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Title: Impact of Phosphorothioate Chirality on Double-stranded siRNAs: A Systematic Evaluation of Stereopure siRNA Designs

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Impact of Phosphorothioate Chirality on Double-stranded siRNAs: A Systematic Evaluation of Stereopure siRNA Designs

Sukumar Sakamuri,* Laxman Eltepu, Dingguo Liu, Son Lam, Bryan R Meade, Bin Liu, Giuseppe Dello Iacono, Ayman Kabakibi, Lena Luukkonen, Tom Leedom, Mark Foster, and Curt W Bradshaw

Abstract: Oligonucleotides are important therapeutic approaches as evidenced by recent clinical successes with anti-sense oligonucleotides (ASOs) and double-stranded siRNAs. Phosphorothioate (PS) modifications are a standard feature in the current generation of oligo therapeutics but generates isomeric mixtures leading to 2^n isomers. All currently marketed therapeutic oligonucleotides (ASOs and siRNAs) are complex isomeric mixtures. Recent chemical methodologies for stereopure PS insertions have resulted in preliminary rules for ASOs with multiple stereopure ASOs moving into clinical development. Although siRNAs have comparatively fewer PS, the field has yet to embrace the idea of stereopure siRNAs. We investigated whether the individual isomers contribute equally to *in vivo* activity of a representative siRNA. Here, we report the results of a systematic evaluation of stereopure PS incorporation into antithrombin-3 (AT3) siRNA demonstrating that individual PS isomers dramatically affect *in vivo* activity. We evaluated a standard siRNA design having six Phosphorothioate insertions and found only ~10% of the 64 possible isomers are as efficacious as the stereorandom control. Based on our data we conclude that G1*R* stereochemistry is critical, G2*R* is important, G21*S* is preferable and G22 and P1/P2 tolerates both isomers. To our surprise, the disproportionate loss of efficacy for most isomers does not translate to significant gain for the productive isomers, warrants further mechanistic studies.

Since the discovery that double-stranded siRNA can cleave mRNA and inhibit protein translation by Fire and Mello in 1998, this new class of therapeutics has moved towards clinical utility.[1] The advent of double-stranded siRNAs as a modality to harness a natural catalytic pathway called RNAi excited scientific and business communities alike because of its implications in therapeutics, particularly for targets difficult to drug using small molecules and proteins.[2] Interest in siRNAs has had periodic boom and bust cycles following recognition of and solutions for technical challenges. One of the significant challenges is delivery of these large, charged siRNA molecules across the cell membrane. Both lipid nanoparticle and ligand-based approaches are clinically validated with a recent approval of first RNAi therapeutic, Patisiran, a lipid complex from Alnylam and numerous GalNAc-targeted siRNAs in clinical trials.[3]

Naturally occurring siRNA molecules have two complementary strands (19 base pairs) with an additional two unpaired bases at the 3' ends, so called 21/21. A wide variety of variations were explored in early optimization including significant modifications in size (19- to 27-mers) and shape (DsiRNA, asymmetric and blunt ended designs).[4] In addition, the siRNA components such as sugars, nucleobases and phosphate backbone were subjected to modifications in an effort to impart nuclease stability, proper strand loading etc.[5] Ever since Kornberg and De-Clercq's early work on phosphorothioate nuclease stability, thiol modification of phosphodiester (PO) became a critical component of siRNA designs.[6] Phosphorothioates are the most atom economical modification to increase nuclease stability and, as recently reported, contribute binding to various proteins and transporters which influences biodistribution and efficacy.[7] One underappreciated outcome of PS insertions is the introduction of stereoisomers compared to the non-stereomeric PO backbone (Figure 1). Standard oligo synthesis lacks stereocontrol[8a] but the issue was ignored primarily due to the success of PS containing oligonucleotides in clinic and the lack of practical stereopure PS synthetic methodology. Stereopure PS containing oligonucleotides became a reality with the development of oxazaphospholidine methods.[8] The anti-sense oligonucleotide field was first to build on this opportunity by exploring structure activity relationships and began defining rules for stereopure PS insertions.[9]

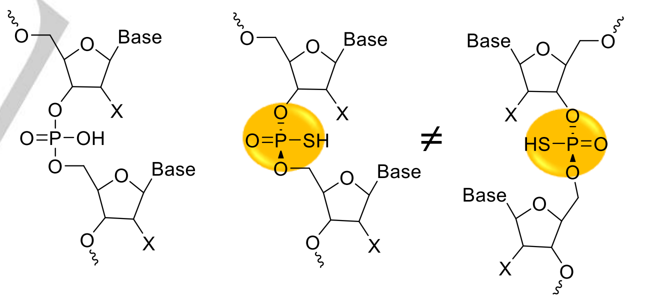

Figure 1. Generic Examples of Phosphodiester and Phosphorothioate Oligonucleotides. Phosphorothioate Isomers Shown in Highlighted Circles.

siRNAs require fewer PS insertions than ASO with 15-20% of the phosphate backbone at the 5' and/or 3' terminus of the passenger (P-strand, sense) and guide (G-strand, antisense) strands. Alnylam's antithrombin-3 (AT3) siRNA design is shown in Figure 2 with four guide strand PS insertions at G1, G2, G21 and G22, and two passenger strand PS insertions at P1 and P2.[3a, 10] For ease of comparison, we used this design for our stereopure PS evaluations. To our surprise there was no systematic evaluation of stereopure PS insertions into siRNA designs. Preliminary work by Wave Life Sciences where a single stereopure PS was introduced into the strands with limited *in vitro* data suggested a potential benefit.[11] Earlier work primarily

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Supporting information for this article is given via a link at the end of the document.

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focused on DNA, methods to generate stereopure PS insertions, with minimal/no systematic work carried out on double-stranded siRNAs.[8c, 9b, 12] This prompted us to systematically evaluate the effect of PS chirality on the function of double-stranded siRNA and identify a practical method to synthesize stereopure PS containing oligonucleotides. This article summarizes our efforts on the former topic and the latter is forthcoming.[16b]

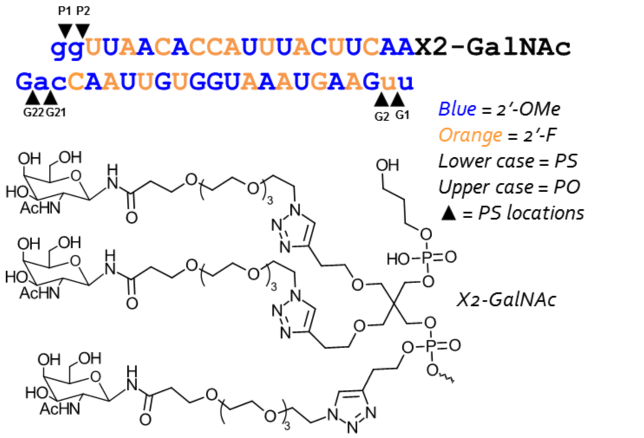
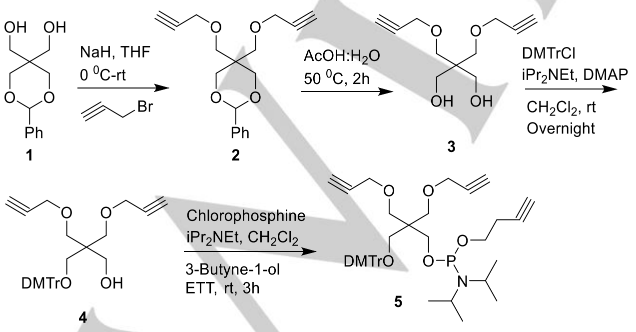
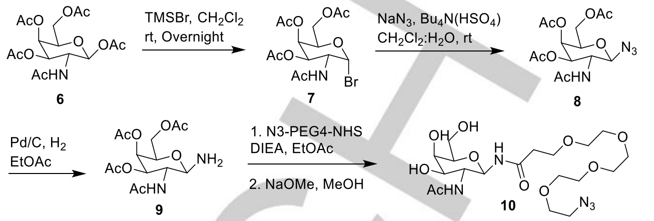


Figure 2. Structures of siRNA and X2 linker-GalNAc. P1, P2, G1, G2, G21 and G22 Identify PS Locations. Structure of X2-GalNAc Used in this Study is Attached to Passenger Strand 3'-End.

For our current investigation, we evaluated an AT3 siRNA as shown in Figure 2 which features fully 2'-modified sequences having six phosphorothioates, two at 5'- end of the passenger strand, the 5'-, and 3'- ends of the guide strand.[10] Monomeric GalNAc ligands were linked to novel X2 linker via click conjugation.[13] Synthesis of X2-linker phosphoramidite **5** and monomeric GalNAc-azide **10** is illustrated in Scheme 1 and 2 respectively. Experimental protocols along with assembly of oligonucleotides, click conjugation and biological testing can be found in the supporting information. We used Wada's oxazaphospholidine P(III) chemistry, well known for excellent diastereoselectivity ($\geq$99%) to insert stereopure PS into 2'-substituted (2'-OTBDMS, 2'-OMOE, 2'-OCEM and 2'-Deoxy) oligonucleotides.[8b-f]



Scheme 1. Synthesis of X2-Phosphoramidite (**5**).



Scheme 2. Synthesis of Monomeric GalNAc-Azide (**10**).

Our systematic investigation started with guide (G)-strand PS insertions followed by passenger (P)-strand insertions and then combining G-strand and P-strand variations to generate stereopure siRNAs. Synthesis of all sixty-four (2^6) stereopure isomers generated from 6 PS insertions is required to establish the role of chirality at individual locations as well as interdependency from different locations. The X2-linker insertion also created a new phosphotriester stereocenter. To rule out any potential role of phosphotriester chirality on P-strand 3'-GalNAc conjugates we determined that chiral phosphotriester-GalNAc conjugates had no measurable effect on biological outcome (unpublished data). It's important to highlight that our X2-linker and GalNAc monomer were synthesized in 4 and 5 simple steps respectively. Trivalent GalNAc conjugate is made by click reaction of monomer GalNAc-azide (**10**) to X2-linked oligonucleotide.

All isomers of AT3 siRNA-GalNAc conjugates and controls were tested *in vitro* in primary mouse hepatocytes and *in vivo* (protocols described in supplementary information). Our preliminary observations of *in vitro* and *in vivo* data suggested similar trends (Figure 5a and Table 2, supplementary information), but the narrow range of *in vitro* data precluded meaningful conclusions. We primarily limited the *in vitro* data to general comparisons.

We opted to focus on *in vivo* studies which demonstrate a combination of stability and duration of action to understand potential therapeutic benefits. In early experiments, we compared the impact of compounds on mouse hepatocyte AT3 gene expression as well as on secreted AT3 protein in the circulation. We found nearly perfect correlation between the two parameters and thus limited all subsequent experiments to measuring plasma AT3 activity. We tested all compounds *in vitro* at three concentrations (1, 3.3, and 10.0 nM) and dosed *in vivo* at 0.25 mg/kg which represented the EC_{50} value for AT3 mRNA knockdown of the racemic control SB1391. Statistical analysis on some of the compounds efficacy is included in the supplementary information.

We first explored guide-strand PS insertions at G1 and/or G2 (Table 1). Unexpectedly, the *in vivo* data revealed nearly half of the isomers were not efficacious or poorly efficacious (Figure 3 and Table 2) indicating a critical role for guide-strand 5'- PS stereochemistry for the sequence evaluated. In particular, G1*S*

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PS stereochemistry is detrimental to the efficacy and was common to all less efficacious/inactive molecules. We can infer that half of all isomers (32/64) in a typical SB1391 design contribute very little to *in vivo* pharmacology. It is important to note some of these isomers with G1*S* PS stereochemistry show moderate *in vitro* activity (supplementary information, Table 2). It is known that *R*- and *S*- PS isomers of DNA have different nuclease stability and binding characteristics.[6c] Our data highlight the efficacy of stereopure PS insertion at guide-strand G1 position does not depend entirely on nuclease stability. It is possible 5' guide-strand PS stereochemistry could influence the G1 phosphorylation required for RISC loading or Ago2 binding.

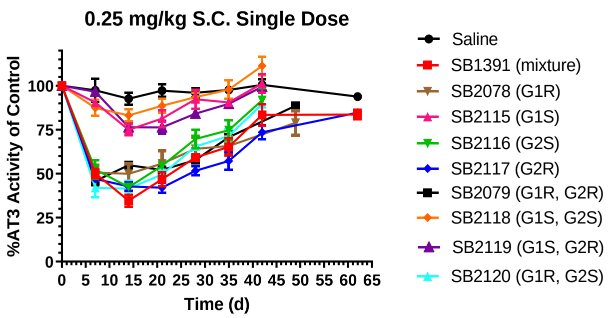

Figure 3. Mouse *In Vivo* Efficacy Data of G-Strand 5'-Stereopure PS Insertions Show G1*S* is Detrimental to Efficacy.

Next, we examined stereopure PS insertions at guide-strand 3'-locations, G21 and G22 in isolation with all other PS racemic. The impact of PS stereochemistry in this data set (Figure 4 and Table 2) was less pronounced than guide-strand 5'-end modifications but mirrors likely higher nuclease stability of *S*-isomers as the *S*- isomer combinations were found to be more efficacious with longer duration of action. For example, SB2214 (*SS* isomer) and SB2217 (*RR* isomer) have similar *in vitro* potency but significantly different *in vivo* profiles (Table 2 and Figure 4). In addition, as an independent location or in combination with other G-strand 3'- stereopure PS positions, G21*S* was preferred over G21*R* (Figure 4SI, Figure 5SI, Supplementary info).

Since both 5'- and 3'- G-strand isomers contributed to *in vivo* profile, we next combined stereopure insertions at all four locations while maintaining a racemic passenger strand. Strikingly, twelve of the sixteen siRNA isomers made, only three stereopure G-strands SB2221 (G1*R*, G2*R*, G21*R*, G22*S*), *SB2222 (G1R, G2R, G21S, G22R) and SB2223 (G1R, G2R, G21S, G22S)* showed comparable or superior efficacy versus racemic control SB1391 (Figure 5 and Table 2). The remaining isomers were not efficacious or less efficacious implying the *in vivo* activity of the racemic mixture significantly relies upon a fraction of the total drug product. All molecules with poor efficacy contain G1*S* stereochemistry, further confirming our learning from Figure 3. Based on this data we concluded that G1*R* stereochemistry is critical, G2*R* is important, G21*S* is preferable and G22 tolerates *R*- and *S*- isomers.

As the siRNA field continues to modify the pattern of 2'-F and 2'-OMe ribose substitutions, generally increasing the 2'-OMe/2'-F ratio[14], we optimized 2'-OMe substitutions on the same AT3 siRNA sequence. We investigated whether the superior chiral PS performance would hold for SB2333 with only seven 2'-F substitutions versus nineteen in SB1391 (Table 1 and Figure 6). Molecule SB2477 incorporated the improved 2'OMe/2'-F ratio and the chiral PS pattern from SB2222 (G1*R*, G2*R*, G21*S*, G22*R*; Table 1). SB2477 recapitulated the strong *in vivo* efficacy of SB2222 suggesting the translatability of our findings to the current 2'-mod pattern (Figure 7 and Figure 7SI in Supplementary info).

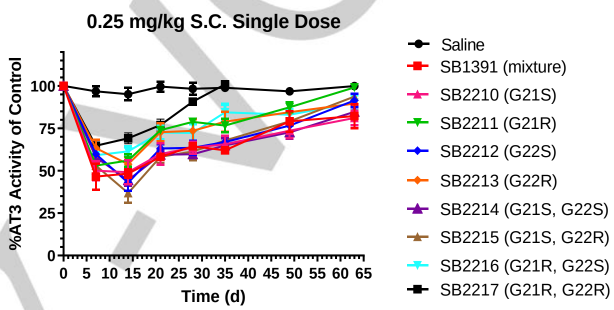

Figure 4. Mouse *In Vivo* Efficacy Data of G-Strand 3'-Stereopure PS Insertions Show G21*S*,G22*S* Preferred Orver *R*-Isomers.

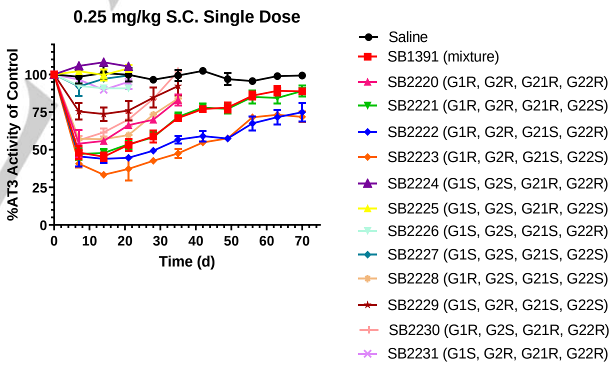

Figure 5. Mouse *In Vivo* Efficacy Data of Steropure G-Strand PS Insertions Demonstrate only SB2221, SB2222 and SB2223 Show Comparable or Superior Efficacy Than Sterorandom control (SB1391).

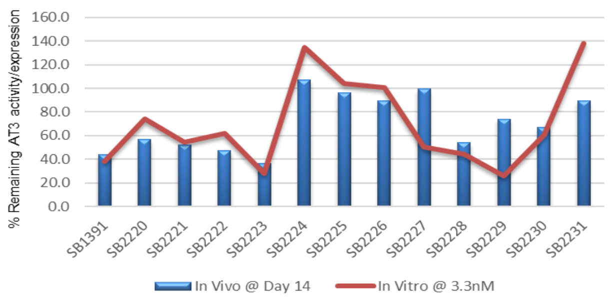

Figure 5a. *In Vitro and In Vivo* Data (Mouse) Comparison of Steropure G-Strand PS Insertions Show Similar Trends.

P4458/G3504	ggUUAACACCAUUUACUUCAAX2-GalNAc GacCAAUUGUGGUAAAUGAAGuu	SB-1391
P5727/G5728	ggUUAACACCAUUUACUUCAAX2-GalNAc GacCAAUUGUGGUAAAUGAAGuu	SB-2333 SB 2'Mod

Figure 6. AT3 siRNA Sequences With Varying 2'-OMe Modifications.

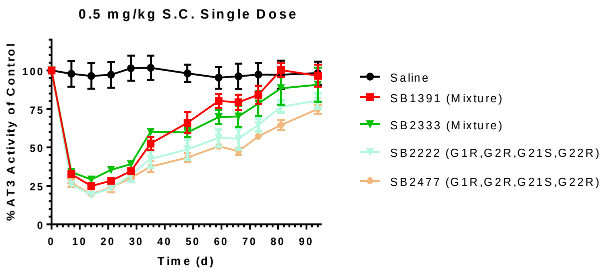

Figure 7. Mouse *In Vivo* Efficacy Data Comparison of Steropure G-Strand PS Insertions With Two Different 2'-Mod Patterns Confrim Translatability of Our Learnings .

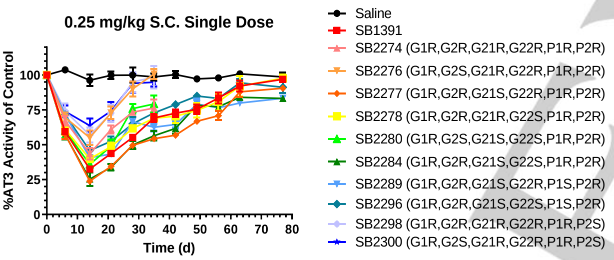

Figure 8a. Mouse *In Vivo* Efficacy Data of Steropure siRNAs Show Only a Small Number of Isomers Contribute to Efficacy.

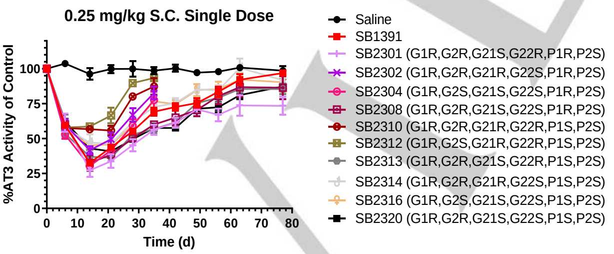

Figure 8b. Mouse *In Vivo* Efficacy Data of Steropure siRNAs Show Only a Small Number of Isomers Contribute to Efficacy.

Finally, we prepared a variety of single isomer siRNAs (6 chiral PS insertions) by combining stereopure passenger-strands and stereopure guide-strands (Figure 8a, Figure 8b and Table 2). Based on our findings that G1*S* is detrimental to efficacy, we limited this set to G1*R* containing isomers reducing the total compounds to 32, of which 25 were synthesized. Consistent with earlier data, a significant number of stereopure siRNAs have poor efficacy when compared to the stereorandom control SB1391. Only a subset of stereopure siRNAs (SB2277, SB2284, SB2301, SB2308, SB2313 and SB2320) were comparable in efficacy to the control. To our surprise, the disproportionate loss of efficacy for most isomers did not translate to significant gain for the active isomers. It is possible that extracellular stability, unknown uptake mechanisms, limited Ago2 binding/loading, selective transport of preferred isomers out of the endosome and other factors may be influencing the stereopure siRNA efficacy and warrants further mechanistic investigation. We also confirmed the impact of increased 2'-OMe/2'F ratio and chiral PS insertions of key AT3 sequence and an additional gene target (PCSK9) in cynomolgus monkey primary hepatocytes (Table 1 and Table 3).

Since it appears that only ~10% of the 64 possible isomers disproportionately contribute to efficacy, future studies would be important to understand underlining reasons to help design better molecules. As advances in siRNA dosing and delivery beyond liver into more technically demanding tissues emerge, the contribution of isomers to clinical profile may increase in importance.[15]

In summary, our systematic evaluation of stereopure PS insertions into double-stranded siRNA demonstrated only certain isomers have comparable efficacy to the stereorandom control. Based on our data we conclude that G1*R* stereochemistry is critical, G2*R* is important, G21*S* is preferable, G22 and P1/P2 tolerates both isomers. Importantly, the approaches translate across two different sequences and ribose 2'-modification patterns using 2'-Fluoro and 2'-OMe substitutions. In addition, recent progress towards simplified synthetic methods[16] could complement our studies and are a step in the right direction towards stereopure siRNA therapeutics.

Keywords: Oligonucleotides • Stereopure Phosphorothioates • Stereopure siRNA Design •

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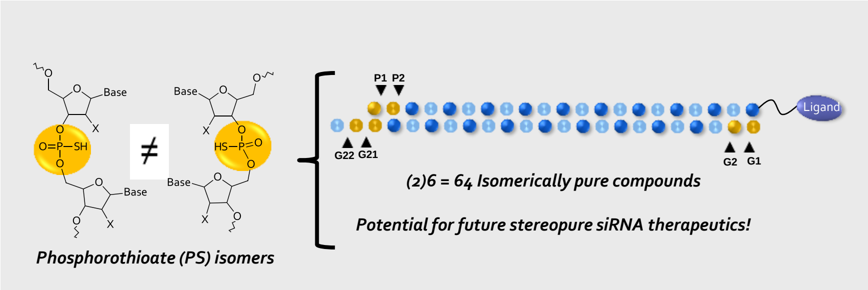

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Impact of Phosphorothioate Chirality on Double-stranded siRNAs: A Systematic Evaluation of Stereopure siRNA Designs

Current siRNA designs contain phosphorothioate modifications for nuclease stability generating isomeric mixtures. We carried out first systematic evaluation to understand the impact of stereopure phosphorothioate incorporations into siRNA and found only ~10% of the 64 possible isomers disproportionately contribute to efficacy. We defined preliminary design rules for stereopure siRNAs.