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Properties of Stereopure Phosphorothioate Groups in RNA-PROTACs

Mathilde Vincent[§] and Jonathan Hall*

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Abstract: Proteolysis-targeting chimeras (PROTACs) are an emerging therapeutic modality that enable the degradation of target proteins *via* the endogenous ubiquitin–proteasome pathway. We are applying this concept to the degradation of RNA-binding proteins (RBPs), which often lack drug-like binding pockets for small molecules. When targeting RBPs with RNA-PROTACs, the targeting ligand consists of short, chemically modified oligoribonucleotides that are iso-sequential with endogenous RNA consensus sequences. Specifically, we are investigating the phosphorothioate (PS) backbone, in which a sulfur atom replaces a non-bridging oxygen in the RNA phosphodiester linkage. PS modifications enhance stability against nucleases, but introduce chirality at the phosphorus atom, when introduced using conventional synthesis reagents, generating diastereoisomers whose stereochemistry can significantly enhance RNA–protein interactions.

Keywords: Oligonucleotides · Phosphorothioate · PROTACs · RNA-binding proteins · Stereochemistry

Mathilde Vincent received her MSc in Chemistry-Chemical Engineering from CPE Lyon. She is a PhD student in the group of Prof. Jonathan Hall at ETH Zürich and investigates the influence of phosphorothioate group stereochemistry in RNA-protein interactions.

Jonathan Hall received his PhD at Imperial College in London in 1988. He did post-doctoral work with J.-M. Lehn in Strasbourg and with Y. Kishi in Cambridge (USA). He joined Novartis in Basel in 1992. In 2007 he moved to ETH Zurich as Professor of Pharmaceutical Chemistry. A principal goal of his group is to help bring RNA as a drug and a target into mainstream pharmaceutical research.

1. RNA-PROTACs to Degrade RNA-binding Proteins

Proteolysis-targeting chimeras (PROTACs) are a novel drug modality designed to degrade targeted proteins of interest (POI) by sequestering the endogenous ubiquitin–proteasome system (UPS).[1] These heterobifunctional molecules consist of three elements: a ligand for the POI, a binder for an E3 ligase, and a linker connecting the two. PROTACs bring the POI and the E3 ligase into close proximity, forming a ternary complex that enables the transfer of ubiquitin onto the surface lysine residues of the POI. Polyubiquitination of the POI then signals the cell's protein-recycling machinery, the 26S proteasome, to degrade the POI. As opposed to classical small-molecule inhibitors, which rely on an 'occupancy-driven' approach, PROTACs follow an 'event-driven' mechanism, eliminating the POI from cells in a catalytic manner.[2] The first peptide-based PROTAC was reported in 2001 by Crews and coworkers, who demonstrated the degradation of methionine aminopeptidase-2 (MetAP-2).[3] Since then, small-molecule PROTACs with improved pharmacokinetic and pharmacodynamic properties have emerged, enabling rapid expansion of the field. To date, more than 30 PROTACs have advanced into clinical trials. In 2025, a major milestone was reached when the American Food and Drug Agency accepted a new drug application for the orally bioavailable PROTAC Vepdegestrant (ARV-471) for the treatment of patients with estrogen receptor ER+/HER2- or metastatic breast cancer.[4]

Our lab has demonstrated the targeted degradation of RNA-binding proteins (RBPs) using an RNA PROTAC-based strategy; specifically, we showed that the cytosolic form of the Lin28 RBP can be selectively degraded through the rational design of an RNA-PROTAC (Fig. 1A).[5] Several classes of oligonucleotide-based PROTACs have since been reported.[6] RBPs play key roles in cellular homeostasis, and the dysregulation of several RBPs, such as TDP-43 and FUS, is implicated in neurodegenerative diseases.[7] Targeting RBPs with small molecules is challenging because they lack small-molecule binding pockets.[8] However, a short oligoribonucleotide ligand that mimics their RNA-binding element (RBE) can be used as the targeting warhead. Since RNA is quickly degraded by nucleases *in vivo*, chemical modifications are required to improve its stability while maintaining binding affinity to the RBP. One of the most widely used modifications is the phosphorothioate (PS) linkage, in which a sulfur atom replaces a non-bridging oxygen in the phosphodiester backbone (Fig. 1B).[9] PS groups are widely incorporated into oligonucleotide therapeutics, as they enhance nuclease stability and biodistribution.[10] Despite its simplicity, PS modifications introduce chirality at the phosphorus atom, creating a mixture of discrete diastereoisomers in the oligonucleotide. Since its introduction in the late 1960s, efforts have been made to better understand the role of PS stereochemistry in terms of activity, stability, and overall therapeutic efficacy. This short article aims to provide a concise, non-exhaustive overview

Correspondence: Prof. Dr. J. Hall, E-mail: jonathan.hall@pharma.ethz.ch
Department of Chemistry and Applied Biosciences, ETH Zurich, Zurich, CH-8093, Switzerland

of: (i) how to introduce PS modifications into an oligonucleotide in a stereopure manner; and (ii) how the stereochemistry of PS-modified oligonucleotides influences their interactions with proteins.

2. Stereocontrolled Chemical Synthesis of PS Oligonucleotides

When PS groups are introduced into 2'-*O*-modified oligonucleotides using conventional phosphoramidite chemistry, a mixture of diastereoisomers is typically obtained. Tetrazole is commonly employed as a weakly acidic activator and nucleophilic catalyst to activate the phosphoramidite, however, repeated nucleophilic attack at the P(III) stereocenter by the activator results in the formation of an *Sp*/*Rp* diastereomeric mixture. Depending on the activator and the 2′-modification, a bias toward one configuration may be observed (Fig. 1B, left).[11] Various chemical strategies have been developed to enable the synthesis of stereocontrolled PS linkages. For instance, HPLC-based methods have demonstrated the feasibility of diastereomeric separation, however, these approaches are generally limited to oligonucleotides containing a single PS modification,[12] or not more than five PS linkages.[13] Since the early 2000s, multiple groups have pursued the synthesis of stereopure PS oligonucleotides with high stereoselectivity and moderate yields. Among these, the bicyclic oxazaphospholidine (OAP) approach is one of the most extensively developed methods (Fig. 1B, right), employing an auxiliary derived from chiral 1,2-amino alcohols.[14]

3. Influence of PS Stereochemistry on Protein Binding

3.1 *Chemical Properties of the PS Moiety*

In a PS linkage, the negative charge is preferentially localised on the sulfur atom.[15] In addition, PS groups are generally more hydrophobic and less hydrophilic than their PO counterparts. This difference arises in part from the lower electronegativity of sulfur relative to oxygen (2.58 *vs.* 3.44 on the Pauling scale), which reflects the larger size of sulfur (atomic radius: 88 pm *vs.* 48 pm). As a result, sulfur is more polarizable, meaning that its electron cloud can be more readily distorted. Changes in negative charge localisation and the bulkier nature of sulfur compared to oxygen alters the steric and electrostatic environment of the phosphorothioate linkage, such that one diastereomer may exhibit enhanced interactions with protein residues. This suggests that certain diastereomers could display superior binding affinity towards the protein of interest.

3.2 *PS Modifications in Bacterial DNA*

Beyond its use in medicinal chemistry, PS modifications also occur naturally in bacterial DNA, where they are thought to participate in host defence mechanisms.[16] Similar to modifications observed in DNA and RNA nucleobases during transcription and translation, such as methylation, bacteria can introduce backbone modifications into DNA. The *dnd* gene cluster mediates the stereospecific installation of PS linkages into double-stranded DNA (dsDNA) at defined sequence motifs. These PS modifications enable specific recognition by the type IV restriction endonuclease (REase) ScoA3McrA, which cleaves DNA 16–28 nucleotides distal from the PS linkage. Structural studies by Liu *et al*. revealed the molecular basis for this stereospecific recognition.[17] The crystal structure of the sulfur binding domain (SBD) of ScoA3McrA in complex with *Rp* PS dsDNA shows a unique hydrophobic cavity that encapsulates the sulfur atom (Fig. 2C). Several hydrophobic interactions are formed between the sulfur atom and His116, Arg117, and Tyr164 residues as well as with the pyrrolidine ring of Pro165. In contrast, *Sp* PS-modified dsDNA is not cleaved, highlighting the importance of PS stereochemistry in biological applications.

3.3 *Stereopure PS Linkages in Oligonucleotide Therapeutics*

Aptamers and decoy oligonucleotides must exhibit high affinity for their target proteins. Researchers have investigated whether a specific PS diastereomer can enhance protein binding. In one study, Yamasaki *et al.* used the transcription factor SATB1, which is associated with various types of cancer.[18] The DNA-binding motif CUTr1 of SATB1 was co-crystallized with a dodecamer dsDNA containing six racemic PS linkages. This enabled quantification of stereochemical preferences at individual PS positions. The largest difference in diastereomer population was observed at the G1C2 PS linkage, where the *Rp* configuration was favoured at a 4:1 ratio (Fig. 2A). In this arrangement, the sulfur atom forms hydrophobic contacts with Leu404 and Leu422, while the oxygen engages in a hydrogen

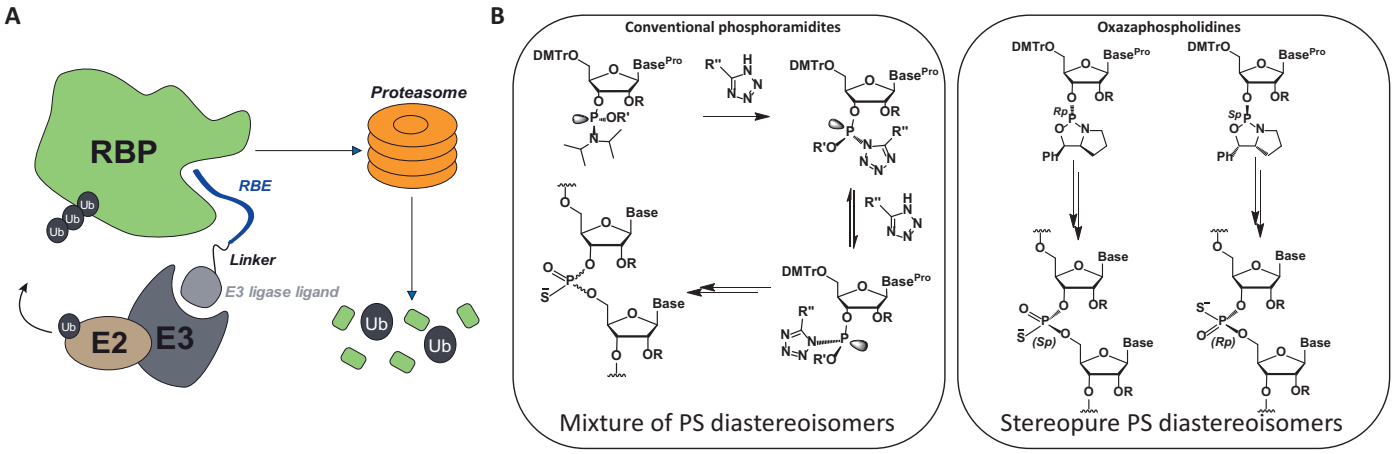

Fig. 1. **PS-modified RNA-PROTAC**. A) Schematic representation of an RNA-PROTAC. The construct consists of a chemically modified RNA sequence (the mimic of the RNA-binding element (RBE), depicted in blue) that binds to an RNA-binding protein (RBP). A linker (shown in black) connects the RBE to an E3 ligase ligand (grey). Formation of the ternary complex enables ubiquitin transfer, tagging the RBP for degradation by the 26S proteasome. B) Chemical structures of conventional phosphoramidites typically used in solid-phase oligonucleotide synthesis, which result in the formation of multiple diastereoisomers for a given phosphorothioate-modified sequence. R: CH_3 or $CH_2CH_2OCH_3$, R′ = CH_2CH_2CN; R″ = SCH_2Ph. The box on the right depicts oxazaphospholidine (OAP) phosphoramidites, which bear a chiral auxiliary that enables high PS stereoselectivity when used with a less nucleophilic activator.

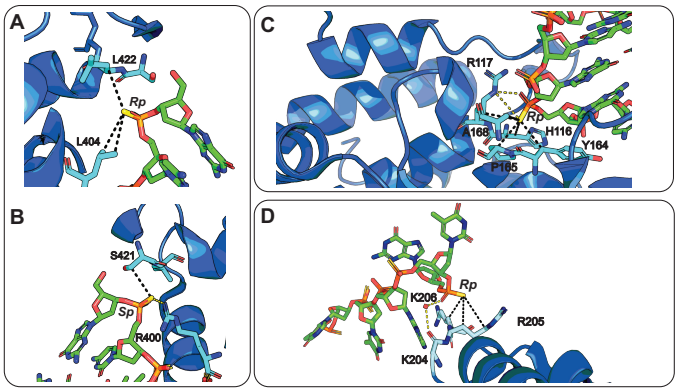

Fig. 2. **Structural studies on stereopure PS-containing oligonucleotides and their interactions with proteins**. A) X-ray structure of a PS-modified DNA dodecamer bound to the CUTr1 protein. The *Rp* PS linkage between G1 and C2 forms hydrophobic contacts with L404 and L422. B) Same complex showing the *Sp* PS configuration, which instead forms a salt bridge with R400 (PDB: 6LFF). C) *Rp* PS modified dsDNA bound to the SBD of ScoMcrA (PDB: 5ZMO). Only the *Rp* configuration fits into the hydrophobic cavity. The sulfur atom creates hydrophobic interactions with P165, A168, R117, H116, and Y164. D) ASO co-crystallized with AnxA2 (PDB: 7ZVN). The *Rp* PS linkage at dT14-dG15 shows enhanced interactions between the sulfur atom and K206 and R205, while the pro-*Sp* oxygen forms a water-mediated contact with the backbone amide of K204. Proteins are shown as blue cartoons and DNA in green. Sulfur atoms are shown in yellow. Dashed black lines indicate hydrophobic interactions, dashed yellow lines electrostatic interactions. Structures were visualized using PyMOL (Schrödinger, LLC, version 3.1.6.1).

bond with Asn425. In contrast, the *Sp* isomer (Fig. 2B) exhibits fewer hydrophobic interactions and instead forms a salt bridge between the sulfur atom and Arg400, resulting in overall weaker binding. In a related study, the Antp homeodomain was co-crystalized with dsDNA containing a single stereopure *Rp* or *Sp* PS linkage.[19] Fluorescence anisotropy measurements revealed approximately two-fold stronger binding for the *Rp* isomer. Complementary NMR data suggested that enhanced affinity arises from favourable interactions between the sulfur atom and a nearby Lys side chain (K57).

Given that the fate of oligonucleotide drugs can be strongly influenced by their interactions with proteins, further investigations into the enhanced binding affinity of PS antisense oligonucleotides (ASOs) have been conducted. The DNA-binding transcription factor PC4 was co-crystallized with a PS ASO.[20] This study revealed that PS groups lead to enhanced binding affinity through dual electrostatic and hydrophobic interactions between the sulfur atom and arginine and lysine residues. In another study using annexin A2 (AnxA2), a non–EF-hand calcium-binding protein lacking a canonical DNA/RNA-binding domain, researchers confirmed that PS–protein interactions are generally driven by van der Waals contacts between the sulfur atom and the hydrophobic portions of lysine and arginine side chains (Fig. 2D).[21]

Other studies have demonstrated that PS stereochemistry at specific backbone positions can significantly influence RNA/DNA–protein interactions. For instance, the *Sp* PS isomer generally exhibits higher nuclease resistance because the sulfur atom in this configuration can disrupt the enzyme's catalytic site.[22] In an early study of RNA hairpins bound to the MS2 coat protein, six out of thirteen single PS substitutions significantly affected binding affinity, with one *Rp*-modified position showing a 20-fold increase compared to the unmodified RNA.[23] However, PS stereochemistry does not universally dictate protein binding. For example, fully modified all-*Rp* and all-*Sp* PS dT19 oligonucleotides exhibited similar binding affinities to recombinant CD4 protein, laminin, fibronectin, and basic fibroblast growth factor.[24]

4. Conclusions

Previous work has established the importance of controlling the stereochemistry of the PS moiety to fine-tune its interactions with nearby amino acid residues. The sulfur atom, being more hydrophobic and polarizable than oxygen, can engage in favourable interactions with hydrophobic residues, such as with the methylene groups of arginine and lysine side chains. In addition, despite this increased hydrophobic character, the PS group retains a negative charge and is thus capable of forming electrostatic interactions and H-bonds with polar residues. Overall, these findings underscore the importance of carefully evaluating the impact of PS stereochemistry within the RNA-binding element of an RNA-PROTAC, as its effects appear to be context-dependent.

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