



Cite this: DOI: 10.1039/d6cb00038j

Received 31st January 2026,  
Accepted 23rd February 2026

DOI: 10.1039/d6cb00038j

rsc.li/rsc-chembio

## Synthesis of siRNAs containing carbocyclic nucleotides and the role of cyclopentane conformation in RNAi activity

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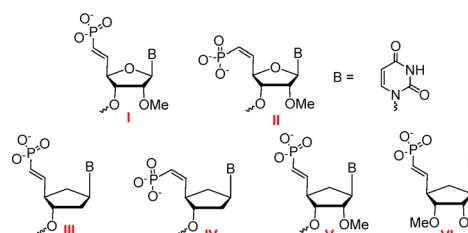
**5'-(E)- and 5'-(Z)-vinylphosphonate carbocyclic DNA and 5'-(E)-vinylphosphonate 2'- and 3'-O-methyl carbocyclic RNAs were incorporated at 5' termini of antisense strands of small interfering RNAs. All but the 3'-O-methyl carbocyclic analogue resulted in gene silencing activity better than the siRNA lacking a 5' phosphate in cells and in mice.**

Chemical modification is necessary to ensure metabolic stability, specificity, and efficient delivery of small interfering RNAs (siRNAs).<sup>1–3</sup> The RNA-induced silencing complex (RISC), which contains the endonuclease Ago2, mediates the gene silencing activities of siRNAs, and the reported high-resolution structures of these complexes have afforded insights into the mechanism of RNA interference (RNAi).<sup>4–6</sup> In order to be loaded into the RISC, the antisense strand of the siRNA must be 5' phosphorylated.<sup>7</sup> When the antisense strand of a synthetic siRNA has a 5'-terminal residue that cannot be enzymatically phosphorylated, chemical incorporation of a natural 5'-monophosphate is ineffective due to rapid dephosphorylation by lysosomal acid phosphatases encountered by the siRNA during entry into cells *via* endocytosis.<sup>8</sup>

Incorporation of the metabolically stable phosphate mimic 5'-(E)-vinylphosphonate (5'-(E)-VP, **I**, Fig. 1) at the 5' terminus of the antisense strand enhances RISC loading and siRNA potency, but the corresponding (Z) isomer (5'-(Z)-VP, **II**) does not.<sup>8–12</sup> Crystal structures of Ago2 loaded with an antisense strand modified with 5'-(E)-VP (**I**) revealed that the 5'-nucleotide binding pocket, which involves residues of the MID and PIWI domains of Ago2, accommodates the 5'-(E)-VP (**I**) moiety but not the (Z) isomer (**II**).<sup>5,13</sup> The combination of 5'-(E)-VP (**I**) in the antisense strand with targeting ligands in the sense strand such

as triantennary *N*-acetylgalactosamine (GalNAc) for liver and 2'-*O*-hexadecyl lipid for central nervous system result in efficacious siRNAs.<sup>14,15</sup> Though siRNAs with antisense strands modified with the 5'-(Z)-VP (**II**, Fig. 1) do not mediate gene silencing,<sup>16</sup> siRNAs carrying 6'-(E)- and 6'-(Z)-VP (which are corresponding methylene homologues of 5'-(E)- and 5'-(Z)-VP, respectively) have comparable potency to siRNAs modified with 5'-(E)-VP (**I**) in mice.<sup>17</sup> Gene silencing was more efficient when the antisense siRNA strand was modified with 5'-VP nucleosides that adopt a South C2'-*endo* pucker than with a 5'-VP nucleoside that adopts a North C3'-*endo* pucker.<sup>18</sup>

In our effort to expand the toolbox of modifications for siRNAs, we previously evaluated biophysical properties of carbocyclic RNAs (car-RNAs).<sup>19</sup> In this non-natural nucleic acid, the 4'-oxygen is replaced by a methylene group. Incorporation of a car-RNA residue does not alter the structure of an RNA duplex, and the 2'-OH group has higher p*K*<sub>a</sub> and lower nucleophilicity than the ribose sugar, which explains their improved nuclease resistance.<sup>20,21</sup> The (E)-VP analogue of car-RNA with a 2'-*O*-methyl (2'-OMe) sugar (**V**, Fig. 1) was mentioned in recently published patents,<sup>22,23</sup> but the effect of this analogue on RNAi activity has not been reported. Here, we evaluated the effects of VP-modified carbocyclic DNA (car-DNA) and car-RNA residues



**Fig. 1** 5'-(E)- and 5'-(Z)-VP-modified nucleotides previously tested in the context of siRNAs (**I** and **II**, respectively) and 5'-(E)- and 5'-(Z)-VP-modified nucleotides of car-DNA (**III** and **IV**, respectively) and 5'-(E)-VP-modified nucleotides of 2'- and 3'-OMe-car-RNA (**V** and **VI**, respectively) tested here.

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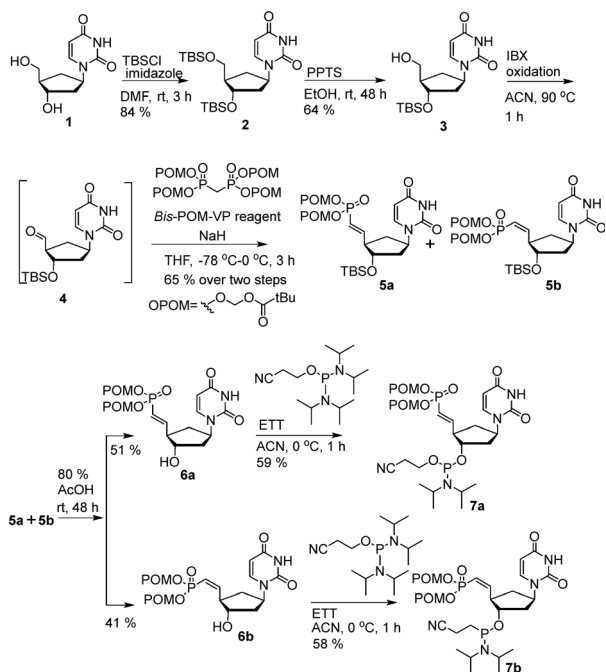


(Fig. 1, III-VI) at the 5' end of the antisense strand on siRNA potency in cell culture and in mice.

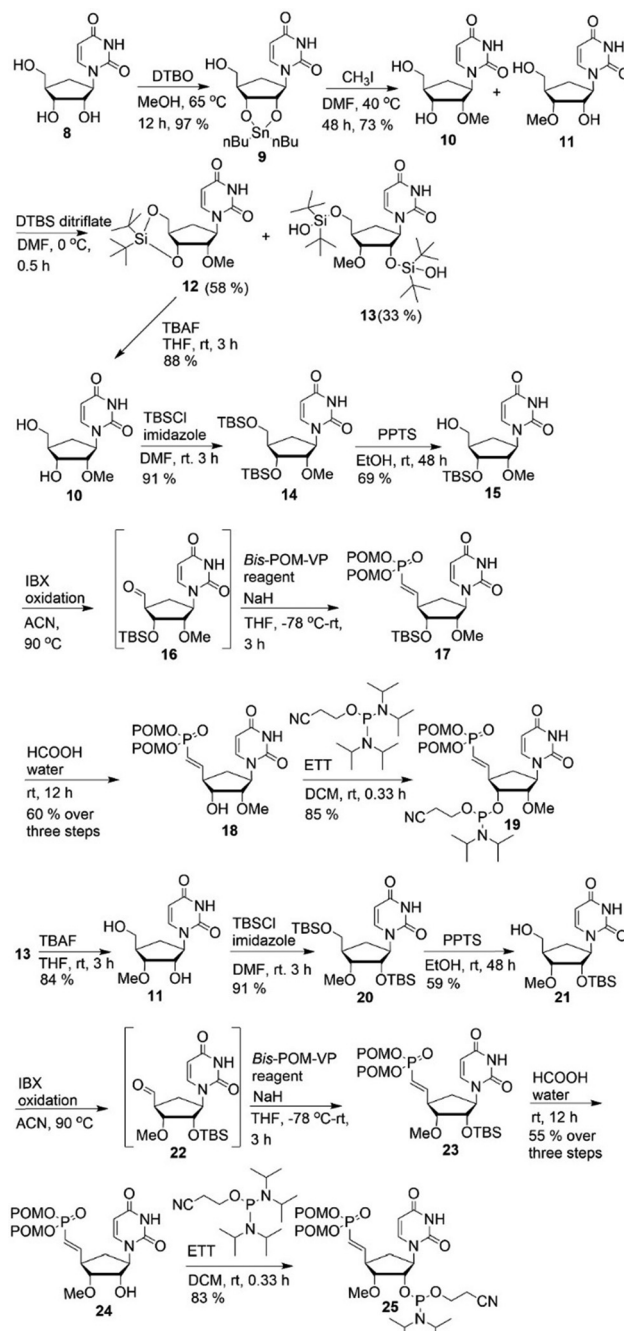
For the syntheses of VP-analogues of car-DNA nucleosides, we started from 2'-deoxycarbocyclic uridine nucleoside **1**,<sup>24,25</sup> which can be easily synthesized from the previously described car-U-RNA<sup>19</sup> following a reported procedure.<sup>26</sup> Compound **1** was reacted with excess *tert*-butyldimethylsilyl (TBS) chloride to afford 5',3'-bis-OTBS product **2**. Pyridinium *p*-toluenesulfonate-mediated selective removal of the 5'-OTBS yielded **3**. Compound **3** was oxidized using 2-iodoxybenzoic acid to afford the aldehyde **4**, which was used without further purification in a Wittig-type reaction in the presence of tetrakis[(pivaloyloxy)-methyl]methylenediphosphonate under basic conditions to afford a mixture of stereoisomers **5a** and **5b**. After 3'-OTBS removal under acidic conditions, an attempt to separate the *E* and *Z* isomers resulted in the pure **6a**. However, **6b** was obtained as an inseparable mixture with 10% **6a**. Compound **6a** and the partially pure **6b** were then phosphitylated to afford the phosphoramidites **7a** and **7b**, respectively (Scheme 1).

For the syntheses of VP analogues of 2'- and 3'-OMe-car-RNA, we converted **8**, synthesized as described,<sup>19</sup> into the corresponding organo-tin derivative **9** under Moffatt conditions.<sup>27</sup> Compound **9** was separated into isomers **10** and **11** by a silylation-desilylation strategy *via* **12** and **13**. The corresponding VP analogues were synthesized as described in Scheme 1 to afford phosphoramidites **19** and **25** (Scheme 2).

The phosphonate-protected phosphoramidites **7a**, **7b**, **19**, and **25** were incorporated at the 5' ends of antisense siRNA strands targeting mouse *Ttr* and *ApoB* mRNAs using standard automated solid-phase oligonucleotide synthesis procedures (Table 1 and Table S1). The 3' termini of the sense strands



Scheme 1 Syntheses of 5'-VP-car-DNA phosphoramidites **7a** and **7b**.



Scheme 2 Syntheses of 5'-VP-2'-OMe-car-RNA and 5'-VP-3'-OMe-car-RNA phosphoramidites **19** and **25**.

were conjugated to triantennary GalNAc. Strands were chemically modified with 2'-fluoro-RNA, 2'-OMe-RNA, and phosphorothioate backbone linkages as previously described.<sup>1,2</sup> Antisense strands that carried the novel car-DNA analogues were prepared with a phosphorothioate linkage and without. As controls, antisense strands without a 5' phosphate and with a 5'-(*E*)-VP (**I**) were prepared. **si-1** and **si-2**, without a 5' phosphate and with a 5'-(*E*)-VP (**I**), respectively, were used as the controls for siRNAs targeting *Ttr*, and **si-9** and **si-10** served as controls for *ApoB*-targeting siRNAs.



Table 1 siRNAs used in this study

siRNA	5' Antisense strand modification	Sense/antisense strand sequences (5'-3') <sup>a</sup>
si-1	None	A●a●CaGuGuUCUuGcUcUaUaAL u●U●aUaGaGcAagaAcAcUgUu●u●u
si-2	5'-(E)-VP	A●a●CaGuGuUCUuGcUcUaUaAL I●U●aUaGaGcAagaAcAcUgUu●u●u
si-3	5'-(E)-VP-car-DNA	A●a●CaGuGuUCUuGcUcUaUaAL III●U●aUaGaGcAagaAcAcUgUu●u●u
si-4	5'-(E)-VP-car-DNA	A●a●CaGuGuUCUuGcUcUaUaAL III●U●aUaGaGcAagaAcAcUgUu●u●u
si-5	5'-(Z)-VP-car-DNA	A●a●CaGuGuUCUuGcUcUaUaAL IV●U●aUaGaGcAagaAcAcUgUu●u●u
si-6	5'-(Z)-VP-car-DNA	A●a●CaGuGuUCUuGcUcUaUaAL IV●U●aUaGaGcAagaAcAcUgUu●u●u
si-7	5'-(E)-VP-2'-OMe-car-RNA	A●a●CaGuGuUCUuGcUcUaUaAL V●U●aUaGaGcAagaAcAcUgUu●u●u
si-8	5'-(E)-VP-3'-OMe-car-RNA	A●a●CaGuGuUCUuGcUcUaUaAL VI●U●aUaGaGcAagaAcAcUgUu●u●u
si-9	None	C●c●UgGaCaUUCaGaAcAaGaAL u●U●cUuGuUcUgaaUgUcCaGg●g●u
si-10	5'-(E)-VP	C●c●UgGaCaUUCaGaAcAaGaAL I●U●cUuGuUcUgaaUgUcCaGg●g●u
si-11	5'-(E)-VP-car-DNA	C●c●UgGaCaUUCaGaAcAaGaAL III●U●cUuGuUcUgaaUgUcCaGg●g●u
si-12	5'-(E)-VP-2'-OMe-car-RNA	C●c●UgGaCaUUCaGaAcAaGaAL V●U●cUuGuUcUgaaUgUcCaGg●g●u
si-13	5'-(E)-VP-3'-OMe-car-RNA	C●c●UgGaCaUUCaGaAcAaGaAL VI●U●cUuGuUcUgaaUgUcCaGg●g●u

<sup>a</sup> Top and bottom rows show sense and antisense strand sequences, respectively. Upper case italics indicate 2'-fluoro RNA; lower case indicates 2'-OMe modification; I–VI are modifications shown in Fig. 1; L indicates triantennary GalNAc; and ● indicates a phosphorothioate linkage.

The siRNAs were first evaluated in a gene silencing assay in primary mouse hepatocytes under free uptake conditions. At the lower doses tested, 1 nM for *Ttr*- and 20 nM for *ApoB*-targeted siRNAs, the controls carrying the 5'-(E)-VP (**I**; **si-2** and **si-10**) were more potent than the controls lacking a 5' phosphate analogue (**si-1** and **si-9**) (Fig. 2A and C). The siRNAs modified with 5'-(E)-VP-2'-OMe-car-RNA (**V**; **si-7** and **si-12**) and 5'-(E)-VP-car-DNA (**III**; **si-3** and **si-11**) were similar in potency to control siRNAs modified with 5'-(E)-VP (**I**) (Fig. 2A and C). The siRNAs functionalized with 5'-(Z)-car-DNA (**IV**; **si-5** and **si-6**) had potencies equivalent to that of **si-2**, the control modified with 5'-(E)-VP (**I**) (Fig. 2B). This was unexpected as a previous report demonstrated that siRNA functionalized with the 5'-(Z)-VP isomer **II** was not active at the concentrations evaluated here.<sup>16</sup> There were no differences in potency between siRNAs modified with 5'-(E)-car-DNA (**III**) with and without phosphorothioate linkages between the first and second residues of the antisense strand (**si-3** vs. **si-4**, respectively) (Fig. 2B). This was expected as the car-DNA should enhance nuclease resistance.<sup>21</sup>

The siRNAs modified with 3'-OMe-car-RNA (**VI**; **si-8** and **si-13**), a modification with a 2'-5' linkage, were even less potent than controls **si-1** and **si-9**, which do not have a 5' phosphate (Fig. 2A). siRNAs with 2'-5' linkages have been evaluated in the past for RNAi activity. 2'-5'-linked DNA with a 5'-(E)-VP showed enhanced RNAi activity compared to the corresponding 5'-OH compound.<sup>11</sup> On the other hand, 2'-5'-linked RNA, which has reduced immunostimulatory effects compared to RNA, showed

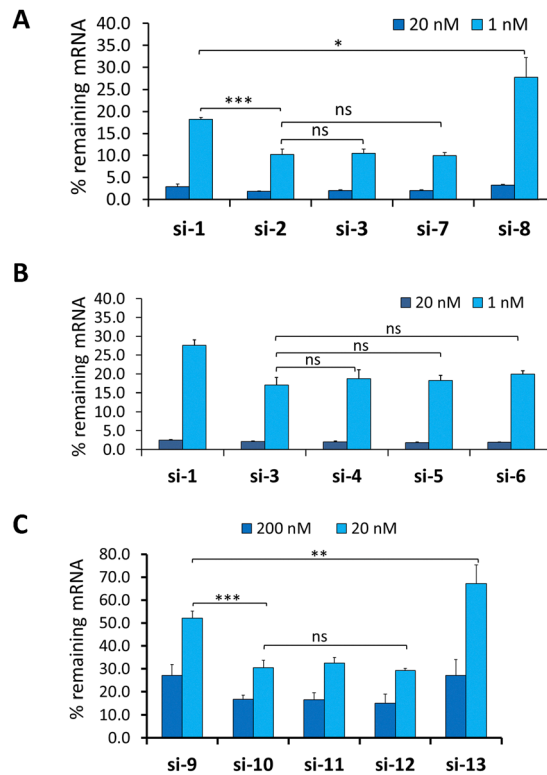


Fig. 2 (A and B) Percent *Ttr* mRNA remaining in mouse hepatocytes after treatment with indicated siRNAs. Panels A and B show data from separate experiments. Primary mouse hepatocytes were cultured with siRNAs under free uptake conditions for 48 h. *Ttr* mRNA was quantified using a QuantiGene Singleplex assay, and percent RNA remaining relative to samples treated with control, non-targeted siRNA was determined. Averages  $\pm$  standard deviations are plotted ( $n = 3$ ). \* $p < 0.05$ , ns means not significant; student's *t*-test was used. (C) Analysis of silencing by *ApoB*-targeting siRNAs in cultured primary mouse hepatocytes. Primary mouse hepatocytes were cultured with siRNAs under free uptake conditions for 48 h. *ApoB* mRNA was quantified using a QuantiGene Singleplex assay, and percent RNA remaining relative to samples treated with control, non-targeted siRNA was determined. Averages  $\pm$  standard deviations are plotted ( $n = 3$ ). \* $p < 0.05$ , ns means not significant; student's *t*-test was used.

nuclease resistance but reduced the Argonaute-2 loading when it was placed at the position 1 of the antisense strand even though the sugar ring had a favorable “clover leaf” bend and *C2'-endo* conformation.<sup>29</sup> Moreover, multiple 2'-5'-linked RNA modifications in the antisense strand significantly reduced activity.<sup>28,29</sup> However, a single 2'-5'-linked RNA modification at position 7 of the antisense strand seed region was recently shown to mitigate off-target effects arising from miRNA-type interactions with non-targeted mRNAs while maintaining the on-target activity.<sup>30</sup>

Next, we determined the potencies of selected *Ttr*-targeted siRNAs in mice. Mice were treated subcutaneously with a dose of  $0.4 \text{ mg kg}^{-1}$ , and TTR protein was quantified in serum over time. **si-3**, which is modified with 5'-(E)-VP-car-DNA (**III**), and **si-7**, which is modified with 5'-(E)-VP-2'-OMe-car-RNA (**V**), had potencies equivalent to that of **si-2**, which carries 5'-(E)-VP (**I**), and were more efficacious than the non-phosphorylated control **si-1**



(Fig. 3). Consistent with the *in vitro* data, the siRNA with the 5'-(*E*)-VP-3'-OMe-car-RNA (**VI**) modification, si-8, was less potent than both control siRNAs (Fig. 3).

We used computational modelling to evaluate how modified carbocyclic 5'-VP analogues interact with the Ago2 MID domain. The complex between miR-20a, which has a 5' UMP, served as the reference structure (PDB ID 4f3t).<sup>4</sup> UCSF Chimera was used to install modified residues at the 5' terminus of the RNA.<sup>31</sup> All models were energy-minimized until conversion with the AMBER ff14 force field as implemented in UCSF Chimera.<sup>32</sup> The sugar of 5'-(*E*)-VP-car-DNA (**III**) is accommodated within the MID domain binding site and adopts the C2'-*endo* pucker (Fig. 4A). The sugar of the 5'-(*Z*)-VP-car-DNA (**IV**) adopts the C3'-*exo* pucker (Fig. 4B). The conformations of both these residues are very similar to the conformation of the 5' UMP in the crystal structure. The 5'-(*E*)-VP-2'-OMe-car-RNA (**V**) also adopts the C2'-*endo* sugar pucker (Fig. 5A). The conformations of these 5'-VP residues also closely correspond to that of the 5'-(*E*)-VP-2'-OMe uracil in the complex of a modified strand bound to Ago2, which was previously analysed by crystallography (PDB ID 5t7b), although in that structure, the pucker is C1'-*exo* ( $\Delta P = 50^\circ$ ).<sup>33</sup> In the crystal structures and our models, the uracil stacks favourably with Y529 Ago2. The siRNA modified with 5'-(*E*)-VP-3'-OMe-car-RNA (**VI**) was less active than the other modified siRNAs tested, and **VI** is not well accommodated

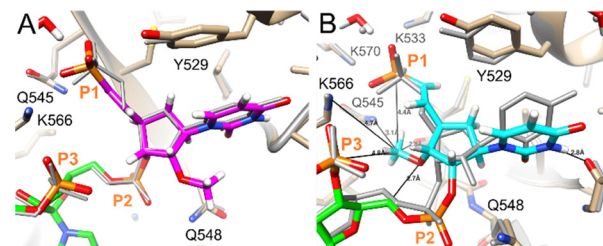


Fig. 5 Models of antisense strands modified with (A) 5'-(*E*)-VP-2'-OMe-car-RNA **V** in pink and (B) 5'-(*E*)-VP-3'-OMe-car-RNA **VI** in cyan lodged at the Ago2 MID domain binding site. The RNA in the crystal structure (PDB ID 4f3t) is shown as a grey wire.

in the MID domain binding site; there are short contacts to phosphates (sum vdW = 2.8 + 2 = 4.8) and a short O3' distance of 2.7 Å to C5' of the second nucleotide in the antisense strand (Fig. 5B). Moreover, neither the VP moiety nor the base are planar, and the sugar pucker of the carbocyclic ring is C1'-*exo* (Southeast). The interaction with Y529 Ago2 is also disrupted in the 5'-(*E*)-VP-3'-OMe-car-RNA (**VI**) model, although the uracil base does form a hydrogen bond to the main chain of the local Ago2 loop (*via* N3H).

In summary, we report the syntheses of four carbocyclic phosphoramidites and their incorporation at the 5' termini of antisense strands of siRNAs. Modification of siRNA with either isomer of 5'-VP-car-DNA (**III** or **IV**) or with 5'-(*E*)-2'-OMe-car-RNA (**V**) resulted in siRNAs with potencies comparable to the siRNA with an antisense strand modified with 5'-(*E*)-VP (**I**) and more active than the siRNA with an antisense strand lacking a 5' phosphate. The 5'-(*E*)-3'-OMe-car-RNA (**VI**) analogue was less active than the siRNA with an antisense strand lacking a 5' phosphate. Interestingly, the siRNA modified with 5'-(*Z*)-VP-car-DNA (**IV**) was as potent as the siRNA modified with the 5'-(*E*) analogue (**III**). The former was not tested in mice due to poor yields. The activity of the siRNA modified with the (*Z*) isomer was unexpected as siRNAs with an antisense strand carrying the 5'-(*Z*)-VP (**II**) moiety on a 2'-OMe sugar are not active at the concentrations tested here.<sup>5,13</sup> Molecular modelling studies showed that the car-DNAs **III** and **IV** as well as 5'-VP-2'-OMe-car-RNA (**V**) fit well inside the Ago2 MID domain binding pocket. However, the 5'-(*E*)-VP-3'-OMe-car-RNA (**VI**) has steric clashes that stem from the 3'-OMe group even though the sugar pucker of the carbocyclic ring is C1'-*exo*. Given the high metabolic stability of car-RNA and car-DNA analogs<sup>21</sup> and the unexpected silencing activity of 5'-(*Z*)-VP-car-DNA (**IV**), these new VP analogues should prove useful in development of more efficacious RNAi therapeutics.

## Conflicts of interest

There are no conflicts to declare.

## Live subject statement

All animal studies were conducted following the animal welfare regulations of the state of Bavaria (Germany) and the European

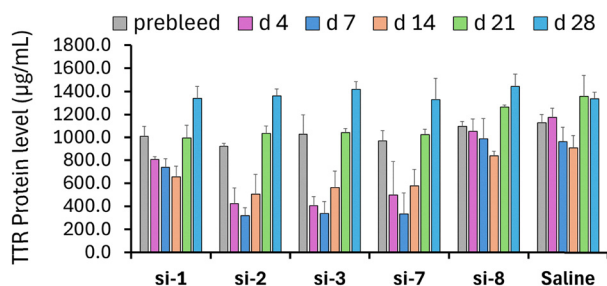


Fig. 3 Levels of TTR protein in serum of mice dosed subcutaneously with 0.4 mg kg<sup>-1</sup> indicated siRNA. TTR protein was quantified at the indicated days after dosing using an ELISA assay. Plotted are averages  $\pm$  standard deviations normalized to pre-dose levels in individual animals ( $n = 3$ ).

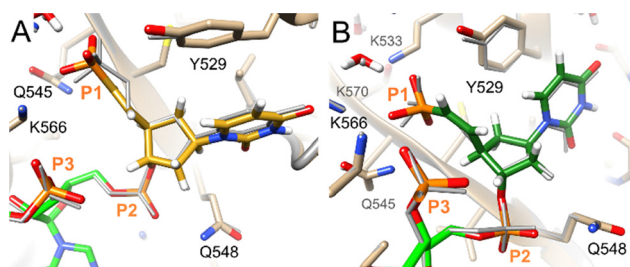


Fig. 4 Models of antisense strands modified with (A) 5'-(*E*)-VP-car-DNA **III** in golden and (B) 5'-(*Z*)-VP-car-DNA **IV** in dark green lodged at the Ago2 MID domain binding site with the RNA in the crystal structure of the miR-20a and Ago2 complex (PDB ID 4f3t) shown as a grey wire.



Union (guideline 2010/63/EU). Protocols were approved by the government of lower Franconia (Approval Nr. 55.2.2-2532-2-1548-20). This has been included as part of the SI.

## Data availability

The data supporting this article has been included as part of the supplementary information (SI). Supplementary information: Synthesis of building blocks and oligonucleotide characterization are available in the published version. See DOI: <https://doi.org/10.1039/d6cb00038j>.

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