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Structure-Guided Control of siRNA Off-Target Effects

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Supporting Information

ABSTRACT: Short interfering RNAs (siRNAs) are promising therapeutics that make use of the RNA interference (RNAi) pathway, but liabilities arising from the native RNA structure necessitate chemical modification for drug development. Advances in the structural characterization of components of the human RNAi pathway have enabled structure-guided optimization of siRNA properties. Here we report the 2.3 Å resolution crystal structure of human Argonaute 2 (hAgo2), a key nuclease in the RNAi pathway, bound to an siRNA guide strand bearing an unnatural triazolyl nucleotide at position 1 (g1). Unlike natural nucleotides, this analogue inserts deeply into hAgo2's central RNA binding cleft and thus is able to modulate pairing between guide and target RNAs. The affinity of the hAgo2–siRNA complex for a seed-only matched target was significantly reduced by the triazolyl modification, while the affinity for a fully matched target was unchanged. In addition, siRNA potency for off-target repression was reduced (4-fold increase in IC_{50}) by the modification, while on-target knockdown was improved (2-fold reduction in IC_{50}). Controlling siRNA on-target versus microRNA (miRNA)-like off-target potency by projection of substituent groups into the hAgo2 central cleft from g1 is a new approach to enhance siRNA selectivity with a strong structural rationale.

Short interfering RNA (siRNA)-triggered gene knockdown via the RNA interference (RNAi) pathway is routinely used to study gene function, and advanced-stage clinical trials are underway for siRNA-based therapeutics.[1,2] However, the natural RNA structure is insufficient for therapeutics, and modifications are required to stabilize siRNAs against nuclease digestion, facilitate delivery to target tissues, and reduce off-target effects.[3] Indeed, because the guide strand of an siRNA can function like a natural microRNA (miRNA), siRNAs often repress hundreds of off-target transcripts complementary to only the seed region (nucleotides 2–8) of the guide strand.[4] Thus, the development of modifications that avoid miRNA-like off-targeting is an active area of research.[5−7] Importantly, recent advances in structural studies of key protein–RNA complexes of the RNAi pathway have enabled structure-guided optimization of siRNA properties.[8,9]

We recently reported a structure-guided computational screening strategy to discover new modifications for siRNA guide strands.[10] This approach identified nucleoside analogue replacements for the 5′-most nucleotide of the guide strand (i.e., guide nucleotide 1, g1), which is bound in a pocket found in the MID domain of hAgo2, the key nuclease in the RNAi pathway. hAgo2 binds pU and pA preferentially over pG and pC at the guide strand 5′ end.[11] Interactions with the edge of the base via a rigid loop in the protein (i.e., the nucleotide selectivity loop) explain this selectivity.[9,11] Our approach identified a triazolyl nucleotide analogue (1-ER triazole I) that performs well at g1 (Figure 1).[10] However, this analogue differs substantially from a natural nucleobase, and its precise mode of binding to hAgo2 had not been determined. Furthermore, how this unusual nucleotide analogue might be accommodated in hAgo2–guide–target complexes was unknown. Here we report the 2.3 Å resolution crystal structure of hAgo2 bound to a guide strand bearing 1-ER triazole I at g1. Comparison with reported structures of the

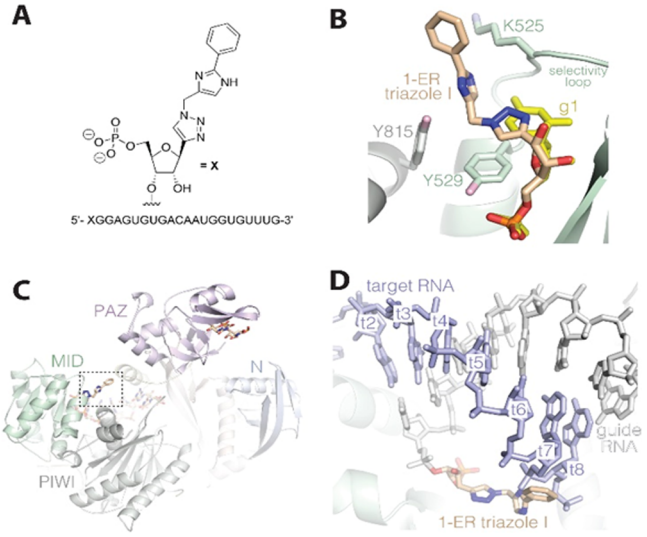

Figure 1. (A) 1-ER triazole I modification at the miR122 g1 position. (B) Binding mode of 1-ER triazole I at g1 in human Ago2 (g1 uridine shown in yellow for comparison). (C) 1-ER triazole I at g1 (inside the dashed box) extends into the Ago2 central cleft. (D) Superposition of the hAgo2/1-ER triazole I structure with the hAgo2 miRNA recognition complex reveals a likely clash between the nucleoside analogue and the target (t) strand.[9]

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hAgo2−guide−target ternary complex suggested that this and closely related analogues may be able to modulate interactions with target RNAs bound to the guide seed region.[9] Indeed, binding studies revealed that a 1-ER triazole I modification on g1 significantly reduced the affinity of the hAgo2−guide complex for a seed-paired-only RNA (miRNA target) with only marginal impact on binding and cleavage of a fully complementary RNA (siRNA target). Moreover, RNAi experiments showed that triazole g1 modifications increase siRNA potency while significantly reducing miRNA-like off-targeting in live human cells.

For crystallization in complex with hAgo2, we synthesized a 22 nucleotide guide RNA (corresponding in sequence to human miR-122) with a 1-ER triazole I nucleotide at g1 (Figure 1A).[8] Recombinant hAgo2 was loaded with the modified guide RNA, purified using a complementary 2′-*O*-methyl capture oligonucleotide, and crystallized as described previously.[9,12] Protein atoms from the original hAgo2 structure (PDB ID 4OLA) were used as the initial model of the complex, and the modified guide RNA was added during iterative rounds of model building and refinement. In the refined structure, the 5′ phosphate and ribose of the triazolyl nucleotide occupy the same position as natural g1 nucleotides, and the triazole ring stacks against the phenyl ring of Y529 like the natural nucleobases at g1 (Figure 1B). However, unlike natural nucleobases, the triazole ring does not directly contact the nucleotide selectivity loop (residues 523−527).[11] The imidazole and phenyl rings of the analogue are sandwiched between Y815 and the aliphatic portion of K525 (Figure 1B). In this binding mode, the imidazole and phenyl rings of the nucleotide analogue extend into the hAgo2 central cleft, where the guide strand pairs with complementary target mRNAs (Figure 1C).[9] Indeed, superimposing the nucleotide analogue structure onto the structure of the hAgo2−miRNA recognition complex (i.e., seed-only complementarity between the guide and target) reveals a steric clash between the analogue's imidazole and phenyl rings and the sugar−phosphate backbone of the target RNA between target strand positions 7 and 8 (t7 and t8) (Figure 1D).

We previously suggested that the central cleft of hAgo2 must open substantially during the transition from seed pairing to extended target pairing to allow a fully complementary target RNA to bind.[9] We therefore predicted that 1-ER triazole I might be better accommodated in siRNA recognition complexes than in miRNA recognition complexes. To test this hypothesis, we used an equilibrium binding assay to measure the affinity of hAgo2 loaded with a 1-ER triazole I-modified guide RNA for target RNAs with Watson−Crick complementarity to nucleotides 2−8 (seed-only target, miRNA-like recognition) or 2−21 (full-target, siRNA-like recognition). For comparison, we also measured binding of the same target RNAs to hAgo2 loaded with the equivalent unmodified guide RNA (Figure 2). For the siRNA-like target, guide strand modification had no measurable effect on the binding affinity (unmodified, K_d = 0.16 ± 0.02 nM; 1-ER triazole I-modified, K_d = 0.16 ± 0.01 nM) (Figure 2). Furthermore, 1-ER triazole I did not substantially alter the in vitro slicing activity of the guide-loaded hAgo2 complexes (Figure 2). This is consistent with our previously reported RNAi results showing that 1-ER triazole I is well-tolerated at the g1 position.[10] In contrast, the triazolyl nucleotide reduced the binding affinity to the miRNA-like target 2.5-fold compared with the unmodified guide (unmodified, K_d = 0.31 ± 0.03 nM; 1-ER triaozle I-modified, K_d = 0.76 ± 0.04 nM).

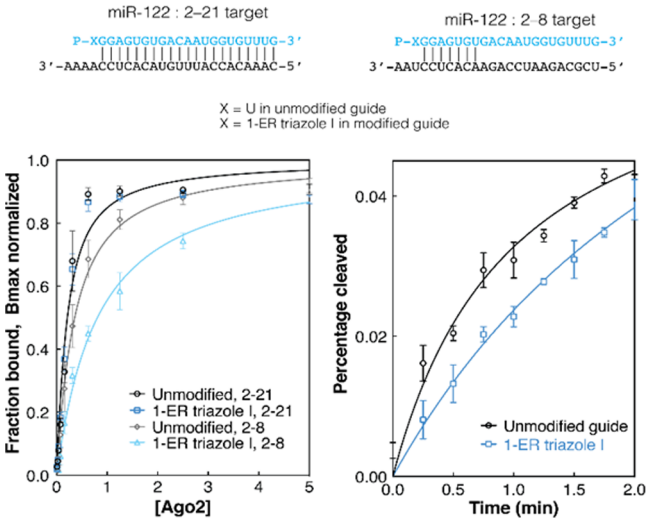

Figure 2. Binding and slicing activity of hAgo2 loaded with miR-122 bearing the 1-ER triazole I modification at g1. (left) Plot of the fraction of RNA bound as a function of guide-loaded hAgo2 concentration in nM. (right) Plot of percent target RNA cleaved as a function of time.

To determine the effect of this modification on miRNA-like off-targeting in RNAi experiments, we used an siRNA targeting the human PIK3CB messenger RNA (mRNA). This siRNA has been shown to knock down PIK3CB mRNA levels in HeLa cells as well as other endogenous transcripts with complementarity to the guide strand seed region, including the YY1 and FADD mRNAs (Figure 3A).[4] To facilitate our analysis, we generated

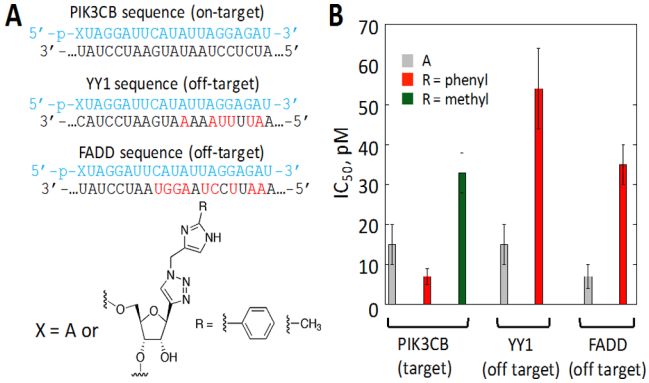

Figure 3. RNAi activity of siRNA with the 1-ER triazole I modification placed at the guide strand 5′ end and evaluated for on-target and two miRNA-like off-target sequences. (A) Sequences of PIK3CB siRNA guide strand with on-target and two off-target sequences. (B) IC_{50} values for target knockdown with unmodified (A) or modified (R = phenyl; R = methyl) siRNAs.

siRNA off-target activity reporter plasmids with copies of the YY1 and FADD sequences inserted into the 3′ UTR of the *Renilla* luciferase gene. These plasmids also encode firefly luciferase as a transfection control. In earlier work, we showed that 1-ER triazole I increases the extent of target knockdown by the PIK3CB siRNA when positioned at g1.[10] Indeed, using half-maximal inhibitory concentration (IC_{50}) values as a measure of siRNA potency, we found that the modification results in a reduction in IC_{50} for on-target knockdown from 15 ± 5 pM (unmodified) to 7 ± 2 pM (1-ER triazole I-modified) (Figure 3B). We also prepared and tested a new g1 modification wherein a methyl group replaces the phenyl group found in 1-ER triazole I

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to test the importance of the phenyl group in target knockdown (Figure 3A). The resulting siRNA had a measured IC_{50} of 41 ± 15 pM, i.e., it was approximately 6-fold less active than 1-ER triazole I-containing siRNA (Figure 3B), suggesting that the interaction of the phenyl group with the aliphatic portion of the K525 side chain contributes to siRNA efficiency (Figure 1B).

Importantly, the 1-ER triazole I modification *increased* the measured IC_{50} for off-target knockdown for both the YY1 and FADD reporter sequences (Figure 3B). For the YY1 off-target reporter, unmodified PIK3CB siRNA had an IC_{50} of 14 ± 5 pM whereas the modified siRNA had an IC_{50} of 54 ± 10 pM. For the FADD sequence, the modification increased the IC_{50} from 8 ± 3 pM (unmodified) to 35 ± 5 pM (modified). Thus, as predicted from our structural and binding studies, the 1-ER triazole I modification selectively reduces miRNA-like off-target knockdown activity while improving on-target knockdown activity. Interestingly, no off-target knockdown was observed for the siRNA bearing the methyl analogue up to a concentration of 2 nM (see Figure S1 in the Supporting Information). Thus, the methyl analogue reduced the siRNA off-target potency by over 2 orders of magnitude while reducing the on-target potency by only a factor of 3 compared with the unmodified siRNA (X = A; Figure 3B).

An important consideration in the development of therapeutic siRNAs is the seemingly unavoidable issue of off-targeting. Because siRNAs must function through hAgo2, all siRNAs will also act as miRNAs, which require only complementarity to the guide RNA seed region to recognize and silence their targets.[13] Thus, any given siRNA will typically repress dozens of unintended genes through miRNA-targeting mechanisms.[14] This complication has long been recognized as the major caveat in knockdown experiments using siRNAs in mammalian cells,[15,16] and it can be mitigated in part by introducing modifications into the siRNA seed region in order to weaken seed pairing and differentially destabilize interactions with seed-only matched targets.[5−7,17] However, biochemical and single-molecule studies have shown that the seed region plays a major role in target searches by hAgo2 and that seed pairing provides most of the affinity for stable target binding.[18−22] Here we leveraged the recent advances in structural characterization of hAgo2−guide[8] and hAgo2−guide−target complexes[9] to identify a class of g1 nucleotide analogues that can specifically reduce off-targeting without modification of the siRNA seed. Moreover, our results directly demonstrate that hAgo2 function can be modulated by projection of substituent groups into the hAgo2 central cleft, thus revealing a new approach to tuning target selectivity and controlling human RNAi.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b06137.

Experimental methods, supplementary figures and table, and ^{1}H and ^{13}C NMR data (PDF)

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Notes

The authors declare no competing financial interest.

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