Impact of 2'-*C*-methylpyrimidine nucleosides on the stability of the i-motif and the inhibitory properties of modified siRNAs

María Dellafiore,[a] Anna Aviñó,[b,c] Adele Alagia,[b,c] Raimundo Gargallo,[d,e] Carlos González, [e, f] Javier M. Montserrat,[g] Adolfo Iribarren,[h*] Ramon Eritja.[b,c*]

a *INGEBI (CONICET), Vuelta de Obligado 2490, 1428 Buenos Aires, Argentina,* ***b*** *Networking Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Jordi Girona 18-26, E-08034 Barcelona. Spain,* ***c*** *Institute for Advanced Chemistry of Catalonia (IQAC)Spanish Council for Scientific Research (CSIC)Jordi Girona 18-26, 08034 Barcelona, Spain;* ***d*** *Department of Chemical Engineering and Analytical Chemistry, University of Barcelona, Martí i Franquès, 1-11, E-08028 Barcelona, Spain;* ***e*** *BIOESTRAN, associated unit UB-CSIC;* ***f*** *Institute of PhysicalChemistry "Rocasolano", CSIC, Serrano 119, E-28006 Madrid, Spain;* ***g*** *Universidad Nacional de General Sarmiento, J.M. Gutiérrez 1150, 1613 Los Polvorines, Argentina;* ***h*** *Universidad Nacional de Quilmes, Roque Saenz Peña 352, 1876 Bernal, Argentina*

[*]Corresponding authors: E-mail: A. Iribarren: airibarren@unq.edu.ar, R. Eritja: recgma@cid.csic.es

INTRODUCTION

2'-*C*-methylpyrimidines are modified nucleosides that have been previously used to modify the core of ribozymes and DNAzymes.[1] In relation to the nucleoside puckering control, it has been shown that the introduction of carbon substituents at the 2'-position of deoxynucleosides deeply affects the sugar conformational state.[2] The sugar puckering equilibrium in solution for the (2'*R*)-2´-deoxy-*C*-methyl nucleoside is shifted mainly to the C2'-*endo* conformation, while the (2'*S*)-epimer preferentially adopts the C3'-*endo*. In addition, 2'-deoxy-2'-*C*-methyl analogs improved the resistance against nucleolytic degradation when they were incorporated into DNA sequences. In this work we have explored the use of 2'-*C*-methylpyrimidines in the context of two different oligonucleotide structures: i-motifs[3] and siRNAs[4]. I-motifs are tetraplex structures present in telomeres and promoter regions of oncogenes. The possibility of producing nanodevices based on these structures with pH-sensitive functions has triggered the interest for modified oligonucleotides with improved properties. In this work the effect of (2'*S*)-2'-deoxy-2'-*C*-methyl-cytidine on the stability of intramolecular i-motifs related to vertebrate telomere was investigated by means of spectroscopic methods (UV, CD and NMR). Regarding siRNA, several studies have shown that duplexes with high silencing potency can only tolerate a limited number of modifications and that the effect of these changes depends on the location within the molecule. Here, we show the results of the incorporation of 2'-*C*-methylpyrimidines in the 3'-overhang region of the sense and antisense strands and in the seed region of siRNA duplexes directed against *Renilla* luciferase.

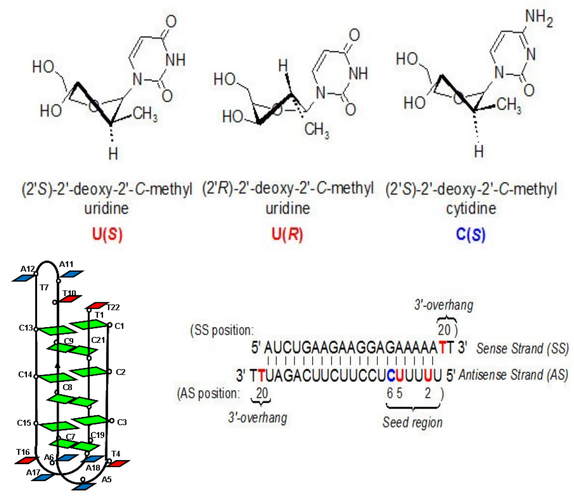

Figure 1. 2'-*C*-pyrimidines in i-motif and siRNA.

RESULTS

i-motif

We replaced dC by CMeUp in different positions of the C-core stretches of the C-rich fragment of the vertebrate telomere $(C_3TA_2)_3C_3T$. We selected single substitutions at 5'-terminal stretch (VT1CMeUp), internal positions (VT2MeCUp) or double substitution involved in the same internal C-CH+ base pairs (VT2,14CMeUp) (Table 1).

Table 1. Sequences used in this study. (2'S)-2'-deoxy-2'-*C*-methyl-cytidine = (C_{Me}Up)

Name	Sequence
VTWT	CCCTAACCCTAACCCTAACCCT
VT1 C_{Me}Up	CCCTAACCCTAACCCTAACCCT
VT2 C_{Me}Up	CCCTAACCCTAACCCTAACCCT
VT2,14 C_{Me}Up	CCCTAACCCTAACCCTAACCCT

i-motif	pH	Tm (°C)	ΔTm (°C)	pH	Tm (°C)	ΔTm (°C)	pH	Tm (°C)	ΔTm (°C)
T-NM	5,5	43,4	-	6,0	26,7	--	6,5	--	--
T-C(S)1	5,5	43,5	0,1	6,0	27,0	0,3	6,5	∞15	--
T-C(S)2	5,5	44,8	1,4	6,0	28,1	1,4	6,5	∞20	--
T-C(S)2-C(S)14	5,5	45,5	2,1	6,0	30,2	3,5	6,5	∞20	--

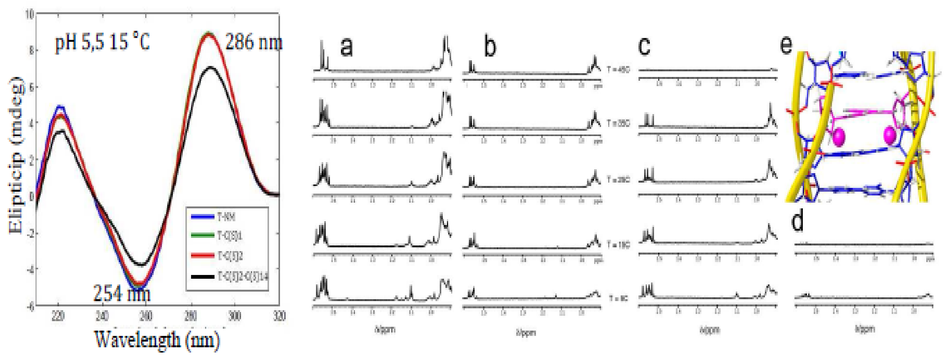

Figure 2. i-Motif modified with 2'-*C*-methylcytidine. Melting temperature (Tm, ºC) at pH 5.5; 6.0 and 6.5, Circular dichroism and exchangeable ^{1}H-NMR region of VT2,14CMeUp at different temperatures at pH 5 (a) and pH 7 (c), and the unmodified human telomeric sequence at pH 5 (b) and pH 7 (d). Model of VT2,14CMeUp based on the structure of the telomeric sequence. (2'*S*)-2'-deoxy-2'-*C*-methyl-cytidine are shown in magenta with 2'-CH_3 groups displayed as spheres (e).

siRNA

(2'*S*)-2'-deoxy-2'-*C*-methyluridine and (2'*R*)-2'-deoxy-2'-*C*-methyluridine were incorporated in the 3'-overhang region of the sense and antisense strands and in positions 2- and 5- of the seed region of siRNA duplexes directed against *Renilla* luciferase, while (2'*S*)-2'-deoxy-2'-*C*-methylcytidine was incorporated in the 6- position of the seed region of the same constructions. Dual luciferase reporter assay in transfected HeLa cells was used as model system to measure IC_{50} values of 24 different modified duplexes.

Table 2. Tm and IC_{50} values for the unmodified and modified siRNA duplexes.

Duplex	Antisense strand	Sense strand	IC_{50} (pM)	Tm (°C)
D1	ASLucWT	SLucWT	7.9 ± 2.0	**70.1**
D2	ASLucU(R)$_{20}$	SLucWT	5.0 ± 0.4	**70.0**
D3	ASLucU(S)$_{20}$	SLucWT	4.0 ± 0.2	**70.2**
D4	ASLucU(S)$_2$	SLucWT	10.2 ± 0.8	**69.4**
D5	ASLucU(R)$_2$	SLucWT	11.7 ± 0.5	**69.1**
D6	ASLucU(S)$_5$	SLucWT	8.7 ± 1.2	**65.5**
D7	ASLucU(R)$_5$	SLucWT	9.9 ± 0.3	**67.9**
D8	ASLucWT	SLucU(S)$_{20}$	16.7 ± 1.2	**70.5**
D9	ASLucWT	SLucU(R)$_{20}$	21.4 ± 3.2	**69.9**
D10	ASLucU(S)$_{20}$	SLucU(S)$_{20}$	9.8 ± 0.6	**68.9**
D11	ASLucU(S)$_{20}$	SLucU(R)$_{20}$	19.1 ± 2.1	**69.3**
D12	ASLucU(R)$_{20}$	SLucU(S)$_{20}$	10.7 ± 2.0	**69.8**
D13	ASLucU(R)$_{20}$	SLucU(R)$_{20}$	7.4 ± 1.0	**71.0**
D14	ASLucU(S)$_2$	SLucU(S)$_{20}$	63.0 ± 6.4	**69.4**
D15	ASLucU(S)$_2$	SLucU(R)$_{20}$	39.1 ± 3.2	**69.6**
D16	ASLucU(R)$_2$	SLucU(S)$_{20}$	8.9 ± 2.5	**69.4**
D17	ASLucU(R)$_2$	SLucU(R)$_{20}$	9.1 ± 1.3	**69.0**
D18	ASLucU(S)$_5$	SLucU(S)$_{20}$	35.4 ± 12.1	**65.6**
D19	ASLucU(S)$_5$	SLucU(R)$_{20}$	24.7 ± 4.0	**65.1**
D20	ASLucU(R)$_5$	SLucU(S)$_{20}$	27.7 ± 7.8	**67.6**
D21	ASLucU(R)$_5$	SLucU(R)$_{20}$	8.6 ± 1.1	**67.2**
D22	ASLucC(S)$_6$	SLucWT	24.6 ± 4.8	**64.7**
D23	ASLucC(S)$_6$	SLucU(S)$_{20}$	37.1 ± 5.3	**68.6**
D24	ASLucC(S)$_6$	SLucU(R)$_{20}$	58.4 ± 8.2	**65.4**

Table 2. Synthesized modified strands of siRNA directed against *Renilla reniformis* luciferase.

siRNA strand	Sequence (5'- 3')
ASLucWT	UUUUUCUCCUUCUUCAGAUTT
ASLucU(*S*)$_2$	U**U(*S*)**$_2$UUUCUCCUUCUUCAGAUTT
ASLucU(*R*)$_2$	U**U(*R*)**$_2$UUUCUCCUUCUUCAGAUTT
ASLucU(*S*)$_5$	UUUU**U(*S*)**$_5$CUCCUUCUUCAGAUTT
ASLucU(*R*)$_5$	UUUU**U(*R*)**$_5$CUCCUUCUUCAGAUTT
ASLucU(*S*)$_{20}$	UUUUUCUCCUUCUUCAGAU**U(*S*)**$_{20}$T
ASLucU(*R*)$_{20}$	UUUUUCUCCUUCUUCAGAU**U(*R*)**$_{20}$T
ASLucC(*S*)$_6$	UUUUU**C(*S*)**$_6$UCCUUCUUCAGAUTT
SLucWT	AUCUGAAGAAGGAGAAAAATT
SLucU(*S*)$_{20}$	AUCUGAAGAAGGAGAAAAA**U(*S*)**$_{20}$T
SLucU(*R*)$_{20}$	AUCUGAAGAAGGAGAAAAA**U(*R*)**$_{20}$T

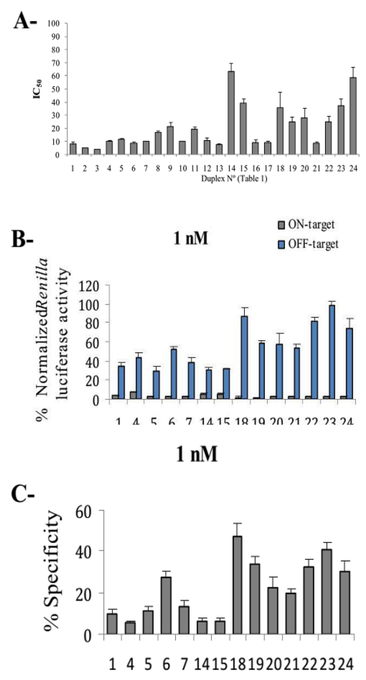

Figure 3. IC_{50} and melting temperatures of duplexes D1-D24, siRNA activity (as % Normalized *Renilla* luciferase activity) and specificity.

CONCLUSIONS

i-Motifs: In conclusion, we studied the effect of the replacement of ~~the~~ dC by C_{Me}Up in the ability to form intramolecular i-motifs. The relative stability of the modified structures have been analyzed by UV, CD and NMR spectroscopies, showing that C_{Me}Up residues induce a stabilization of the i-motif being the C_{Me}Up: C_{Me}Up pair more stable than the C_{Me}Up: dC pair. This stabilization could be used to modulate the stability of i-motif structures at mild acidic to neutral pH values

siRNA: Duplexes containing (2'*S*)- and (2'*R*)-2'-deoxy-2'-*C*-methyluridine in the 3'-overhang region of the antisense strand yielded lower IC_{50}s than the unmodified control siRNA, suggesting an increased affinity to RISC as observed for other thymine derivatives. No obvious correlation could be found between the thermal stability and the IC_{50}. The presence of the 2'-*C*-methyl nucleosides has a strong impact in the ON/OFF-target selectivity when located at the seed positions. Modifications in position 2- of the seed region had an unfavorable effect on specificity while modifications in positions 5- and 6- had a positive effect on the strand preference obtaining an increase of 2-4 times in selectivity compared with the unmodified siRNA. This is the first time than these differences in the selectivity have been observed for conformationally restricted nucleosides. The most interesting modified siRNA duplexes (D6 and D21) had antisense strands with similar silencing potency and higher specificity. These observations may be useful for the design of more effective siRNA-based drugs.

REFERENCES: 1) See for example: a) L. Robaldo, F. Izzo, M. Dellafiore, C. Proietti, P. V. Elizalde, J. M. Montserrat, A. M. Iribarren. *Bioorg. Med. Chem. 20*, 2581-2586, **2012**. b) L. Robaldo, A. Berzal-Herranz, J. M. Montserrat, A. M. Iribarren. *ChemMedchem 9*, 2271-2277, **2014**. 2) L Robaldo, R Pontiggia, S Di Lella, D A. Estrín, J W. Engels, A M. Iribarren, J M. Montserrat. *J. Phys. Chem. B*, *117,* 57-69, **2013**. 3) A. Aviñó, M. Dellafiore, R. Gargallo, C. González, A. M. Iribarren, J. M. Montserrat, R. Eritja. *ChemBioChem 18*, 1123-1128, **2017**. 4) M. Dellafiore, A. Aviñó, A. Alagia, J. M. Montserrat, A. M. Iribarren, R. Eritja. *ChemBioChem 19*, 1409-1413, **2018**.

AKNOWLEDGMENTS: This study was supported by the Spanish Ministry of Education (CTQ2014-52588-R), the Generalitat de Catalunya (grant 2014 SGR 187); Argentine Ministry of Science and Technology (PICT-2008-2194) and Universidad Nacional de Quilmes (1400/15) CIBER-BBN is an initiative funded by the VI National R&D&I Plan 2008–2011, IniciativaIngenio 2010, Consolider Program, CIBER Actions and are financed by the Instituto de Salud Carlos III with assistance from the European Regional Development Fund. MD was recipient of an EMBO short term fellowship (ASTF 515-2015).