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Tuning RNA Interference by Enhancing siRNA/PAZ Recognition

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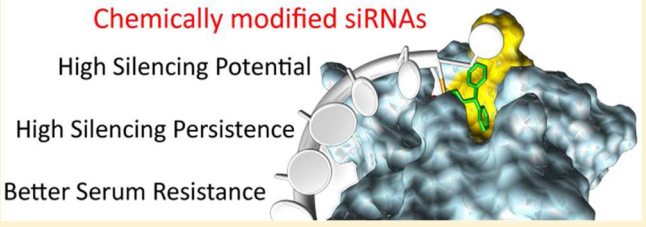
S *Supporting Information*

ABSTRACT: Chemically modified siRNAs were synthesized to enhance the corresponding silencing activities. The introduced modifications endowed siRNAs with high silencing effect, long RNAi persistence, and better serum resistance. Theoretical data allowed us to correlate the observed siRNAs interfering performance with the peculiar interactions with PAZ.



KEYWORDS: *RNA interference, modified siRNAs, molecular modeling, siRNA/PAZ interactions, gene silencing*

RNA interference (RNAi) is a biological process whereby double-stranded RNAs (dsRNAs) silence gene expression in a sequence-specific manner.[1] The mechanism that mediates gene silencing involves the processing by the enzyme Dicer of dsRNA into small interfering RNAs (siRNAs). These are 21−22 nucleotides long dsRNA containing a 2 nucleotide (nt) overhang at the 3′-end. In vivo, siRNAs associate with the RNA-induced silencing complex (RISC), which contains the signature component of the RNAi machinery, Argonaute 2 (Ago 2).

The siRNA strand with the thermodynamically less stable 5′-end is preferentially incorporated as the guiding or antisense strand (AS) of RISC,[2] while the passenger or sense strand (SS) of the siRNA duplex is cleaved by Ago 2 and liberated from the complex.[3] Then, the activated RISC, containing the AS, binds the fully complementary target RNA and leads to its cleavage by Ago 2, thus preventing its translation into the corresponding protein.[4]

The Argonaute proteins are, indeed, core components of RISC and are made up by PAZ, Mid, and PIWI domains. X-ray structural analysis and NMR studies have revealed that the 3′-overhang region of the AS of siRNAs is recognized by the PAZ domain with its 2 nt 3′-overhang being lodged into a binding pocket mainly composed of hydrophobic amino acids.[5]

Given their critical biological role, siRNAs have considerable potential as new therapeutic tools for intractable diseases considering that they can be rationally designed and synthesized if the sequences of the disease-causing genes are known.[6] Nevertheless, much has to be done to fulfill the tremendous expectations placed on this research area.

In this respect, chemical modification of siRNAs is now suggested as a demand to enhance their potential in vivo. In particular, modified siRNAs can feature longer half-lives,[7,8] amenability of conjugation with specific carriers,[9,10] and higher potencies resulting in lower doses required to reach the silencing effect.[7] Moreover, selective loading of the AS into RISC is essential for avoiding undesirable side effects, while the release of the 3′-end from the PAZ binding pocket during the cleavage of the mRNA is crucial to perform RNase activity.[11] In this respect, experiments on cell cultures have demonstrated that the conjugation of siRNAs with different aromatic residues at 3′-end modulates the silencing activity, increases the nuclease resistance, and influences the affinity of the 3′-overhang portion for the PAZ.[12−14]

According to the so-called "two state model"[15] the 2 nt 3′-overhang of the AS is buried in the PAZ binding pocket so that, at first, its base pairing with the RNA target is prevented. Then, the aforementioned RNA region is dislodged from PAZ allowing the base pairing with the target mRNA in the PIWI domain. The 3′-region of the AS would, then, reanchor the PAZ domain with the release of the cleaved target. Therefore, the above-described model suggests that a fine-tuning of the interactions between the PAZ and the siRNAs 3′-overhang can result in an efficient silencing effect

On the basis of these studies, we decided to investigate the gene silencing activity of siRNAs bearing different aromatic residues (namely, tyramine, diphenylpropylamine, and tryptamine, **a**, **b**, and **c**, respectively; Scheme 1) at the 3′-end of the AS and/or SS. The reason for using these aromatic moieties was dictated by the necessity of selecting different shapes and

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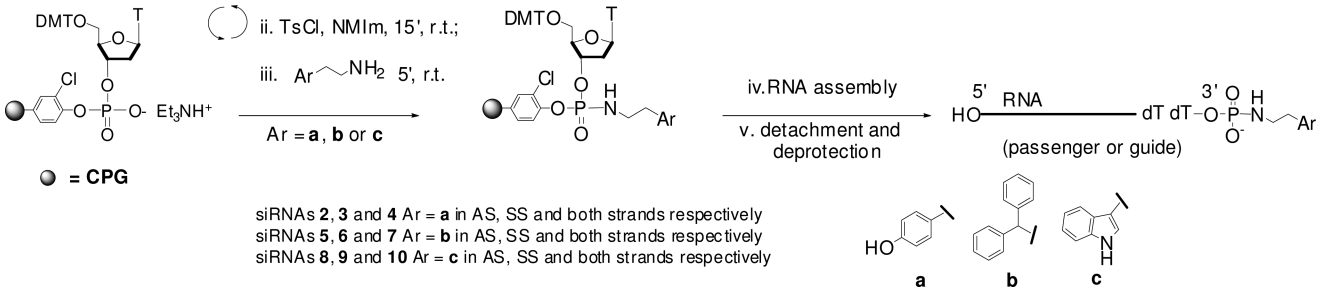
Scheme 1. Functionalization of CPG Support with Aromatic Derivatives a, b, or c



electronic properties while featuring an appropriate size for the 3′-overhang region of siRNAs.[13] Moreover, we chose to introduce the aromatic group at the 3′-region of RNA strands ending with a dTdT overhang, as it has been reported that multiple addition of 2′-deoxynucleotides or of aromatic residues to the 3′-end can be tolerated by the RNAi machinery.[12−14] On the other hand, a primary amino function was required for the introduction of the aromatic moieties on functionalized CPG support[16] through phosphoramidate linkage (Scheme 1).

A solid phase approach that allows for the selective detachment of 3′-conjugated RNA strands was employed to synthesize the 3′-conjugated ASs and SSs. Unmodified RNAs were synthesized by standard procedures. The RNA strands were prepared so as to target firefly luciferase mRNA encoding for a bioluminescent protein commonly used as a reporter in gene inhibition assays[6] (AS 5′-CGAAGUAUUCCGCGUACG-TT-3′; SS 5′-CGUACGCGGAAUACUUCGATT-3′) and combined to form siRNAs **2**−**10** (Scheme 1).

Since it has been demonstrated that a RNA-like A conformation is required for effective gene silencing,[8] we first assessed whether the modified duplexes retain such a conformation. Circular dichroism (CD) spectra of the duplexes containing aromatic residues at 3′-end exhibited very similar profiles to that of the unmodified siRNA despite the presence of a hypochromic effect (see the Supporting Information), which is maximized for **5**. This behavior suggests an interaction of **b** with the bases of the RNA duplex.[17]

CD thermal denaturation experiments were also carried out to probe the thermal stability of **1**−**10** demonstrating that this was lower in **2**−**10** with respect to that of **1**, especially when the aromatic moiety was located on the AS or both strands of siRNAs (see the Supporting Information). Indeed, this was somehow expected since it has been reported that most modifications of 3′-overhang region (i.e., deletion of nucleosides or substitution with modified nucleobases, conjugation to 3′-end with lipophilic or aromatic groups) influence the thermal stability of RNA duplexes.[18] On the other hand, there is no obvious correlation between the overall duplex Tm and the associated siRNA's gene silencing activity. Rather, specific regions of siRNA duplexes have different tolerance toward stabilization and destabilization resulting in position-specific changes of activity upon incorporation of chemical modification that affect thermal stability. Overall, these results suggested that the presence of an aromatic residue at 3′-end of one or both strands poorly influences the siRNA's thermodynamic stability.

Subsequently, the ability of native siRNA **1** and modified siRNAs **2**−**10** to inhibit luciferase expression in HeLa cells at different concentrations (10, 1, and 0.1 nM) was probed. To this aim, the luciferase activities were determined 48 h after transient transfection of each siRNA along with the reporter plasmids, one encoding firefly luciferase (Luc), responsive to siRNAs, and one encoding Renilla luciferase (Rl) to normalize the data. Modified siRNAs effectively reduced firefly luciferase activity in a dose-dependent manner (Figure 1a) and appeared to have comparable or better silencing effect with respect to the unmodified siRNA **1**.

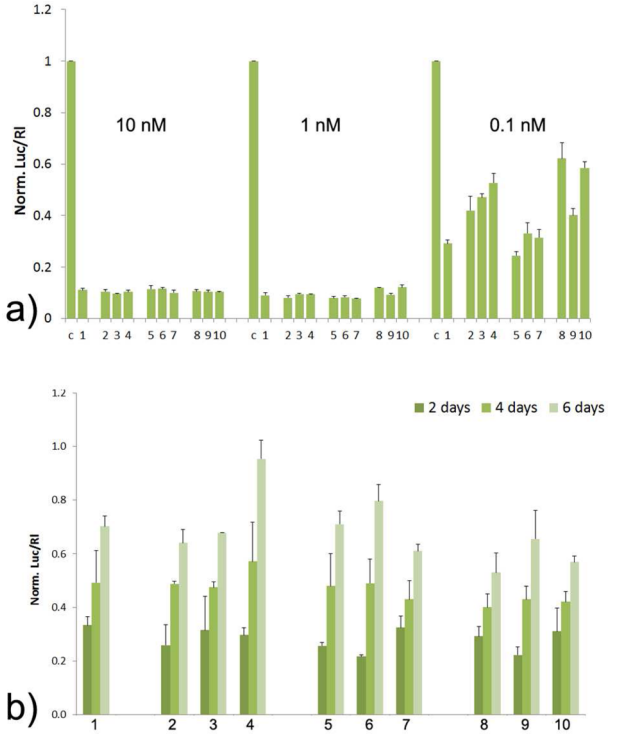


Figure 1. RNAi activity of modified siRNAs. (a) Dual Luciferase assay at 10, 1, and 0.1 nM transfected siRNAs. (b) Time−course experiments. The experimental conditions are described in the Supporting Information.

When a lower concentration (0.1 nM) of siRNAs was tested, some differences could be observed and **5**, bearing residue **b** on the AS, demonstrated better silencing activity if compared to the unmodified siRNA (Figure 1a, $p < 0.001$). Time−course experiments were also carried out to measure the persistence of silencing effect (Figure 1b). In this case, HeLa cells stably expressing firefly luciferase gene[19] were transfected with 20 nM siRNAs along with plasmid encoding Rl luciferase and luciferase activities at different days after transfection were analyzed. In particular, conjugation with **b** at 3′-overhang of AS or SS (**5** and **6**, $p < 0.05$ and $p < 0.001$, respectively) and with **c**

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on SS (**9**, $p < 0.05$) demonstrated a slight but significant increase in silencing activity, as compared to **1**, at 2 days. During the complete time−course experiment, **7**, **8**, and **10** reduced luciferase activity more than unmodified siRNA, even after 6 days (Figure 1b; **7** and **8** vs **1**, $p < 0.05$; **10** vs **1**, $p < 0.01$).

Improving the biostability is also crucial for therapeutic purposes of synthetic siRNAs. Therefore, the nuclease resistance of **2**−**10** was also investigated through 100% fetal bovine serum (FBS) experiments using unmodified siRNA as control (Figure 2). SiRNA **1** appeared to be partially degraded

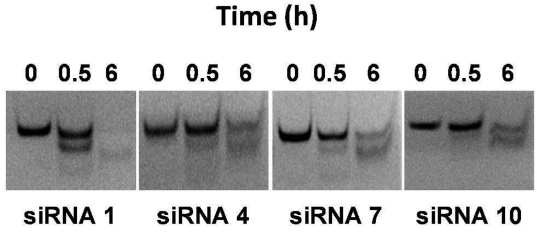

Figure 2. Serum stability of unmodified and selected modified siRNAs. The entire analysis is reported in the Supporting Information.

already after 30 min as well as siRNAs featuring only one modified strand. After 6 h of incubation, siRNA **1** was completely degraded, whereas **4**, **7**, and **10** featuring both modified strands (and at a lower extent siRNA **5**; see the Supporting Information) demonstrated an enhanced resistance to degradation with a significant population of full-length siRNAs.

Indeed, the differences observed in the performances of modified siRNAs, in terms of efficiency, potency, persistence, and biostability, should be ascribed to the nature of the aromatic moieties and to the site of conjugation.[12−14] These features can both influence the siRNA duplexes asymmetry and/or the strength of AS/PAZ domain interactions.

When **a** or **b** or **c** were conjugated to the 3′-end of SS, comparable silencing effects could be observed with respect to the unmodified siRNA (Figure 1a). In this case, these chemical modifications could influence the thermodynamic asymmetry of the siRNAs allowing the "opening" of the duplex and thus the selective loading of AS by RISC. On the contrary, the introduction of **b** and **c**, but not **a**, at the AS 3′-overhang improved the RNAi activity (see the higher silencing potency of **5** in Figure 1a at 0.1 nM and the more persistent silencing effect of **7**, **8**, and **10** in Figure 1b at 6 days). In this respect, given the previously described role of this RNA region in the silencing process, the better performances of **5**, **7**, **8**, and **10** should be ascribed to the modulation of the interactions with the PAZ domain.

To rationalize these latter data, molecular docking simulations were attained using the published X-ray structure of the Ago 2 PAZ domain of human eIF2c1 in complex with a 9-mer siRNA-like duplex (PDB: 1SI3)[5] employing the software AutoDock4.2 (AD4) as the search engine.[20] The modified siRNA ASs were constructed starting from the RNA cocrystal conformation where each nucleotide was mutated to obtain the target sequence, while at the dTdT 3′-overhang the **a**, **b**, and **c** moieties were attached. These groups as well as the dTdT 3′-overhang were free to move during the simulations. Well-defined binding poses were achieved (see the Supporting Information) where the three ASs adopted a conformation similar to that of the cocrystal RNA, while the terminal aromatic moieties were able to establish different interactions with a wide aromatic gorge of the PAZ domain (Figure 3). In particular, the **a** group was able to establish a H-bond interaction with Y336 backbone CO and well-oriented π-stacking and T-shaped interactions with Y309 and F292 side chains, respectively (Figure 3a). The same charge transfer interactions are also established and even maximized by the **b** group (Figure 3b) that feature an additional phenyl ring, which can provide a supplementary T-shaped contact with F292. In this respect, **b** should allow for a tighter binding (see Table S3 in the Supporting Information) with the Ago 2 PAZ domain so as to modulate the rates of dislodging and lodging of AS in and from the PAZ domain providing a further determining factor for siRNA efficiency and potency. This should explain why **5** is more efficient in inhibiting luciferase activity at low concentrations (0.1 nM) than the corresponding **a** analogues (**2**). Interestingly, analysis of the results achieved for the siRNA bearing the **c** moiety at the 3′-overhang of the AS (**8**) demonstrated that this aromatic group is predicted to preferentially occupy a shallower portion of the aforementioned aromatic gorge so that the indole ring is more solvent exposed (Figure 3c). In such a position, this aromatic ring can H-bond with H269 side chain and establish a cation−π interaction with R275. Indeed, the different nature of the siRNA/PAZ interactions could favor a slower release of AS, thus giving a reason for the longer persistence of the interfering effect by **8** and **10**. For the latter and for **7**, the persistent interfering activity should also be ascribed to the improved biostability.

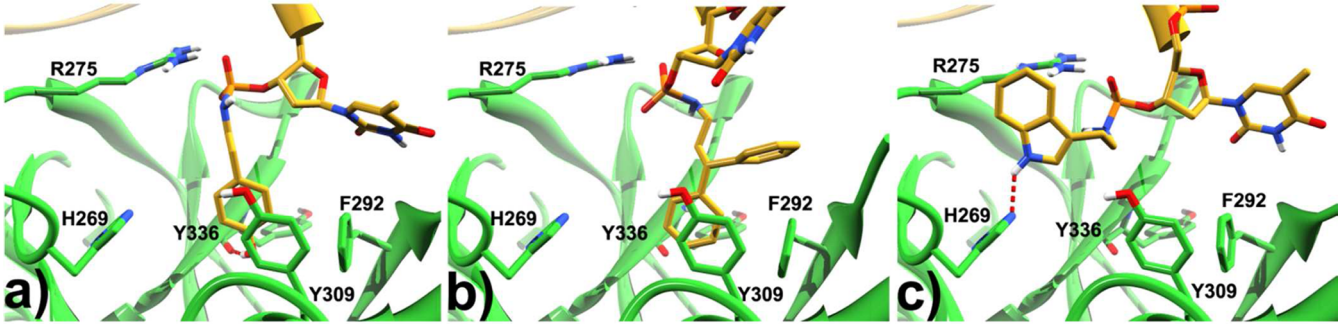

Figure 3. Close view of the predicted siRNA/PAZ complexes. The chemically modified siRNAs (bearing the **a**, **b**, and **c** moieties at the 3′ overhang: **a**, **b**, and **c**, respectively) are represented as yellow sticks, while the PAZ domain is depicted as green sticks and ribbons. H-bonds are represented as dashed red lines.

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In summary, we synthesized conjugated siRNAs possessing aromatic residues at the 3′-end of SS and/or AS. It was demonstrated that all modified siRNAs had a silencing efficiency similar to that of the unmodified one outlining that the reported chemical modifications are compatible with the RNAi machinery. The differences observed in the silencing activities of conjugated siRNAs add further information on the nature of the PAZ−siRNA interactions. In particular, it has been demonstrated that incorporation into the siRNA of tryptamine and of diphenylpropylamine at the 3′-end of AS improved the silencing activity. While the siRNA bearing the diphenylpropylamine at the 3′-end of the AS strand displayed enhanced silencing activity in terms of dose required to reach inhibition effect, the single modification with **c** of the AS strand and the double modification (AS and SS strands) with either **b** or **c** conferred to the corresponding siRNAs a more persistent silencing effect. As already described by other authors,[12−14] also in our case, the chemical modification of siRNAs at the 3′-end of SSs and ASs resulted in improved stability in serum.

The different performances observed for modified siRNAs, in terms of efficiency, potency, persistence, and biostability, can be correlated with the presence of the attached aromatic moieties and with the site of conjugation. The theoretical results presented herein highlight that the potency and the persistence of modified siRNAs are also ascribable to the AS/PAZ interactions even if the mere presence of an aromatic moiety at the 3′-end of the AS strand does not always imply higher silencing potential. In fact, the extension of the aromaticity of these moieties as well as their three-dimensional shape can be key elements for an effective interaction with PAZ. In this scenario, it can also be suggested that modulating the charge-transfer interactions with the protein binding site (i.e., electron-withdrawing substituents on the aromatic ring) would result in improved interfering activities thanks to the stabilization of siRNA/PAZ interactions. All in all, these data should stimulate the rational design of chemically modified siRNAs featuring better binding profiles and pave the way for a fine-tuning of their interfering activity.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic procedures, details of biological assay, and molecular modeling calculations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

RNAi, RNA interference; dsRNAs, double-stranded RNAs; siRNAs, small interfering RNAs; RISC, RNA-induced silencing complex; Ago 2, Argonaute 2; AS, antisense strand; SS, sense strand; FBS, fetal bovine serum; AD4, AutoDock4.2

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