG 3′ | ND | 0.056 ± 0.002 |
| Dz-10LNA | 5′ TGGCTGGA $G_1$ $G_2$ $C_3$ $T_4$ $A_5$ $G_6$ $C_7$ $T_8$ $A_9$ **$C_{10LNA}$** $A_{11}$ $A_{12}$ $C_{13}$ $G_{14}$ $A_{15}$ ACATATAG 3′ | ND | 0.103 ± 0.005 |
| Dz-13LNA | 5′ TGGCTGGA $G_1$ $G_2$ $C_3$ $T_4$ $A_5$ $G_6$ $C_7$ $T_8$ $A_9$ $C_{10}$ $A_{11}$ $A_{12}$ **$C_{13LNA}$** $G_{14}$ $A_{15}$ ACATATAG 3′ | ND | ND |

[a] The core sequence is indicated in italic and the modified position is marked with bold fonts.

*C*-methyl modification, and due to the fact that the 10–23 DNAzyme core structure remains unknown, we carried out a preliminary in silico study of the conformational behavior of 2′-deoxy-2′-*C*-methylnucleosides when forming part of a single stranded loop structure. We have previously reported that the isolated nucleosides have a practically frozen conformation depending on the 2′-C-configuration.[22] While the molar fraction of (2′*S*)-2′-deoxy-2′-*C*-methyluridine in the north conformation is practically one (Fig. 1), the (2′*R*)-isomer exists almost exclusively in the south conformation. This behavior can be explained by the tendency to accommodate the 2′-methyl group in an equatorial position. As suggested by the experimental data shown above, 2′-*C*-methylnucleosides may have some degree of conformational flexibility when incorporated into nucleic acid structures. To carry out the in silico study, first (2′*S*)- and (2′*R*)-2′-deoxy-2′-*C*-methyluridine conformations were optimized using MP-2 methods and the resulting geometries were minimized using molecular dynamics, including water solvents molecules with a TIP3P model. Figure 1 shows the experimental and the calculated dihedral and pseudorotational angles for (2′*S*) and (2′*R*)-2′-deoxy-2′-*C*-methyluridine. The results clearly indicated a south like conformation for (2′*R*)-configuration and a north like one for the (2′*S*)-isomer. With these results in hand, a library for the modified nucleosides was prepared. Then an NMR determined stem–loop structure with a single chain of three Ts and one dU was selected from the NDB database (ndb-id: 1II1). This structure was used as template for further replacement of dU by (2′*S*) and (2′*R*)-2′-deoxy-2′-*C*-methyluridine using in silico calculations. Molecular dynamic experiments were run for 15 ns, leaving the first 5 ns for thermal stabilization. Table 4 shows the residence times in the different sugar conformations (pseudorotational angles *P*), expressed as relative percentages, for the three assayed nucleotides in position 9. The sequence with dU has a pseudorotational angle consistent with an east conformation as main state (loop 1-$dU_9$, Table 4), but 2′-*C*-methylnucleosides showed a different behavior. While (2′*S*)-2′deoxy-2′-*C*-methyluridine alternated between the north and east conformations with similar residence times (loop 1-$U_{9R}$, Table 4), the (2′*R*)-isomer preferred the south conformation as in the case of the isolated nucleoside.

**Table 4**
Molecular dynamics experiments for (2′*R*) and (2′*S*)-2′-deoxy-2′-*C*-methyluridine in stem–loop structures

| Loop name | Sequence | |
|---|---|---|
| Loop 1-$dU_9$ | 5’ AGGATCC *TdU₉TT* GGATCCT 3’[a] | ACGATCC $^{T}X_{9}$ TCCTAGG $_{T}{}^{T}$ |
| Loop 1-$U_{9S}$ | 5’ AGGATCC *TU₉ₛTT* GGATCCT 3’ | |
| Loop 1-$U_{9R}$ | 5’ AGGATCC *TU₉ᵣTT* GGATCCT 3’ | |

| Loop 1-$dU_9$ | | Loop 1-$U_{9S}$ | | Loop 1-$U_{9R}$ | |
|---|---|---|---|---|---|
| P | % | P | % | P | % |
| 75<P<125 | 78 | 80<P<120 | 46 | 125<P<175 | 75 |
| -50<P<0 | 22 | -40<P<20 | 54 | 0<P<50 | 25 |

[a]The core sequence is indicated in italic and the modified position is marked with bold fonts.

These findings suggest that although an almost exclusive conformation is present for isolated 2′-*C*-methylnucleosides, they can be flexible enough when they are part of a loop structure to reach other conformational states, feature not possible for LNA or 2′-anhydro nucleosides. This may partially account for the experimental data here reported, suggesting that at least partial flexibility is needed for the maintenance of DNAzyme activity.

We are aware that there are other factors that may be playing an important role in the final active conformation of the DNAzyme catalytic core, but they are difficult to rationalize due to the lack of structural data.

### 3.5. Modified 10–23 DNAzymes activity/stability relationship

In order to preliminarily evaluate the stability against nucleolytic degradation, Dz-4S7R8S, Dz-4S7S8R and Dz as control, were isotopically labeled with $^{32}P$ and incubated with endonuclease RQ1. The surviving full length percentage was determined at 15 min (Fig. 2A), being as expected, the modified DNAzymes more stable than the unmodified one.

Taking into account that the introduction of the modified nucleosides in the catalytic core produced a decrease in the hydrolytic activity and an improvement of the stability, and considering that the DNAzyme activity in cellular systems will depend on both

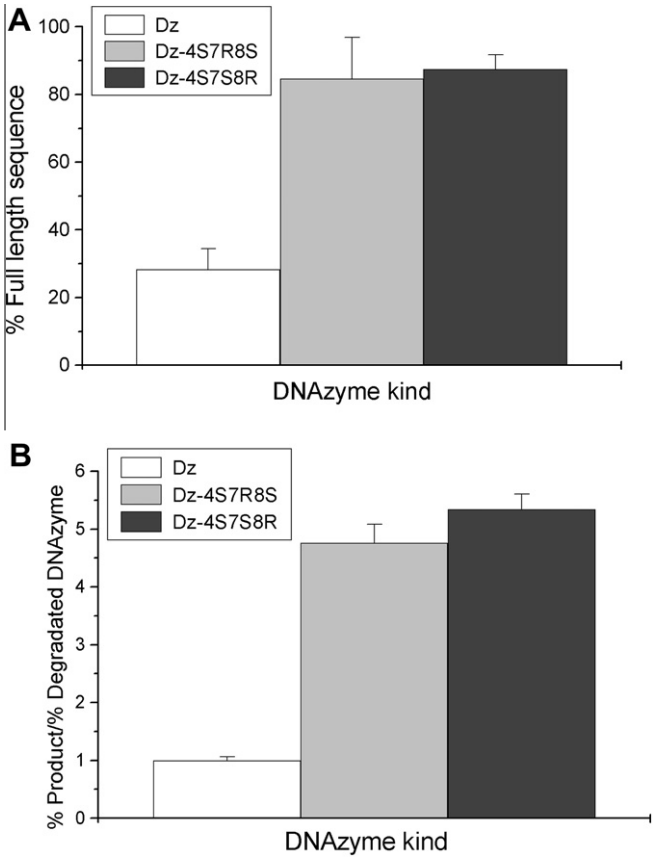


**Figure 2.** (A) Percentage of DNAzyme full length sequence after endonuclease degradation. (B) Activity/stability relationship for Dz, Dz-4S7R8S and Dz-4S7S8R.

| Name | DNAzyme sequence |
|---|---|
| Dz-3 | 5′ ATGAAACA $G_1G_2C_3T_4A_5G_6C_7T_8A_9C_{10}A_{11}$ $A_{12}C_{13}G_{14}A_{15}$ CAACGTGG 3′[a] |
| Dz-3M[b] | 5′ ATGAAACA $G_1G_2C_3T_4A_5G_6C_7T_8A_9C_{10}A_{11}$ $A_{12}C_{13}C_{14}A_{15}$ CAACGTGG 3′[b] |
| Dz-3-4S7R8S (Dz-3-A) | 5′ ATGAAACA $G_1G_2C_3$ ***$U_{4S}$*** $A_5G_6$ ***$C_{7R}U_{8S}$*** $A_9$ $C_{10}A_{11}A_{12}C_{13}G_{14}A_{15}$ CAACGTGG 3′[c] |
| Dz-3-4S7S8R (Dz-3B) | 5′ ATGAAACA $G_1G_2C_3$ ***$U_{4S}$*** $A_5G_6$ ***$C_{7S}U_{8R}$*** $A_9C_{10}$ $A_{11}A_{12}C_{13}G_{14}A_{15}$ CAACGTGG 3′ |

[a] The core sequence is indicated in italics, the phosphorothioate position are marked with underlined fonts and the 2'-*C*-methylnuclesides are indicated with bold fonts. [b] $G_{14}$ was mutated to $C_{14}$.

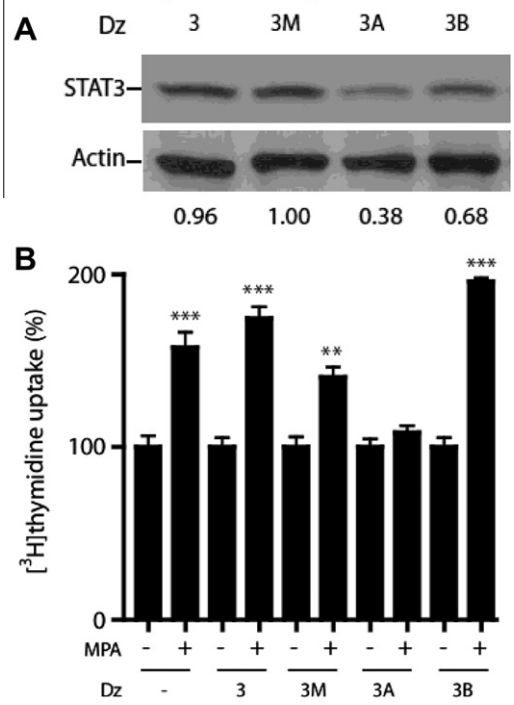


**Figure 3.** (A) Downregulation of Stat3: densitometry values of a representative experiment of Stat3 western blot referenced to actin as loading control. (B) Prevention of MPA-induced proliferation in breast cancer cells: [$^3$H]thymidine uptake was used as a measure of DNA synthesis. Data are presented as percentage ± standard deviation (*** $p$ <0.001, ** $p$ <0.01).

parameters, the relationship between the activity (at 1 mM $Mg^{2+}$ concentration) and the stability (measured as the proportion of the DNAzyme degraded) was calculated at 15 min., and normalized versus the unmodified sequence (Fig. 2B). The best ratio was achieved for the Dz-4S7S8R, being five times better than the unmodified DNAzyme.

### 3.6. Inhibition of Stat3 expression and breast cancer cell proliferation

There is extensive data suggesting that Stat3 promotes human breast cancer progression. Stat3 was found to participate in the expression of genes that control critical cellular functions such as proliferation, survival, self-renewal, angiogenesis, metastasis, inflammation, and immune response. In agreement with these data, we have previously demonstrated that Stat3 is an obligatory requirement for progestin-induced breast cancer cell proliferation.[35] Therefore, we prepared a set of 2′-*C*-methylmodified DNAzymes against Stat3 and tested their ability to inhibit breast cancer growth. To improve the stability toward exonucleases for the cell culture experiments, two phosphorothioate modifications were introduced at both ends of the DNAzymes. First, in order to verify that the behavior of these sequences carrying different recognition arms was similar to that of the previously studied DNAzymes, their stability and activity were tested. As expected, the same tendencies as those previously described in Sections 2.2 and 2.4 were obtained (see Supplementary data). Then, we performed western blot analysis of protein extracts from T47D human breast cancer cells transiently transfected with 2′-*C*-methyl DNAzymes: Dz-3, Dz-3M, Dz-3A and Dz-3B (Fig. 3). Our results show, that Dz-3B yielded only a 32% of Stat3 downregulation while Dz-3A induced a 62% inhibition of Stat3 expression as compared to cells transfected with Dz-3M. This last sequence carries a modification ($G_{14}$ to $C_{14}$) that abolishes the catalytic activity and therefore serves as a potential antisense control (Fig. 3A). In accordance with these results, no effect in progestin-induced proliferation as measured by [$^3$H]thymidine incorporation assays was observed employing Dz-3B (Fig. 3B) but transfection of T47D cells with Dz-3A resulted in complete abrogation of progestin-induced proliferation (Fig. 3B).

## 4. Conclusions

(2′*R*)-2′-Deoxy-2′-*C*-methyluridine, (2′*S*)-2′-deoxy-2′-*C*-methyluridine, (2′*R*)-2′-deoxy-2′-*C*-methylcytidine and (2′*S*)-2′-deoxy-2′-*C*-methylcitidine were introduced in one, two and three positions of the 10–23 DNAzyme catalytic core. In the case of DNAzyme Dz-4S7R8R, 72% of the activity was maintained at 1 mM $Mg^{2+}$. When conformationally locked nucleosides, like LNA-C or anhydrouridine, were introduced at the same positions, no catalytic activity was observed. These facts could be partially explained by the conformational flexibility showed by 2′-*C*-methylnucleosides when incorporated into loop environments. In relation to DNAzyme stability, triple modified molecules Dz-4S7R8S and Dz-4S7S8R were

three times more resistant against endonuclease degradation and have four to five times higher activity/resistance ratios than non-modified sequences. A DNAzyme carrying three 2′-*C*-methylpyrimidines (Dz-3A) was tested against Stat 3 showing a significant inhibition of Stat3 expression.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2012.02.047.

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