Stereochemical bias introduced during RNA synthesis modulates the activity of phosphorothioate siRNAs

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An established means of improving the pharmacokinetics properties of oligoribonucleotides (ORNs) is to exchange their phosphodiester linkages for phosphorothioates (PSs). However, this strategy has not been pursued for small interfering RNAs (siRNAs), possibly because of sporadic reports that PS siRNAs show reduced inhibitory activity. The PS group is chiral at phosphorous (Rp/Sp centres), and conventional solid-phase synthesis of PS ORNs produces a population of diastereoisomers. Here we show that the choice of the activating agent for the synthesis of a PS ORN influences the Rp/Sp ratio of PS linkages throughout the strand. Furthermore, PS siRNAs composed of ORNs with a higher fraction of Rp centres show greater resistance to nucleases in serum and are more effective inhibitors in cells than their Sp counterparts. The finding that a stereochemically biased population of ORN diastereoisomers can be synthesized and exploited pharmacologically is important because uniform PS modification of siRNAs may provide a useful compromise of their pharmacokinetics and pharmacodynamics properties in RNAi therapeutics.
Chemically modified oligoribonucleotide (ORN) drugs represent an emerging class of pharmaceuticals of which, arguably, the most important is the small interfering RNAs (siRNA). siRNAs are duplexes composed of complementary ORNs of ~21 nucleotides (nt), with 2-nt overhangs. One of the ORNs (the ‘guide’ strand) directs the RNA-induced silencing complex (RISC) to cleave complementary mRNA targets and thereby represses gene expression, whereas the other ORN (passenger strand) is discarded. For most applications in vivo (passenger strand) is discarded. For most applications, we investigate whether the control of stereochemistry of PS ORN strands in a siRNA can be exploited to improve their pharmacological properties. We show here that the choice of the activating agent during synthesis of a PS RNA shifts the Rp/Sp ratio in the PS linkages throughout an ORN and when assembled into duplexes, this bias transforms the PK and PD activities of PS siRNAs. To our knowledge, this account represents the first demonstration of how a stereochemically biased population of PS siRNAs can be prepared and exploited to optimize its pharmacological profile. The findings of the study suggest that the PS modification of siRNA drugs may provide a useful compromise of PK and PD properties for in vivo applications. In addition, they encourage continued efforts towards the synthesis of full stereochemically pure PS ORNs.

Results
Tetroazole activators bias Rp/Sp ratios in PS dinucleotides. Solid-phase synthesis protocols of ORNs typically use tetroazole reagents to facilitate the attack of the ribose 5’-hydroxyl group on the activated phosphorous centre (Fig. 1). Tetrozaoles act as weakly acidic and nucleophilic catalysts during the displacement of diisopropylamine at the PIII-stereocentre by the 5’-hydroxyl

Figure 1 | Mechanism and stereochemical course of phosphoramidite coupling during solid-phase ORN synthesis. Epimerization at P occurs at the tetroazole stage (R, Si-tert-BuMe2; R’, CH2CH2CN; R”, H or SCH2Ph; B, nucleobases adenine, guanine, cytosine and uracil; CPG, controlled pore glass solid support).
group of the support-bound ribonucleoside. The final oxidative sulfurization step is stereoretentive, at least in the case of oligodeoxyribonucleotides (ODNs)19,20.

We began the investigation with a study of PS diribonucleotides, following the example of a limited investigation using 2′-O-methoxymethyl (MOE) ribonucleotides21. We synthesized PS uridine dinucleotide (UsU) using standard 2′-O-tert-butylimidethylsilyl (TBDMS)-protected phosphoramidites and 5-benzylthio-1-H-tetrazole (BTT) as an activator. The diastereoisomeric products were separated using reverse-phase high-performance liquid chromatography (RP-HPLC), which showed an ~2:1 ratio of diastereoisomers (Fig. 2a). The major isomer was assigned the Rp stereochemistry, as incubation of an equimolar mixture of isomers with nuclease P1 (nP1) attenuated the slower migrating (slow) isomer was assigned the inactive activator, dicyanoimidazole was investigated but gave unsatisfactory product yields. We determined the diastereoisomeric ratios of the products using the nP1/RP-HPLC method (Supplementary Fig. 5). For BTT the respective Rp isomer was the dominant product in all cases except GsG (Fig. 3a, Supplementary Table 2), whereas for tetrazole the trend was reversed, that is, the Sp isomers were the major products (Fig. 3b, Supplementary Table 3). The difference in the product profiles may represent different states of the thermodynamic equilibria between tetrazolides 3 and 4 before coupling; however, a kinetic resolution of the rapidly equilibrating 3 and 4 following a Curtin–Hammett principle cannot be discounted (Fig. 1). To further investigate the importance of the 2′-substituent on the Rp/Sp product ratio, we synthesized six UsU-containing PS dinucleotide from DNA and from 2′-F-, 2′-O-Me-, MOE-, 2′-O-[(triisopropylsilyl)oxy]methyl (TOM)- and 2′-O-bis(2-acetoxyethoxy)methyl (ACE)-substituted phosphoramidites, using BTT and tetrazole. In each case we assumed that the stereochemical outcome of the coupling reaction was independent of the Rp/Sp isomeric mixture of the starting phosphoramidites, as shown for 2′-O-TBDMS diribonucleotides (Fig. 2c,d) and for tetrazole-mediated coupling of DNA26.

The 2′-O-TOM protecting group, which presents a relatively low steric hindrance to phosphoramidite coupling27, produced a slight excess of the faster HPLC-running isomer (presumably Rp), whereas tetrazole was unselective. In the case of DNA, which differs from RNA by its C2′-endo conformation and

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**Figure 2 | Properties of PS diribonucleotides.** (a) HPLC chromatogram of BTT-synthesized UsU. (b) HPLC chromatogram of an equimolar mixture of UsU before and after incubation with nP1. (c) Diastereoisomer ratios of BTT-synthesized UsU from epimeric (mixure) and from stereopure U-phosphoramidites: fast-migrating phosphoramidate isomer (fast) and slow-migrating isomer (slow). (d) Diastereoisomer ratios of BTT-synthesized CsC and AsA starting from epimeric (mixture) and stereopure phosphoramidites (fast-migrating phosphoramidite isomer (fast) and slow-migrating isomer (slow)). (e) Diastereoisomer ratios of BTT-synthesized octanucleotides containing single PS dinucleotide units. Error bars are s.d. of three independent experiments. Norm, normal.
its lack of a 2'-substituent on ribose, BTT and tetrazole produced similarly weak Rp-selectivity (Fig. 3d). For the three 2'-O-alkyl substituents (2'-O-Me, 2'-MOE, 2'-O-ACE) stereoselectivities midway between those of 2'-O-TBDMS and 2'-O-TOM were observed. We also synthesized all 16 dinucleotides of DNA and 2'-O-Me using BTT, as well as four dinucleotides with both activators to confirm that the product ratios for TsT and 2'-O-Me UsU were not outliers for the dinucleotide series and were highly reproducible (Supplementary Fig. 6, Supplementary Note 1).

For the coupling of a single nucleotide in a PS ORN the difference in the stereochemical ratios arising from the use of the two activators is small. For example, AsA synthesized using BTT has an Rp/Sp ratio of ~1.2, whereas with tetrazole this ratio is reduced to ~0.8 (Fig. 3a,b); for tetrazole especially, diastereoselective excesses only exceeded 20% for 6 of the 16 dinucleotides (Fig. 3b). Thus, efforts to model such small differences of this solid-phase reaction would be very difficult. However, in an ORN containing 20 PS linkages these small differences are at least partially cumulative and therefore might affect considerably the bulk biophysical properties of an isosequential population of diastereoisomers. To our knowledge, there are no reports on how the physicochemical and biological properties of PS ORNs may be influenced by a variation in the relative population of individual diastereoisomers.

Higher Rp content of PS siRNAs improves cellular activity. To assess the biophysical and biological consequences of the use of different activators during the synthesis of PS ORNs, we prepared 24 PS ORNs and assembled siRNA sequences of various compositions targeting TP53 (p53), LIN28B (Lin28), TGFb1 (TGFβ1), TGFb2 (TGFβ2), RBFOX2 (Fox2) and Renilla luciferase (Ren; Table 1, Supplementary Table 5). SiRen was prepared ‘blunt-ended’.

Assuming that the stereochemical outcome in each coupling step depends only on the sequence of the newly-formed terminal dinucleotide unit (Fig. 2e) and reflects the diasteromeric ratio observed for isolated dinucleotide formation, a theoretical total Rp content can be calculated for each PS siRNA by dividing the sum of the individual Rp ratios for all the dinucleotide units (Fig. 3a,b) by the number of nucleotide linkages (Table 1). We then measured the melting temperatures (Tm) of the duplexes (Supplementary Figs 7–10). It has been reported that short ODNs11 and ORNs composed of stereopure Rp strands form more stable duplexes with complementary PO ORNs than their Sp analogues, or even their PO counterparts7,28. In our case, the PO siRNAs showed the highest Tms of the three formats (Table 1). The Tms of all siRNAs synthesized using BTT (PSBTT siRNA) were 5–6°C higher than those of siRNAs synthesized using tetrazole (PStet siRNA). These data are consistent with a higher Rp content (~60%) of the PSBTT siRNA and support our hypothesis that the stereoselectivity observed during the formation of isolated dinucleotides (Fig. 3a,b) extends to the synthesis of ~21-nt ORNs.

The two most important pharmacological properties of an siRNA are its stability to nucleases and its potency. We therefore tested the effects of stereochemical bias in PS siRNAs on their stability to nucleases in sera and on their biological activity in cells. Stability of ORNs to serum nucleases is commonly assayed using mammalian sera such as fetal bovine serum (FBS). We incubated BTT-synthesized PS and PO siRNAs targeting Fox2, p53, transforming growth factor β1 (TGFβ1) and TGFβ2 with a solution of 10% FBS in a time-course experiment under conditions to highlight the differences in stabilities to nucleases. We quenched the reactions at regular intervals and analysed the equally loaded mixtures on a non-denaturing gel. Under our conditions the PS siRNAs showed superior stability to their PO counterparts in all the cases (Supplementary Figs 11–14). In two cases (TGFβ2 and p53), we compared the stability of the PSBTT and PStet siRNAs under identical incubation conditions in a detailed time-course experiment (Fig. 4a,b).

Stability of single-stranded PS ODNs to nucleases in sera is reportedly greater for Sp- than for Rp isomers29,30. Also, double-stranded ODNs and ORNs are more resistant than their single-stranded counterparts17,31. We assigned the rapid appearance of
**Table 1 | Properties of PO- and PS siRNAs.**

| Target | Sequence of siRNA | siRNA | % R<sub>p</sub> | T<sub>m</sub> (°C) |
|--------|-------------------|-------|----------------|----------------|
| p53    | 5'<UUGUUUUCAGGAAGUAGUUU| si1   | PO             | 66.3 |
|        | UUAACCAAAGCUCCUCAUCA| si2   | PS<sub>BTT</sub> (62) | 58.0 |
|        | si3               | PS<sub>PS</sub> (45)  | 52.7 |
| Lin28  | 5'<AAUUCUUCAUGAAAGUAGTT| si4   | PO             | 67.2 |
|        | TUUUAGGAAGUACUUUCA| si5   | PS<sub>BTT</sub> (56) | 55.5 |
|        | si6               | PS<sub>PS</sub> (40)  | 50.6 |
| TGFβ1  | 5'<CCACAUUUGUCUUAGCGCUU| si7   | PO             | 74.1 |
|        | UUGGUAUAAAGCGUAGUG| si8   | PS<sub>BTT</sub> (62) | 67.0 |
|        | si9               | PS<sub>PS</sub> (44)  | 61.7 |
| TGFβ2  | 5'<GGAAUUGAGCUAUACUAUUAU| si10  | PO             | 70.8 |
|        | UUCCUAUCUGAUUGUCUUA| si11  | PS<sub>BTT</sub> (65) | 63.0 |
|        | si12              | PS<sub>PS</sub> (47)  | 57.0 |
| Fox2   | 5'<CCUGCGUAAUUGCAUUUUUU| si13  | PO             | 68.5 |
|        | UUGGACCUGAUAAGGUUAAA| si15  | PS<sub>BTT</sub> (67) | 61.7 |
|        | si14              | PS<sub>PS</sub> (47)  | 55.0 |
| Ren    | 5'<GAGCGAGAAGGGCGGAAUUU| si16  | PO             | nd  |
|        | CUCUCUUCGCCGUCUUUA| si17  | PS<sub>BTT</sub> (61) | nd  |
|        | si18              | PS<sub>PS</sub> (43)  | nd  |

BTT, 5-benzylthio-1H-tetrazole; Nt, nucleotide; PO, phosphodiester; PS, phosphorothioate; siRNA, small interfering RNA; tet, 1H-tetrazole; T<sub>m</sub>, melting temperature.

*Upper sequence: siRNA guide strand; lower sequence: passenger strand.

†Nucleotide linkages in both strands of the siRNAs: PS<sub>BTT</sub>, PS<sub>PS</sub>: PS ORNs synthesized with BTT and tetrazole, respectively.

‡Calculated R<sub>p</sub> content of siRNA.

Figure 4 | Pharmacological properties of PO- and PS siRNAs. (a) Stability of PS<sub>BTT</sub>-synthesized (si11) and PS<sub>PS</sub>-synthesized (si12) siTGFβ2 siRNAs to nuclease degradation in FBS: ‘mock’ refers to no FBS treatment. (b) Quantification of bands on western blots cannot be relied upon to determine small differences in the activity. A routine and sensitive method to compare the activity of siRNAs is the luciferase reporter assay, in which a target site for an siRNA can be cloned. We employed six luciferase reporter genes, of which five contained a cloned target site for siRNAs of Table 1 (Supplementary Note 2), and one in which an unmodified Renilla reporter plasmid was used. siRNAs were tested at four concentrations. In 5/6 cases the PO siRNAs (si1, si4) inhibited their targets by 80% at the highest treatment concentrations. Surprisingly, both the PS<sub>BTT</sub> siRNAs (si2 and si5) appeared to inhibit their targets more efficiently than the PS<sub>PS</sub> siRNAs (si3 and si6), albeit to a lower extent than their PO counterparts (some toxicity was observed at the highest concentrations). In other words, the higher content of R<sub>p</sub> linkages in the PS<sub>BTT</sub> siRNA was associated with increased potency.

Many factors affect the potency of siRNAs and include strand selection, duplex stability, the presence of nucleotide motifs and chemical modification of guide and/or passenger strands. For unmodified siRNAs, it is known that specific PO cleavage in the centre of the passenger strand by the RNase H-like Argonaute2 (Ago2) is an important step in the activation of the guide strand. This was elegantly demonstrated employing a PS linkage at the said position: the use of an R<sub>p</sub> linkage partially attenuated, whereas an Sp PS group greatly attenuated passenger strand cleavage. Our own data are consistent with these observations as the higher R<sub>p</sub> PS content in the central position of passenger strands of PS<sub>BTT</sub> siRNAs correlates with higher potency.
of PS siRNAs, we assembled Lin28 and p53 siRNAs, combining a PO ORN with either a PSBTT or a PSTet ORN (Table 2). We measured their Tₘs and tested them in cells in pair-wise comparisons.

For all pairs of siRNAs (si19/si21, si20/si22, si23/si25 and si24/si26), switching the PS backbone from the guide to the passenger strands did not significantly change the Tₘ. However, in each case the potency was clearly highest for siRNAs with a PO guide strand (si19, si20, si23 and si24), regardless of the stereochemistry of the PS passenger strands (Fig. 5e,f). The results suggest that an unmodified guide strand is the dominating factor in the activity of a mixed PO/PS siRNA, consistent with numerous previous accounts on modifications to siRNAs31,41. Finally, we compared the properties of two pairs of siRNAs with a PS guide strand and a PO passenger strand (si21/22 and si25/26). The chimeric siRNAs bearing PSBTT guide strands (si21 and si25) showed the higher Tₘs (3.0–3.3 °C), as well as a superior potency to those comprising PSTet strands (si22 and si26). This is consistent with the properties of their fully phosphorothioated analogues (Table 1, Fig. 5a–d). Furthermore, we compared the properties of two pairs of siRNAs with a PS guide strand and a PO passenger strand (si21/22 and si25/26). The chimeric siRNAs bearing PSBTT guide strands (si21 and si25) showed the higher Tₘs (3.0–3.3 °C), as well as a superior potency to those comprising PSTet strands (si22 and si26). This is consistent with the properties of their fully phosphorothioated analogues (Table 1, Fig. 5a–d). Furthermore, it demonstrates that a bias for PS linkages with Rp stereochemistry also in the PS guide strand is beneficial for potency, in analogy to the effects described for the RNase H-mediated cleavage of mRNAs by stereopure Rp antisense PS ODNs41. Recently, two crystal structures of human Ago2 in complex with RNA guides were published32,43. Both the structures show extensive hydrogen bonding contacts between Ago2 residues and the non-bridging oxygens of all PO linkages at the 5’-end and with one PO group at the 3’-end of the guide42,43. Given the multitude of these interactions it is to be expected that thiolation of POs in these regions of the guide will affect its interactions with Ago2 and therefore also the properties of PS siRNAs in RISC.

A high Rp content of PS siRNAs increases loading into RISC.

To add further insight on the interactions of PS siRNAs with RISC, we transfected HeLa cells with PO, PSBTT and PSTet siRNAs (si1, si2 and si3, respectively) and immunoprecipitated the RISC using an Ago2 antibody. We then performed northern blotting for the presence of the individual guide and passenger strands in the complex, as described previously44. For the wild-type siRNA (si1), the great majority of the siRNA strands were present in complex with Ago2 compared with the input sample (Fig. 6, Supplementary Fig. 17). In line with the aforementioned study, we assumed that the northern blotting detected individual passenger and guide strands (and not siRNA) of si1 because Ago2 rapidly cleaves the passenger strands of siRNAs44, and also because the affinity of Ago2 for single-stranded RNA is much higher than for double-stranded siRNA45. Consistent with the high potency of si1, the guide strand appeared to occupy a much larger fraction in RISC than the passenger, although the affinity of the two northern probes for the guide and the passenger strands may not be equal.

As expected, bands on the blot were much weaker for the PSBTT and PSTet ORNs, possibly because of an overall lower binding affinity of the radiolabelled probes for PS ORNs. For both the PS siRNAs, loading of the siRNA into RISC was incomplete, as seen by residual bands in the input samples. However, a larger proportion (43%; Fig. 6) of the PSBTT guide strand of si2 was taken into the Ago2 complex from the input sample compared with the PSTet guide strand of si3 (31%; Fig. 6). This is consistent with the higher potency of si2 over si3 against both endogenous p53 protein (Fig. 4d) and the luciferase reporter genes (Fig. 5).

Taken together, our results suggest that an Rp bias in both the PS guide and passenger strands contributes to the increased
and despite considerable research, a practicable solid-phase synthesis of stereopure PS oligonucleotides is still unavailable. The pharmacological consequences of uniformly modifying siRNAs with PS groups has not been investigated in detail, probably because of sporadic reports that PS siRNAs in vitro are less active than unmodified siRNAs\textsuperscript{13,14}. However, medicinal chemists are well aware that small reductions in the potency of a drug can be easily compensated by an improved PK profile.

Here we have confirmed that distinct tetrazole activators produce a stereogenic bias during the solid-phase synthesis of PS ORNs. In a siRNA composed of two ORNs and ~40 PS linkages, this bias substantially influences the biophysical and biological properties of the duplex. Specifically, compared with tetrazole, ribonucleoside coupling catalysed by BTT yields a higher fraction of Rp PS linkages in an ORN, as evidenced by elevated T\textsubscript{m}.\textsuperscript{9} Surprisingly, this produces a more potent PS siRNA, albeit with slightly less activity than its PO counterpart. It is likely that other commonly used activating agents, for example, ethylthiotetrazole, may similarly alter these properties and thus, also deserve to be investigated.

Controlling the configuration of phosphorothioate linkages was of central importance in various studies charting the stereochemical course of enzymatic processes. Similarly, we envisage that understanding the synergy and additivity of this motif in a supra(bio-)molecular context may be valuable in the field of siRNA therapeutics. The use of PS\textsubscript{BTT} siRNAs in animal models of disease is currently under investigation.

### Methods

**General.** Solvents for purification and chromatography were purchased as technical grade. For column chromatography SiO\textsubscript{2}-60 (230–400-mesh ASTM; Fluka) was used as the stationary phase. Analytical thin layer chromatography was performed on aluminium plates precoated with SiO\textsubscript{2}-60 F\textsubscript{254} (Merck) and visualized with a ultraviolet lamp (254 nm). Concentration in vacuum was performed at ~10 mbar and 40 °C, drying at ~10 \textsuperscript{-2} mbar and room temperature (rt). NMR (nuclear magnetic resonance) spectra were measured on a Bruker AV400 spectrometer at 1.\textsuperscript{H} and 3.\textsuperscript{13}P NMR spectra are reported as follows: chemical shift in parts per million in CDCl\textsubscript{3} and CD\textsubscript{3}CN.

**Synthesis of ORNs.** Chemicals for oligonucleotide synthesis were purchased from Aldrich and TCI (Sigma-Aldrich Chemie GmbH; D-89555 Steinheim). Phosphoramidites were obtained from Thermo Fisher Scientific (Walther, MA), Glen Research (TOM-U) (Sterling, VA) and from Dharmacon (ACE-U). The activator 5-benzylthiotetrazole (BTT) was purchased from Biosolve (5555 CE Valkenswaard, the Netherlands). All oligonucleotides used in this work were synthesized with a MM12 synthesizer from Bio Automation Inc. (Plano, TX) on 500 Å Unylinker CPG from ChemGenes (Wilmington, MA). The coupling time for phosphoramidites was 2 × 90 s. The oligonucleotides were purified on an Agilent 1200 series preparative HPLC fitted with a WatersXBridge OST C-18 column, 10 × 50 mm, 2.5 μm at 60 °C. The RNA phosphoramidites were prepared as 0.08 M solutions in dry acetonitrile (ACN), the activator BTT (Biosolve BV, 5555 CE Valkenswaard, the Netherlands) was prepared as a 0.24 M solution dry ACN, the activator 1H-tetrazole (Tet; Sigma-Aldrich Chemie GmbH, D-89555 Steinheim) was purchased as a 0.45 M solution dry ACN. Oxidizer was prepared as a 0.02 M I\textsubscript{2} solution in TFH/Pyridine/H\textsubscript{2}O (70:20:10, w/v/v); the sulfurizing reagent was prepared as a 0.50 M solution of 3-(N,N-dimethylaminoethyl)methane-1H,1,2,4-dithiazole-5-thione (DDTT; Sulfurizing Reagent I; Glen Research, Virginia) in dry pyridine/ACN (ACN): 40(40). Capping reagent A was: TFH/lutidine/acetic anhydride (8:1:1) and capping reagent B was: 16% N-methylimidazole/TFH. Deblock solution was prepared as a 3% dichloro acetic acid in dichloroethane. The cleavage, the deprotection of the bases and phosphodiester backbone was done by the incubation of the CPG-support for 2 h, at 65–68 °C, 1.8 bar in gaseous methane.

**Deprotection of 2′-O-TBDMS (tert-butyldimethylsilyl-) was performed by incubation of the ORN for 1.5 h, at 70 °C in a mixture of dry 1-N-methyl-2-pyrrolidone/triethylamine/triethylamine x 3HF.**

**RP-HPLC purification of ORNs.** Running buffer for HPLC purification of single-stranded ORNs (up to 23 nt): buffer A (0.1 M triethylammonium acetate), buffer B (methanol); gradient for the DMT-on purification: 20–60% buffer B over 5 min; gradient for the DMT-off purification: 5–35% buffer B over 5 min. Fractions containing the product were collected and dried in a miVac duo SpeedVac from Genovac. The oligonucleotides were analysed by LC-MS (Agilent 1200/6130) on a Waters Acquity UPLC C-18 column, 2.1 × 50 mm, 1.7 μm, 65 °C.

**Buffer A:** 0.4 M HFIP, 15 mM triethylamine; buffer B: MeOH. Gradient: 7–35% B in 14 min; flow rate: 0.3 ml min\textsuperscript{-1}.
Nuclease degradation assays. To determine the stereochemistry of PS diastereoisomers, an equal quantity of both the diastereoisomers were mixed and a sample was injected onto HPLC to generate a reference chromatogram. Then mixtures of ZnCl₂ (2.5 µl, 5 mM), NaOAc (10 µl, 20 mM), H₂O (6.5 µl; Milli-Q) and 5 µl of the RNA-dinucleotide diastereoisomers (1 mM) were prepared. nP1 1 µl (2.4 U µl⁻¹) was added and the samples were incubated at 37 and 50 °C (Usk, USA, Cu, Ga, C₅ and Ga₃) for 30 min. The reaction was stopped by adding EOH/HCl (1:1, 9 µl). To denature the nP1 enzyme the samples were heated to 95 °C for 3 min. To separate the enzyme from the ORNs the samples were extracted with CHCl₃ (20 µl). Supernatant (20 µl) was injected and analysed by HPLC.

Stability of siRNA in 10% FBS. Solution of the siRNA (25 µl, 50 µM) was diluted by addition of 42.5 µl H₂O (Milli-Q) and mixed with 7.5 µl FBS, Sigma-Alrich, Buchs, CH). The reaction mixture was incubated at 37 °C (treatments were carried out in triplicates) and aliquots of 6 µl were collected, flash frozen in liquid nitrogen and stored at −80 °C. Samples were analysed on a 15% denaturing acrylamide gel. Gels were prepared using 30% acrylamide solution diluted with water (Millipore) and 5 µl Tris-Borate–EDTA buffer, 10% ammonium persulfate solution to start polymerization. DNA loading buffer II was added to the samples without heating before loading onto the gel. The gel was run for 20 min at 50 V followed by 1 h at 90 V. After electrophoresis the gel was stained with GelRed nucleic acid stain (incubation time: 10 min) and analysed on a Bio-Rad Gel DocTM TM System. All samples were assayed in triplicates (Supplementary Fig. 11–14).

Cell culture and transfection of siRNAs. HeLa cells (ATTCC, no. CCL-2; LGC, Molsheim, FR) and HuH7 cells (Cell Lines Service, no. 300156, Eppelheim, DE) were maintained in Dulbecco’s Modified Eagle’s medium (Gibco, Invitrogen, Basel, CH) supplemented with 10% FBS (Sigma-Alrich, Buchs, CH). siRNA against Renilla luciferase was a gift from Table 3 (Ventana Dharmacon (Cham, Switzerland)) and the control siRNA (Con; no. AM4640) was from Ambion (Austin, USA). siRNAs were transfected using Oligofectamine (no. 12252-011, Invitrogen, Basel, CH) that was diluted with 15 µl FBS, Sigma-Aldrich, Buchs, CH) for 30 min. The reaction was stopped by adding EtOH/HCl (9:1, 5 µl). To denature the nP1 enzyme the samples were heated to 95 °C for 3 min. To separate the enzyme from the ORNs the samples were extracted with CHCl₃ (20 µl). Supernatant (20 µl) was injected and analysed by HPLC.

Luciferase assays. HeLa cells were seeded in white 96-well plates and the RNAs were transfected after 4 h. There were no overt differences in cell viability between the cells transfected with PO siRNA and the control cells. All transfections were performed in triplicates. After 24 h DNA transfection (20 ng per well) was transfected using jetPEI (no. 101-100, Polyplus, Illkirch, FR) according to the manufacturer’s protocol. After 48 h, the supernatants were removed and the firefly substrate (15 µl; Dual-Glo Luciferase Assay System, Promega, Dubendorf, CH) was added. The diluted siRNAs were transfected into HeLa cells in 15 cm cell culture dishes (∼1.2 × 10⁶ cells; 60% confluency) and incubated for 24 h. Ago2 immunoprecipitation was performed with modifications as described previously[50]. In brief, PBS-washed cells were collected and lysed in NP40 lysis buffer (50 mM HEPEs pH 7.5, 150 mM KCl, 0.5% NP40, 0.5 mM DTT, 2 mM EDTA and 50 µl 1 M RNAi, complete protease inhibitor (Roche)). Each cleared lysate was equally divided into 20 Allo psi (clone 9A11, Ascenion, Munich, Germany) and control IP samples (non-specific rat serum IgG, Sigma)). Five per cent of (1st and 2nd replicate) of each input sample was collected for later RNA extraction. Then, 40 µl of Prot G Dynabeads (Life Technologies) per 15 cm dish were washed two times with 1 ml of citrate-phosphate buffer (25 mM citric acid, 66 mM Na₂HPO₄ and pH 5.0). The Ago2 or rat IgG control serum antibodies (20 µg per 40 µl of Prot G Dynabeads) were immobilized in total volume of 1,000 µl of citrate-phosphate buffer by gentle rolling for 1 h at 4°C. Thereafter, the beads were washed three times with each 1 ml of NP40 lysis buffer and blocked for 1 h at 4°C with 1 ml of NP40 lysis buffer containing BSA (10 µg ml⁻¹). The beads were washed three times with 1 ml of NP40 lysis buffer and resuspended in 50 µl therein. Lysates were incubated with the Ago2/control antibody-coupled Prot G beads for 1 h at 4°C with gentle rolling and then washed five times with 1 ml of IP wash buffer (50 mM HEPEs, pH 7.5, 300 mM KCl, 0.05% NP40, 0.5 mM DTT and phosphotepseprotease inhibitor (Roche)). Finally, bound RNA was released by the addition of 200 µl proteinase K digest buffer (100 mM Tris–HCl pH 7.5, 150 mM NaCl and 12.5 mM EDTA) containing 240–440 µg proteinase K (recombinant PCR grade solution, (Roche)) at 65°C for 45 min shaking. RNA from Ago2/control IP and input was isolated by chloroform/phenol (Life Technologies) extraction and resuspended in 20 µl PCGR grade water and subjected to northern blot analysis. A single described in ref. 47. One microgram of total input RNA and 10 µl (50%) of IP RNA were denatured in RNA loading buffer for 5 min at 95°C and separated on 15% denaturing polyacrylamide/urea gel electrophoresis. RNA transfer onto neutral nylon membrane (Hybond NX, GE Healthcare) and EDG1/methylimidazole crosslinking was performed as described previously[47]. Detection probe were generated according to ref. 48. PO backbone of nP3 (2.5 µl each; 50 µM stock; guide or passenger strand) and 2′-O-Me anti-miR-21 as normalization control were 5′ phosphorylated using T4 phosphorykinase, 3′-phosphorophate-free (Roche) according to the providers’ protocol with 1 U µl⁻¹ 3′-APT (6600C/mmol Perkin-Elmer) followed by chloroform/phenol purification and used for the detection of the respective counter strand. Membranes were (pre)-hybridized rotating at 40°C overnight. Washed membranes were exposed to Phospho-Imager screen (GE Healthcare) for at least 24 h and the signals were detected by Typhoon scanner device FLA-7000 (GE Healthcare). Densitometric signal quantification was done by using ImageQuant TL software (GE Healthcare). Assays were performed in two independent biological replicates.

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