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siRNA Modified with 2′-Deoxy-2′-*C*-methylpyrimidine Nucleosides

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(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, whereas (2′*S*)-2′-deoxy-2′-*C*-methylcytidine was incorporated in the 6-position of the seed region of the same constructions. A dual luciferase reporter assay in transfected HeLa cells was used as a model system to measure the IC_{50} values of 24 different modified duplexes. The best results were obtained by the substitution of one thymidine unit in the antisense 3′-overhang region by (2′*S*)- or (2′*R*)-2′-deoxy-2′-*C*-methyluridine, reducing IC_{50} to half of the value observed for the natural control. The selectivity of the modified siRNA was measured, it being found that modifications in positions 5 and 6 of the seed region had a positive effect on the ON/OFF activity.

Introduction

Soon after Fire[1] and Mello[2] discovered that gene silencing can be achieved by use of double-stranded RNA (dsRNA), it was found that the same effect could be obtained with short 21-nucleotide duplexes.[3] These dsRNAs, called siRNAs, inspired applications in the fields of gene silencing[3] and therapeutics.[4] When siRNAs were evaluated as potential drugs, they exhibited poor serum stability, unspecific immune stimulation, and OFF-target effects.[5] Fortunately, chemical modifications can contribute to mitigate these disadvantages with a view to in vivo applications.[6]

The incorporation of modified nucleosides into siRNA sequences has been approached in several studies showing, in most cases, that duplexes with high silencing potency can only tolerate a limited number of modifications and that the effects of these changes depend on the location within the molecule.[7] It has been shown that some modifications in the seed region favor the selection of the antisense strand by the RNA-induced silencing complex (RISC) by introducing a thermodynamic asymmetry, whereas changes in the 3′-overhang lead to higher potency and weaker OFF-target effects.[8] It has also been proposed, as a general guideline, that the A-form of the modified helix structure plays an important role.[7] In view of these factors, different modifications in the 2′-position of the ribose moiety have been evaluated: in particular, 2′-*O*-methyl,[9] 2′-deoxy-2′-F in the *ribo*[10] and *arabino*[11] configurations, 2′-*O*-aminoethyl, 2′-aminopropyl, 2′-guanidinoethyl, and 2′-cyanoethyl derivatives.[8] Other modified nucleosides assessed for siRNA performance included phosphorothioates,[12] vinylphosphonates,[13] LNA,[14] and 5-alkyl modifications,[15] among others.[6–8]

With regard to 2′-modified analogues, ribo- and 2′-deoxy-2′-*C*-methylpyrimidine nucleosides have been previously used to enhance oligonucleotide stability in the context of ribozyme[16] and DNAzyme[17] applications, it having been observed that these nucleosides could be incorporated in the catalytic core without significant loss of catalytic activity. It has also been established that incorporation of (2′*S*)-2′-deoxy-2′-*C*-methyluridine residues in RNA duplexes produced an important decrease in the melting point temperatures, whereas circular dichroism experiments indicated that the helix was still A-type, thus suggesting a localized disturbance disorder.[18] These results could find applications in the field of modified siRNA, in view of the previously mentioned importance of the A-form in siRNA performance[7] and the influence of different thermodynamic stabilities in the seed region on strand selection.[19]

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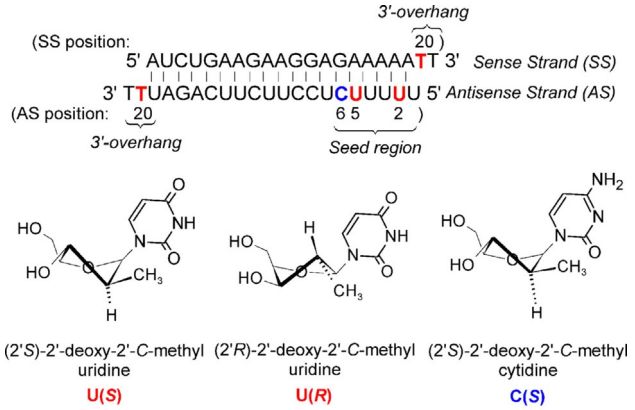
Considering these facts, we selected positions 2, 5, and 6 of the seed region in the antisense strand and the 3′-overhang positions in both the antisense and sense strands of siRNA duplexes targeted against *Renilla* luciferase to evaluate the effects of the incorporation of (2′*R*)- and (2′*S*)-2′-deoxy-2-*C*-methylpyrimidine nucleosides. Silencing efficacy, thermal stability, and ON/OFF target effects were studied by using of the dual luciferase reporter assay in transfected HeLa cells as model.

Results and Discussion

Synthesis of siRNAs incorporating (2′*S*)- and (2′*R*)-2′-deoxy-2′-*C*-methylpyrimidine nucleosides

(2′*S*)-2′-Deoxy-2′-*C*-methyluridine [U(*S*)], (2′*R*)-2′-deoxy-2′-*C*-methyluridine [U(*R*)], and (2′*S*)-2′-deoxy-2′-*C*-methylcytidine [C(*S*), Scheme 1] were prepared and converted into their corresponding phosphoramidites as previously reported.[20] The modified phosphoramidites were used to obtain oligonucleotides through solid-phase synthesis.

A 21-mer siRNA targeting the *Renilla reniformis* luciferase gene was designed.[21] For the antisense strand, positions 2, 5, and 6 of the seed region and position 20 of the 3′-overhang were individually modified with 2′-deoxy-2′-*C*-methylnucleosides. For the sense strand, position 20 of the 3′-overhang was modified. Sequences of the synthesized unmodified and modified strands are shown in Scheme 1. The compositions of the different modified siRNA duplexes are shown in Table 1.



siRNA strand	Sequence (5′- 3′)
ASLucWT	UUUUUCUCCUUCUUCAGAUTT
ASLucU$(S)_2$	U**U(S)$_2$**UUCUCCUUCUUCAGAUTT
ASLucU$(R)_2$	U**U(R)$_2$**UUCUCCUUCUUCAGAUTT
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

Scheme 1. Modified siRNA strands directed against the *R. reniformis* luciferase gene.

Table 1. T_m and IC_{50} values for the unmodified and modified siRNA duplexes.

Duplex	Antisense strand	Sense strand	IC_{50} [pM]	T_m [°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

Thermal stability and silencing potency studies of modified siRNA duplexes

All possible combinations of antisense and sense strands were annealed, and thermal denaturation curves at 260 nm for all siRNA duplexes (D1 to D24, Table 1) were recorded. In order to evaluate the effects of the incorporation of the modified nucleosides on duplex thermal stability, we compared the melting temperatures (T_m) of all of the modified siRNAs with that of the unmodified one (WT, Table 1).

A dual luciferase reporter assay was used as a simplified system to study the correlations between 2′-deoxy-2′-*C*-methylpyrimidine nucleoside position and siRNA potency. HeLa cells were transfected with each siRNA duplex (Table 1) at different concentrations (0, 0.002, 0.008, 0.016, 0.06, 0.16, 0.3, and 1 nM) together with a psiCHECK2-AS plasmid containing *Renilla* and *Firefly* luciferase genes. Luminescence was measured 24 h after transfection. *Renilla* luciferase activity was normalized to *Firefly* luciferase activity, and mock activity (0 mM siRNA) was set as 100%. Half-maximum inhibitory concentrations (IC_{50}) were calculated as indicators of silencing activity and are shown in Table 1 (see also Figure S1 in the Supporting Information).

2′-Deoxy-2′-*C*-methylnucleoside modifications in the 3′-overhang region of the antisense strand (D2 and D3, Table 1) kept siRNA potency (IC_{50} decrease) unchanged or slightly increased relative to the unmodified duplex (D1, Table 1). When the same modifications were located in the sense strand (D8 and D9, Table 1) they increased IC_{50} values (16.7, 21.4, Table 1). When sense and antisense strands were simultaneously modified in the 3′-overhang region (D10, D11, D12, D13, Table 1), the IC_{50} values were in the 7.4–19.1 range (Table 1). If these results are compared with recent findings relating to 3′-modified

siRNAs,[22] some similarities can be found. When, for example, the 2′-deoxy-2′-*C*-methyl-U modification is at the 3′-end of the antisense strand, the IC_{50} is maintained or slightly improved, as in the case of the 2′-*O*-methyl modification.[22] However, when the 2′-deoxy-2′-*C*-methyl-U modification is present at the 3′-end of the sense strand there is a strong decrease in the IC_{50} values that was not observed in the case of the 3′-modified siRNAs.[22] This effect might indicate a preferential binding of the PAZ domain to the antisense strand modified with 2′-deoxy-2′-*C*-methyl-U, but structural studies would be needed to confirm this hypothesis. No correlation could be established between the IC_{50} values of the analyzed sequences and their thermal stability, with little or no difference being observed for the T_m values.

It is known that the 3′-overhang region of siRNA interacts with the Ago2 protein of the RISC, which is involved in the strand selection process.[2,5,6] From the above results it seems that the presence of 2′-deoxy-2′-*C*-methyluridine favors the interaction between the oligonucleotide and the RISC complex, thus having a positive silencing effect when incorporated in the antisense strand (D2, D3, Table 1) and a negative effect when incorporated in the sense strand (D8, D9, Table 1). This influence seems to be independent of the nucleoside 2′-carbon configuration (*R* or *S*). When both sense and antisense strands were modified no correlation could be established between the configuration of the 2′-carbon of the analogue and the IC_{50} of the duplex (D10–D13, Table 1).

The seed region of the antisense strand (Scheme 1) is involved in strand selection by the RISC and in the initial interaction with the target RNA. Therefore, it has been proposed that a mild thermal duplex destabilization in this region favors the selection of the antisense strand. On the other hand, either a strong thermal destabilization or a strong thermal stabilization could interfere with hybridization with the target RNA, reducing the silencing effect.[8] When 2′-deoxy-2′-*C*-methylnucleosides were incorporated at positions 5 or 6 of the antisense strand, thermal destabilization was observed (D6, D18, D19, D22, and D24, Table 1), with T_m values being lower as the modification was positioned closer to the center of the double-stranded region. In the case of duplexes modified at position 2, the increase in IC_{50} values (D4, D5, Table 1) could be a consequence of altered strand selection by the RISC, because the T_m values are similar to that observed in the case of D1. In the case of duplexes modified in positions 5 and 6 (D6, D7 Table 1) the IC_{50} values are similar to or slightly higher than those determined with unmodified siRNA.

When the IC_{50} values of duplexes with simultaneous modification in the seed region of the antisense strand plus modification in the 3′-overhang region of the sense strand (D14–D21, D23–D24, Table 1) were analyzed, no evident relationship with the IC_{50} values observed for the corresponding duplexes incorporating single modifications could be inferred (D4–D9, D22, Table 1). In some cases such as D16, D17, and D21 the IC_{50} values are similar to or slightly higher than those seen with unmodified siRNA but in the rest of the siRNA duplexes the IC_{50} values are much higher (25–63), thus indicating a strong decrease in the silencing potency.

In the cases of D14 to D17, antisense strands modified with the *R* isomer gave better results, contrasting with the similar effects observed in the cases of D4 and D5.

OFF/ON target silencing studies of modified siRNA duplexes

One of the main problems in the use of siRNA is the presence of OFF-target effects, produced by incorrect strand selection by the RISC. In this work, the silencing activity of each strand was evaluated by using the dual luciferase reporter assay. When ON-target activity was assessed, plasmid psiCHECK2-AS was used, whereas when OFF-target activity was evaluated, psiCHECK2-SS plasmid was employed. It is expected that duplexes incorporating modifications at positions 2, 5, and 6 should have the strongest impact in the ON/OFF-target effect. We selected 14 out of the 24 prepared duplexes including both singly (D4, D5, D6, D7, D22, Table 1) and doubly modified strands (D14, D15, D18–D21, D23, D24, Table 1). Figure 1 shows the normalized percentages of *Renilla* luciferase activity and siRNA specificity for the selected duplexes at a concentration of 1 nM. Specificity was calculated as a ratio between the normalized *Renilla* luciferase activity in OFF-target and ON-target effect experiments. siRNA specificity was also tested at 16 pM (see the Supporting Information); at this low concentration most of the specificity is lost, in agreement with the literature.

Results show that the incorporation of 2′-deoxy-2′-*C*-methyl nucleosides in some positions can strongly enhance siRNA specificity (D6, D18, D19, D20, D21, D22, D23, and D24, Figure 1). Comparison of duplexes with potencies similar to that of D1 shows that the incorporation of the analogue can result either in lower specificity (D4) or in higher specificity (D6 and D21). Some duplexes with considerable lower potency than D1 were also less specific (D14 and D15, Figure 1) whereas others, surprisingly, proved to be more specific (D18, D19, D20, D22, D23, and D24, Figure 1).The observed results show a general trend suggesting that modifications in position 2 of the seed region have unfavorable effects on specificity whereas modifications in positions 5 and 6 have positive outcomes. Lower IC_{50} values for duplexes with higher specificity could therefore be a consequence of target hybridization problems evidenced by lower T_m values. If the silencing potency (IC_{50}) and the ON/OFF target selectivity are considered, duplexes D6 and D21 are the most interesting modified duplexes because they have silencing efficiency (IC_{50} = 8.7 and 8.6) similar to that of the unmodified siRNA (IC_{50} = 7.9) but two to three times higher selectivity.

Conclusion

(2′*S*)-2′-Deoxy-2′-*C*-methyluridine and (2′*R*)-2′-deoxy-2′-*C*-methyluridine were incorporated in the 3′-overhang regions of the sense and antisense strands and in the 2- and 5-positions of the seed region, whereas (2′*S*)-2′-deoxy-2′-*C*-methylcytidine was included in the 6-position of the seed region of siRNA duplexes targeting *Renilla* luciferase. 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} values than the

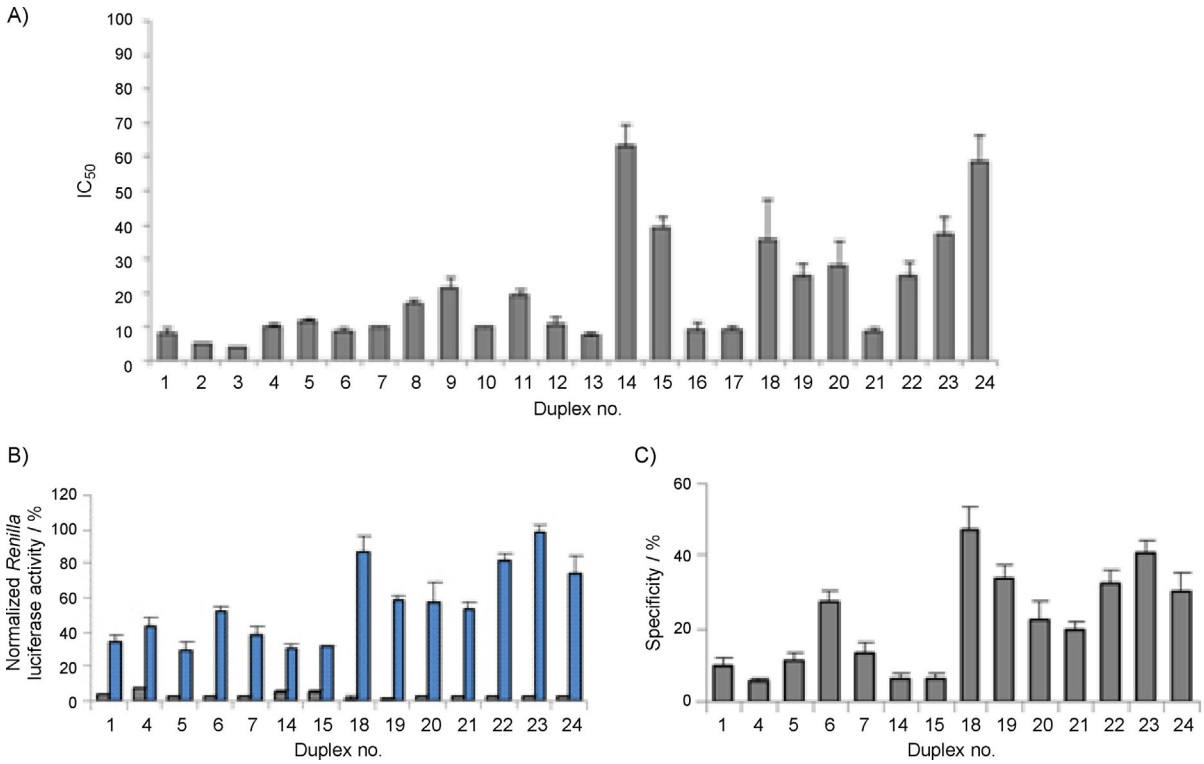


Figure 1. A) Half-maximum inhibitory concentrations (IC_{50} [pM]) of 2′-*C*-methylnucleoside-modified siRNAs (duplexes defined in Table 1). B) ON-target (■) and OFF-target (■) effects. Normalized percentages of *Renilla* luciferase activity. C) Specificity values for siRNA duplexes.

unmodified control siRNA. This suggests that these modified nucleosides have an increased affinity towards the RISC, as also observed for other thymine derivatives.[21–24] No obvious correlation between the thermal stabilities of the modified siRNA duplexes and the IC_{50} values could be found.

The presence of the 2′-*C*-methyl nucleosides has a strong impact on the ON/OFF-target selectivity when they are located at the seed positions. Modifications in position 2 of the seed region had an unfavorable effect on specificity whereas modifications in positions 5 and 6 had a positive effect on the strand preference, producing increases in selectivity of two- to four-fold in relation to the unmodified siRNA. Unfortunately, at lower siRNA concentrations these strand preferences are lost.

Hence, lower potency for modified siRNAs was a consequence of two different circumstances: a decrease in specificity for duplexes incorporating modifications in position 2 and target hybridization problems for duplexes incorporating modifications in positions 5 and 6 of the antisense strand.

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 might be useful for the design of more effective siRNA-based drugs.

Experimental Section

Preparation of modified phosphoramidites: The synthesis of (2′*S*)- and (2′*R*)-2′-deoxy-2′-*C*-methyluridine and (2′*S*)-2′-deoxy-2′-*C*-methylcytidine phosphoramidites was carried out as previously reported.[20a,b]

Oligonucleotide synthesis: The oligonucleotides listed in Table 1 were prepared with a DNA/RNA Applied Biosystems 394 synthesizer (Foster City, CA, USA) by solid-phase 2-cyanoethyl phosphoramidite chemistry on a 0.2 μmol scale, in DMT-ON mode by using LV200 polystyrene supports and 2′-*O*-TBDMS-5′-*O*-DMT-protected phosphoramidites (ChemGenes). The coupling time was 10 min for both natural and modified phosphoramidites.

Deprotection and purification of unmodified and modified RNA oligonucleotides: Each solid support was treated with NH_3 solution (33%, 1.5 mL) and ethanol (0.5 mL) at 55 °C for 1 h. Then, the suspension was cooled to room temperature; the supernatant was separated and concentrated to dryness with use of a Speedvac concentrator. The obtained residue was dissolved in *N*-methylpyrrolidone (115 μL), triethylamine trihydrofluoride (75 μL), and triethylamine (60 μL) and incubated at 55 °C for 2 h. Oligonucleotide purification was carried out by use of RNA purification cartridges (GlenResearch). The modified oligonucleotide structure was confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) spectrometry (Table II-SM, Figures II-X-SM).

siRNA preparation: siRNA duplexes were annealed by using equimolar ratios of the sense and the antisense strands in KOAc (100 mM), HEPES/KOH (30 mM), and $MgCl_2$ (2 mM), pH 7.4, at final concentrations of 20 μM for cell culture studies and 2 μM for thermal denaturation studies. Duplexes were heated at 95 °C for 5 min and allowed to cool slowly to 4 °C.

Thermal denaturation studies: Melting curves of duplex siRNAs were obtained by following change of absorbance at 260 nm versus temperature. Samples were heated from 25 °C to 85 °C, with

a linear temperature ramp of 1 °C min^{-1}, in a JASCO V-650 spectrophotometer (JASCO, Easton, MD, USA) equipped with a Peltier temperature control.

Cell culture: HeLa cells (ATCCCCL2) were maintained in a monolayer culture at exponential growth in high-glucose Dulbecco's modified Eagle medium (DMEM, Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with heat-inactivated fetal bovine serum (10%, Gibco, Life Technologies, Carlsbad, CA, USA). Cells were incubated at 37 °C in a humidified environment under CO_2 (5%) and periodically checked for contamination.

PsiCHECK2 ON/OFF target reporters: To construct the on-target (psiCHECK2-AS) and off-target (psiCHECK2-SS) reporters, 5′-phosphorylated DNA sequences (Sigma–Aldrich) corresponding to the antisense strand target:
5′-TCGAATCAAATCTGAAGAAGGAGAAAAATG, and
5′-GGCCCATTTTTCTCCTTCTTCAGATTTGAT

sense strand target:
5′-TCGACATTTTTCTCCTTCTTCAGATTTGAT, and
5′-GGCCATCAAAATCTGAAGAAGGAGAAAAATG

of the synthesized siRNAs were annealed and inserted into the XhoI and NotI sites of the psiCHECK2 plasmid (Promega, Madrid, Spain). The correct insertion of the sequences was confirmed by sequencing.

Transfection and luciferase assay: For siRNA luciferase assay, HeLa cells were plated in 24-well tissue culture plates at density of 1×10^5 cells per well 24 h before transfection. In dose–response ON-/OFF-target assessment, psiCHECK2 (**AS**) or psiCHECK2 (**SS**, 1 μg) and siRNAs at different concentrations (0, 0.002, 0.008, 0.016, 0.06, 0.16, 0.3, and 1 nM) were co-transfected with Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). The inhibitory effect of siRNAs on *Renilla* protein expression was measured on lysates collected 24 h after transfection by using the dual-luciferase reporter assay system and a Glomax-Multi luminometer (Promega). The activity of *Renilla* luciferase was normalized to that of *Firefly* luciferase and the mock activity was set as 100%. The normalized percentage of activity of *Renilla* luciferase (Y) was plotted against log[siRNA] (X) and fitted to $Y=100/(1+10^{(x-\log IC_{50})})$. IC_{50} was calculated.

The results are representative of at least three independent experiments and each transfection was performed in triplicate. IC_{50} values were calculated by using GraphPad Prism software with the sigmoidal dose–response function.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: deoxymethylpyrimidine bases · nucleosides · siRNA · stability · structure–activity relationships

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