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Expanding the binding space of argonaute-2: incorporation of either *E* or *Z* isomers of 6′-vinylphosphonate at the 5′ end of the antisense strand improves RNAi activity†

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A phosphate or a phosphate mimic at the 5′ terminus of the antisense strand of a small interfering RNA (siRNA) is required for efficient loading into the RISC complex through the MID domain binding pocket of Ago2. Introduction of 5′-*E*-vinylphosphonate improves this binding and siRNA potency, but the *Z* isomer does not. Here, we demonstrate that both the *E* and *Z* isomers of 6′-vinylphosphonate at the 5′ ends of antisense strands of siRNAs have equivalent potencies.

The potency of an siRNA is influenced by the efficiency of loading of the siRNA antisense strand into the RNA-induced silencing complex (RISC). For interaction with the MID domain of Ago2, the endonuclease component of the RISC,[1–3] the 5′ terminus of the antisense strand of the siRNA must be phosphorylated. When the antisense strand of an siRNA has a 5′-terminal residue that cannot be enzymatically phosphorylated, chemical incorporation of a 5′-monophosphate is ineffective due to rapid dephosphorylation by lysosomal acid phosphatases encountered by the siRNA during entry into cells through the endocytic pathway.[4] Incorporation of a metabolically stable 5′-(*E*)-vinylphosphonate (5′-(*E*)-VP, **I**, Fig. 1A), a phosphate mimic, enhances RISC loading of the antisense strand and RNA interference (RNAi) potency;[4–8] however, the *Z*-isomer (5′-(*Z*)-VP, **II**, Fig. 1A) does not.[9] Crystal structures of Ago2 loaded with an antisense strand modified with 5′-(*E*)-VP have elucidated how the 5′-nucleotide binding pocket that involves residues of the MID and PIWI subdomains of Ago2 accommodates the 5′-(*E*)-VP moiety.[10,11] The combination of 5′-(*E*)-VP in the antisense strand along with targeting ligands in the sense strand, like triantennary *N*-acetylgalactosamine (GalNAc), which facilitates uptake by liver hepatocytes,[12] and 2′*O*-hexadecyl lipid, which mediates entry into cells of the central nervous system (CNS),[13] is a proven method for generating efficacious siRNAs.

We previously evaluated the stereochemical requirement for 5′-(*E*)-VP modification as a function of the nature of the sugar.[14–16] The 6′-homologation of nucleosides has been reported,[14–18] and the use of such "extended" backbone linkages in siRNA has been explored to a limited extent.[19,20] The 6′-morpholino at the 5′ end of the sense strand of an siRNA duplex improved the RNAi activity through an improved strand bias relative to the 5′-morpholino analogue.[20–22] We also demonstrated that 6′-morpholino in a locked nucleic acid sugar at the 5′ end of the sense strand synergistically improves metabolic stability and siRNA activity.[23] Here, we describe syntheses of nucleoside building blocks containing *E* and *Z* isomers of 6′-VP (Fig. 1B) and evaluation of the silencing efficacies of siRNAs containing these modifications.

The 6′-VP building blocks were synthesized as shown in Scheme 1. The 6′-OH containing uridine nucleoside **1**[20] was oxidized with Dess–Martin periodinane to afford the 6′-aldehyde **2**, which was coupled with the previously described reagent tetrakis[(pivaloyloxy)-methyl]methylenediphosphonate (bis-POM VP)[9] under basic conditions.

The resulting *E* and *Z* isomers were separated to obtain compound **3** as the major product (56% yield from compound **1**) and compound **4** (11% yield from compound **1**). Desilylation

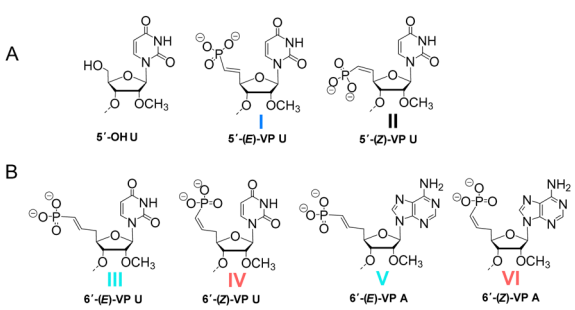

Fig. 1 (A) 5′-OH, 5′-(*E*)-VP (**I**) and 5′-(*Z*)-VP (**II**) and (B) 6′-(*E*)-VP (**III**) and 6′-(*Z*)-VP (**VI**) nucleotides.

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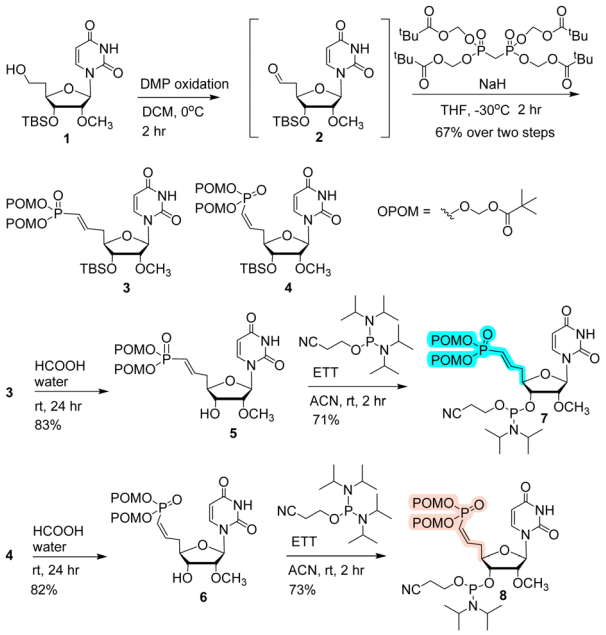
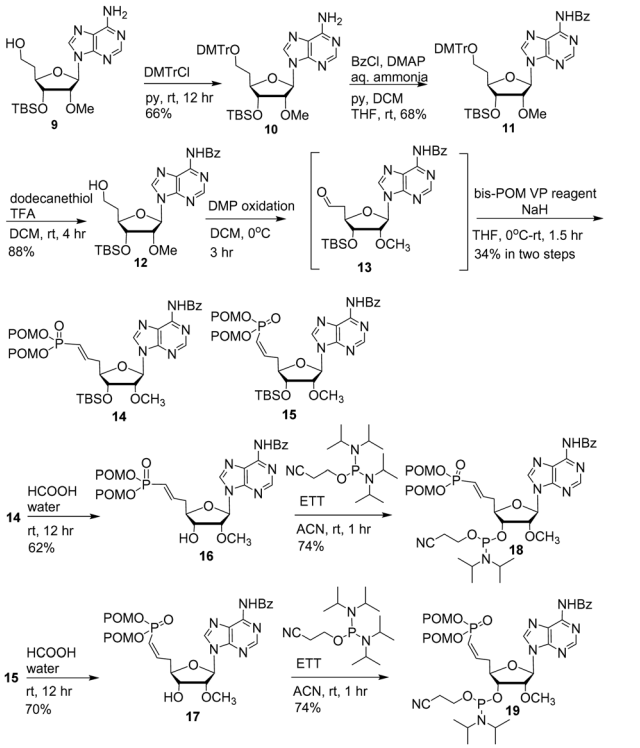


Scheme 1 Synthesis of 6′-uridine-(*E*)- and (*Z*)-VP phosphoramidites **7** and **8**. The functionalizations resulting from couplings with bis-POM VP are highlighted in cyan and soft red.



Scheme 2 Synthesis of 6′-adenosine-(*E*)- and (*Z*)-VP phosphoramidites **18** and **19**.

of the *tert*-butyldimethylsilyl group under 50% aqueous formic acid conditions afforded compounds **5** and **6**, which have free 3′-hydroxyl groups, in good yields. These compounds were then converted to phosphoramidites **7** and **8**, respectively. The adenosine 6′-VP building blocks **18** and **19** were synthesized using similar routes from the reported compound **9**[23] as shown in Scheme 2.

These VP building blocks were incorporated at the 5′ ends of the antisense strand targeting mouse *Ttr*. The no-VP and 5′-(*E*)-VP controls as well as the sense strand with trivalent GalNAc at the 3′ end[12] were also synthesized, and siRNAs were prepared. The sequences, chemical modifications, and silencing activities of the parent and no-VP siRNA have been previously described.[9,24] We also prepared siRNA modified with new VP building blocks targeting *ApoB* and *SOD1* mRNAs as well as appropriate control siRNAs (Table 1 and Tables S1, S2 in the ESI†).

The siRNAs targeting *Ttr* were first evaluated in primary mouse hepatocytes for their abilities to reduce the levels of *Ttr* mRNA under free uptake conditions. As expected, the control 5′-(*E*)-VP-containing **si-2** was more potent than the control no-VP **si-1** (Fig. 2A). The 6′-(*Z*)-VP-modified **si-4** had efficacy comparable to the 5′-(*E*)-VP-containing **si-2**, whereas the 6′-(*E*)-VP-modified **si-3** was slightly more potent than **si-1** but was less active than **si-2** and **si-4** (Fig. 2A). That the siRNA modified with the 6′-(*Z*)-VP isomer had activity comparable to that of the siRNA modified with the *E* isomer was confirmed by analyses of siRNAs targeting another hepatic target *ApoB*, using an siRNA which is intrinsically less potent than *Ttr* siRNA (Fig. S1 and Table S2, ESI†). Interestingly, 6′-VP siRNAs with one phosphorothioate linkage at the 5′ terminus of the antisense strand and those with two phosphorothioate linkages at this terminus had comparable potencies (Fig. S1, ESI†).

Table 1 siRNAs targeting *Ttr* and *SOD1*[a]

Duplex	Sense strand (upper) and antisense strand (lower)[a] (5′–3′)	Target
si-1 (control)	a•a•caguGu*UCU*ugcucuauaaL u•*U*•auaGagcaagaAcAcuguu•u•u	*Ttr*
si-2	a•a•caguGu*UCU*ugcucuauaaL **I**•*U*•auaGagcaagaAcAcuguu•u•u	
si-3	a•a•caguGu*UCU*ugcucuauaaL **III**•*U*•auaGagcaagaAcAcuguu•u•u	
si-4	a•a•caguGu*UCU*ugcucuauaaL **IV**•*U*•auaGagcaagaAcAcuguu•u•u	
si-5 (Control)	c•a•uuuU$_{hd}$AaUCCucacucua•a•a u•*U*•uagAgUGaggaUuAaaaug•a•g	*SOD1*
si-6	c•a•uuuU$_{hd}$AaUCCucacucua•a•a **I**•*U*•uagAgUGaggaUuAaaaug•a•g	
si-7	c•a•uuuU$_{hd}$AaUCCucacucua•a•a **III**•*U*•uagAgUGaggaUuAaaaug•a•g	
si-8	c•a•uuuU$_{hd}$AaUCCucacucua•a•a **IV**•*U*•uagAgUGaggaUuAaaaug•a•g	

[a] Chemical modifications: •, phosphorothioate linkage; lower case, 2′-*O*-methyl; italicized upper case, 2-fluoro; L, trivalent-GalNAc; U$_{hd}$, 2′-*O*-hexadecyl uridine; **I**, 5′-(*E*)-VP; **III**, 6′-(*E*)-VP; and **IV**, 6′-(*Z*)-VP.

This suggests that the 6′-VP modifications provide some additional protection from nuclease-mediated degradation.

Next, the *Ttr*-targeting siRNAs were tested in mice at a single subcutaneous dose of 0.3 mg kg^{-1}. The levels of TTR in serum

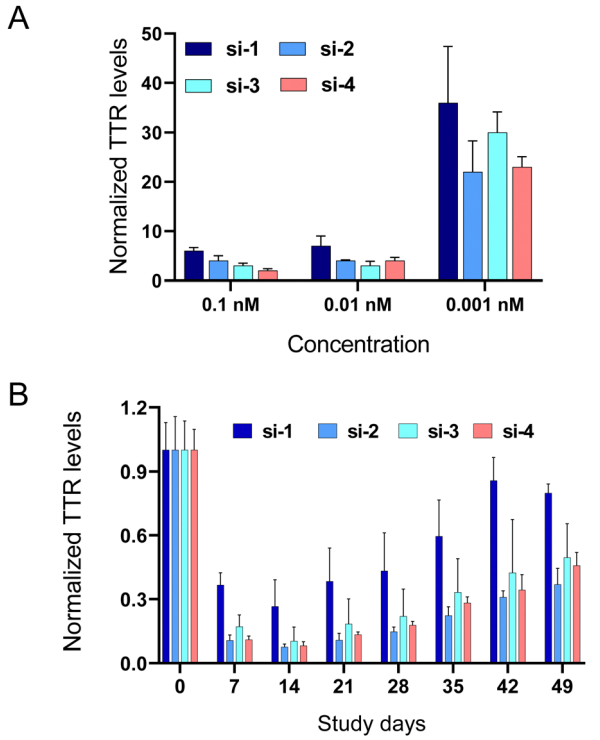


Fig. 2 (A) Percent *Ttr* mRNA remaining after treatment of primary mouse hepatocytes with the indicated siRNAs for 48 hours. Levels of *Ttr* mRNA were quantified using RT-qPCR and were normalized to *Ttr* mRNA in cells treated with a non-targeting siRNA. Plotted are averages ± standard deviation (n = 4). (B) TTR protein in serum of mice treated with a single subcutaneous dose of 0.3 mg kg^{-1} of the indicated siRNAs (n = 3 per group). TTR was measured in serum using a sandwich ELISA assay utilizing an HRP-conjugate antibody and 3,3′,5,5′-tetramethylbenzidine for read-out at 450 nm. All samples were measured in duplicate, and each data point is the average ± standard deviation normalized to the pre-dose level in individual animals.

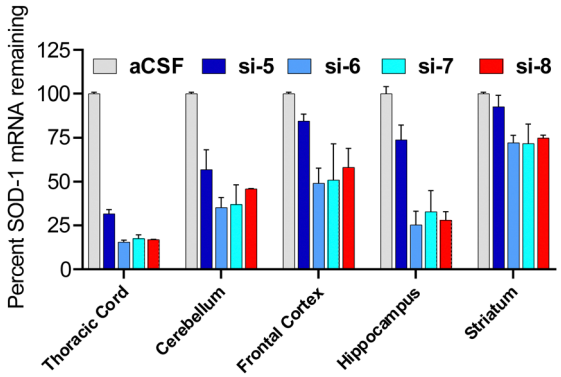


Fig. 3 Percent *SOD1* mRNA remaining on day 14 in rats treated intrathecally with 0.6 mg siRNA in 30 µL (n = 3 per group). *SOD1* mRNA levels were determined in the indicated regions *via* qPCR. Data are plotted relative to *SOD1* levels in rats injected with artificial cerebrospinal fluid (control) with normalization to *PPIB* mRNA levels.

were monitored until day 49 after injection. The VP-modified siRNAs were all more potent throughout the time course than **si-1**, the siRNA lacking a phosphate mimic (Fig. 2B). **si-2** and **si-4** had almost equal siRNA efficacy within the error limit, and the 6′-(*E*)-VP modified **si-3** was slightly less active (Fig. 2B).

We also evaluated *SOD1*-targeted siRNAs in rats. These siRNAs had sense strands conjugated to 2′-*O*-hexadecyl, which facilitates uptake into CNS tissues after intrathecal administration.[13] Rats were given an intrathecal dose of siRNA, and *SOD1* mRNA levels were determined on day 14 post dose. The 5′-(*E*)-VP-labeled control **si-6** was more potent than the no-VP parent **si-5** in all CNS tissues evaluated (Fig. 3) as was previously reported.[13] Both 6′-(*E*)-VP- and 6′-(*Z*)-VP-modified siRNAs (**si-7** and **si-8**, respectively) had potencies similar to that of **si-6**. In summary, evaluation of siRNAs targeting three different mRNAs (*Ttr*, *ApoB*, and *SOD1*) in two tissues (liver and CNS) indicate that both *E* and *Z* isomers of 6′-VP enhance silencing activity when used to modify the 5′ terminus of the antisense strand relative to the control siRNA lacking a phosphate.

Using the structure of Ago2 in complex with miR-20a[3] as a starting point, we built models of RNAs with 6′-(*E*)-VP and 6′-(*Z*)-VP at the 5′ terminus docked to the Ago2 MID binding pocket (Fig. 4). Structures were generated in UCSF Chimera[25] and energy-minimized with Amber.[26] Both 6′-(*E*)-VP and 6′-(*Z*)-VP are accommodated within the binding pocket, although these modifications are inserted more deeply into the binding pocket to accommodate the extended 5′ end than is the phosphate of the 5′ terminus of miR-20a. There are more severe deviations of sugar and backbone orientations for 6′-(*E*)-VP than for 6′-(*Z*)-VP, leading to fewer hydrogen bonds between Ago2 residues and phosphate as well as displacement of a water molecule that is present both in

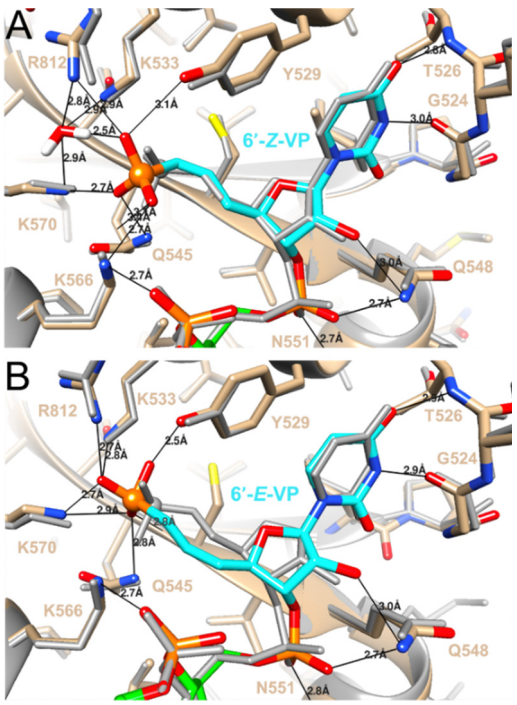


Fig. 4 Computational models of (A) 6′-(*Z*)-VP-U and (B) 6′-(*E*)-VP-U at the 5′ terminus of an RNA strand lodged in the MID binding pocket. VP carbon atoms are highlighted in cyan, and models are superimposed on the crystal structure of the parent complex with UMP at the 5′ end drawn with thin gray bonds (PDB ID 4f3t).[3]

the parent complex and the modeled complex with 6′-(*Z*)-VP. This may explain the observation that 6′-(*Z*)-VP-modified siRNAs targeting *Ttr* match or surpass the activity of the corresponding 6′-(*E*)-VP-modified siRNAs.

In summary, we have designed two new metabolically stable phosphate mimics and have shown that both enhance silencing when conjugated to the 5′ terminus of the antisense strand of siRNAs in both hepatic and extra-hepatic tissues. We synthesized the adenosine and uridine analogues of the 6′-VP phosphoramidites as these two residues at the 5′ terminus of the antisense strand facilitate preferential loading of the antisense strand rather than the sense strand into the RNA-induced silencing complex.[27] Unlike the previously described 5′-VP modifications where the *E* isomer enhances RNAi activity but the *Z* isomer does not[4–8,9] the 6′-VP *E* and *Z* isomers both enhanced silencing relative to siRNAs lacking a phosphate mimic at the 5′ terminus of the antisense strand. We recently demonstrated that siRNAs with GalNAc conjugated to the 3′ terminus of the antisense are as potent as those with GalNAc on the 3′ terminus of the sense strand.[28] We expect that antisense strands functionalized at the 5′ end with 6′-VP and at the 3′ terminus with a targeting ligand will be potent and are conducting experiments to confirm this. The 6′-VP modifications characterized here expand the scope of RNAi therapeutics and further our understanding of the mediators of antisense strand interactions with the MID domain of Ago2.

All authors collectively designed the study and the manuscript was written by contribution from all authors.

Data availability

The data supporting this article have been included as part of the ESI.†

Conflicts of interest

There are no conflicts of interest to declare.

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