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Mechanistic analysis of the enhanced RNAi activity by 6-mCEPh-purine at the 5′ end of the siRNA guide strand

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Abstract

A key approach for improving siRNA efficacy is chemical modifications. Through an in silico screening of modifications at the 5′-end nucleobase of the guide strand, an adenine-derived compound called 6-(3-(2-carboxyethyl)phenyl)-purine (6-mCEPh-purine) was identified to improve the RNAi activity in cultured human cells and in vivo mouse models. Nevertheless, it remains unclear how this chemical modification enhances the siRNA potency. Here, we used a series of biochemical approaches to quantitatively evaluate the effect of the 6-mCEPh-purine modification at each step in the assembly of the RNAi effector complex called RISC. We found that the modification improves the formation of mature RISC at least in two different ways, by fixing the loading orientation of siRNA duplexes and increasing the stability of mature RISC after passenger strand ejection. Our data will provide a molecular platform for further development of chemically modified siRNA drugs.

Introduction

RNA interference (RNAi) is an evolutionarily conserved gene silencing mechanism that is triggered by double-stranded RNAs (dsRNAs). Long dsRNAs are first processed by the RNase III enzyme Dicer into ~21 nt small interfering RNAs (siRNAs), which are then incorporated into the effector
complex called RNA-induced silencing complex (RISC) for direct cleavage of complementary target RNAs [Kobayashi and Tomari, 2016]. In mammals, dsRNAs longer than \( \sim 30 \) nt activate innate immune pathways, which ultimately lead to cell death [Alexopoulou et al., 2001, Barber, 2005, Gantier and Williams, 2007, Estornes et al., 2012]. However, predicted or synthetic \( \sim 21 \) nt siRNA duplexes can mediate efficient RNAi without adverse immune responses [Elbashir et al., 2001, Marques and Williams, 2005], enabling their utilization even in humans. Recently, the first siRNA drug Onpattro, which targets transthyretin (TTR) mRNA and reduces misfolded TTR amyloids, was approved for treating patients suffering hereditary ATTR amyloidosis [Adams et al., 2018].

Chemical modifications of siRNAs are key to improving the efficacy of RNAi, especially in vivo. Indeed, both small RNA strands of Onpattro are heavily, but not completely, modified with 2′-O-methylation in order to improve the chemical stability and silencing activity [Adams et al., 2018]. Rational design of further chemical modifications has promising potentials for future siRNA drug development [Khvorova and Watts, 2017, Egli and Manoharan, 2019, Setten et al., 2019]. To this end, it is critical to understand the precise effect of chemical modifications on the assembly and function of RISC.

RISC contains a member of the Ago subfamily of Argonaute protein family. siRNA duplexes are first loaded into Ago by the aid of the Hsc70/Hsp90 chaperone machinery [Iki et al., 2010, Iwasaki et al., 2010, Johnston et al., 2010, Miyoshi et al., 2010, Tsuboyama et al., 2018]. The resultant complex containing Ago and a siRNA duplex is called “pre-RISC.” Subsequently, one of the two siRNA strands, called the passenger strand, is ejected from Ago, forming “mature RISC” that contains Ago and the single-stranded guide strand [Matranga et al., 2005, Miyoshi et al., 2005, Rand et al., 2005, Leuschner et al., 2006]. The selection of the guide strand is often asymmetric; in general, the strand whose 5′ end is less stably paired is more favored as the guide strand [Schwarz et al., 2003]. Importantly, the direction of duplex loading (i.e., the orientation of the siRNA duplex in pre-RISC) predetermines which of the two strands remains in mature RISC as the guide strand. Mature RISC then recognizes target RNAs complementary to the guide strand and mediates endonucleolytic cleavage, translational repression, and/or deadenylation and decay [Huntzinger and Izaurralde, 2011, Iwakawa and Tomari, 2015]. In humans, there are four Ago paralogs (Ago1–Ago4), among which Ago2 is a major endonuclease or “slicer” for target cleavage [Liu et al., 2004, Meister et al., 2004, Park et al., 2017b].

Ago proteins are characterized by having a bilobed architecture, with one lobe consisting of the N and PAZ domains and the other lobe containing the MID and PIWI domains [Song et al., 2004, Yuan et al., 2005, Wang et al., 2008]. The pocket called “5′ nucleotide-binding pocket” is located at the interface between the MID and PIWI domains, which recognizes the monophosphate and the nucleobase at the 5′ end of the guide strand [Wang et al., 2008, Elkayam et al., 2012, Nakanishi et al., 2012, Schirle and MacRae, 2012]. A previous structural and biophysical analysis of the isolated MID domain of human Ago2 suggested that the 5′ nucleotide-binding pocket has a nucleobase preference for uracil (U) and adenine (A), compared to cytosine (C), and guanine (G) [Frank et al., 2010]. The recognition of the 5′-end nucleobase was also confirmed in the structure of full-length human Ago2 [Elkayam et al., 2012, Schirle and MacRae, 2012].

It is thought that the binding of the 5′ end of the guide strand in the 5′ nucleotide-binding pocket plays a critical role in anchoring siRNAs within Ago. Accordingly, Shinohara et al. hypothesized that chemically modifying the 5′-end nucleobase of the guide strand could be an effective strategy to enhance the siRNA knockdown potency by increasing the affinity of the siRNA duplex in Ago2 [Shinohara et al., 2021]. After an in silico screening of modifications, they designed the compound 6-(3-(2-carboxyethyl)phenyl)purine (6-mCEPh-purine) (Fig. 1A), an adenine-derived nucleotide analog bearing a hydrophobic moiety and an acidic functional group at the position 6, which occupies the empty space around the 6th position of the adenine nucleobase and creates additional interactions in the pocket. As anticipated, Shinohara et al. found that 6-mCEPh-purine markedly enhances RNAi activity in cultured cells and in an in vivo mouse model (IC50 = 0.073 mg/kg for 6-mCEPh-purine and >0.3 mg/kg for A at 168 h after tail vein injection; [Shinohara et al., 2021]). Nevertheless, the molecular mechanisms underlying the observed im-
provement by 6-mCEPh-purine remains unclear. Here, using a series of cell-free biochemical assays, we carefully evaluated the effect of the 6-mCEPh-purine modification at each fundamental step in RISC assembly, and found that 6-mCEPh-purine enhances the formation of mature RISC at least in two different ways. Our analysis provides a molecular platform for further development and optimization of siRNA chemical modifications.

Results

The 6-mCEPh-purine modification enhances target cleavage

As a starting point for understanding how the 6-mCEPh-purine modification enhances RNAi in vitro, we decided to perform a well-established target cleavage assay using a series of siRNA duplexes bearing A, U, 6-mCEPh-purine or G at the 5’ end of the guide strand (Fig. 1B). Changing the guide 5’-end nucleobase inevitably alters the base-pairing state and the thermodynamic asymmetry of the siRNA duplexes, which may affect which strand is chosen as the guide. To equalize this effect, we introduced the universal base 5-nitroindole (Suppl. Fig. 1) to position 19 of the passenger strand, which lies across from the 5’ end of the guide strand [Loakes and Brown, 1994, Kawamata et al., 2011]. We also prepared a 5’-cap-radiolabeled target RNA bearing a complementary sequence to the guide strand, but with an adenine at the position opposite to the guide 5’-end nucleobase (Fig. 1C); this position is called “t1A,” which does not form a base pair with the guide 5’ nucleobase but is instead directly recognized by Ago2
To assemble RISC, we incubated the four siRNA duplexes with different guide 5’-end nucleobases in the lysate of HEK293T cells overexpressing Ago2. We then added the target RNA and examined the time courses of the target cleavage reaction. Predictably, 6-mCEPh-purine showed significantly stronger target cleavage than natural nucleotides (Fig. 1D,E; see Materials and Methods and Supplementary Table 1 for statistical analyses; the same hereinafter), confirming the in cells and in vivo results [Shinohara et al., 2021].

The 6-mCEPh-purine modification improves mature RISC formation

To more directly examine the effect of the 6-mCEPh-purine modification on RISC assembly, we radiolabeled the guide strand 5’ end in the four siRNA duplexes (Fig. 2A) and monitored the formation of pre-Ago2-RISC and mature Ago2-RISC in Ago2-overexpressing HEK293T cell lysate, using a previously established assay that utilizes native agarose gel electrophoresis [Yoda et al., 2010, Kawamata and Tomari, 2011, Kawamata and Tomari, 2011, Kwak and Tomari, 2012]. In this assay, a nonradiolabeled, uncleavable 30-nt 2’-O-methyl target oligonucleotide complementary to the guide strand was included in the reaction mix to trap mature Ago2-RISC (Fig. 2B, also see below). As shown in Figure 2C,D, the amounts of pre-Ago2-RISC were comparable among the four different duplexes. In contrast, siRNAs bearing 5’ A or U produced much more mature Ago2-RISC than that with 5’ G (Fig. 2C,E), consistent with the result of the target cleavage assay (Fig. 1E). Importantly, the 6-mCEPh-purine modification significantly promoted the formation of mature Ago2-RISC compared to nonmodified A (Fig. 2C,E), which also agrees with the observed enhancement in target cleavage (Fig. 1E).

The guide strand selection is slightly enhanced by the 6-mCEPh-purine modification

How does the 6-mCEPh-purine modification promote mature RISC formation without apparent changes in the amount of pre-RISC formed? Given that the guide and passenger strands cannot be distinguished in the pre-RISC signal on the native agarose gel, one possibility is that 6-mCEPh-purine helps the asymmetric selection of the guide strand by fixing the orientation of duplex loading. To investigate this hypothesis, we radiolabeled the passenger strand in the four siRNA duplexes (Fig. 3A), incubated the duplexes in Ago2-overexpressing HEK293T cell lysate together with a 2’-O-methyl oligonucleotide complementary to the passenger strand, and observed the formation of pre-Ago2-RISC and “passenger-derived” mature Ago2-RISC on native agarose gel (Fig. 3B). The amounts of “passenger-derived” mature RISC (Fig. 3C,E) were generally much lower than those of “guide-derived” mature RISC (Fig. 2C,E), indicating that the four duplexes have intrinsically strong asymmetry in guide-strand selection presumably due to the relatively weak thermodynamic stability between 5-nitroindole and natural nucleotides [Loakes and Brown, 1994]. Nevertheless, the 6-mCEPh-purine modification reduced the formation of passenger-derived mature RISC, compared to A, U and G (Fig. 3C,E), suggesting that 6-mCEPh-purine can further enhance the guide strand selection upon duplex loading. However, the observed difference was small, leaving a possibility of another reason(s) for the observed improvement of guide-derived mature RISC formation by 6-mCEPh-purine in Figure 2C.

6-mCEPh-purine enhances mature RISC formation independently of the guide strand selection

Another possibility for the improvement of mature RISC formation is that the 6-mCEPh-purine modification enhances the anchoring of the guide strand in Ago2 after the ejection of the passenger strand. To test this hypothesis, we replaced the 5´ monophosphate of the passenger strand with an amino linker (Fig. 4A), which blocks loading of this strand into the nucleotide-binding pocket of Ago2 [Chiu and Rana, 2002, Czauderna et al., 2003]. Accordingly, the orientation of duplex loading is fixed in such a way that the strand with 5´ 6-mCEPh-purine, A, U or G is always
Figure 2: The 6-mCEPh-purine modification improves mature RISC formation. (A) Four siRNA duplexes used in this set of experiments. Each of them bore different nucleotides (A, U, 6-mCEPh-purine or G) at the 5’ end of the guide strand and 5-nitroindole at position 19 of the passenger strand. The guide strand was radiolabeled at the 5’ monophosphate, whereas the passenger strand had a nonradiolabeled 5’ monophosphate. (B) Scheme of RISC assembly. A nonradiolabeled, uncleavable target oligonucleotide complementary to the guide strand was added to trap mature Ago2-RISC. (C) A representative result of the native agarose gel assay. (D, E) Quantification of pre-Ago2-RISC (D) and mature Ago2-RISC (E) formation. The quantified signals were normalized to the mature Ago2-RISC value of 6-mCEPh-purine at 120 min. The graphs show the average ± SD from three independent experiments using the same set of reagents. The ANOVA P-values are given in insets under the graphs. Note that the ANOVA P-value for pre-Ago2-RISC in D is imprecisely determined, because the distribution of residuals was slightly skewed toward higher values even after log-transformation. The results of pairwise comparison on the effect of 6-mCEPh-purine compared to natural nucleotides are indicated by asterisks (* P < 0.05, ** P < 0.005, *** P < 0.0005). The detailed results of statistical analyses are summarized in Supplementary Table 1.

chosen as the guide (Fig. 4B). Still, we observed enhanced formation of mature RISC with 6mCEPh-purine (Fig. 4C,E), whereas the amounts of pre-RISC formed were similar among the four duplexes (or slightly smaller for 6-mCEPh-purine) (Fig. 4C,D). This observation indicates that the 6-mCEPh-purine modification can enhance mature RISC formation independently of the guide strand selection. Similar enhancement of mature RISC-like signals on the native agarose gel was observed with a different siRNA sequence derived from firefly luciferase (Suppl. Fig. 2), although the identities of the bands remain to be validated.

Different 5’-end nucleotides do not affect the duplex loading efficiency

In all the native agarose gel experiments described above, the formation of pre-RISC was consistently similar among the duplexes bearing the different nucleotides (A, U, G, and 6-mCEPh-
Figure 3: The guide strand selection is slightly enhanced by the 6-mCEPh-purine modification. (A) Four siRNA duplexes used in this set of experiments. Each of them bore different nucleotides (A, U, 6-mCEPh-purine or G) at the 5’ end of the guide strand and 5-nitroindole at position 19 of the passenger strand. The passenger strand was radiolabeled at the 5’ monophosphate, whereas the guide strand had a nonradiolabeled 5’ monophosphate. (B) Scheme of RISC assembly. A nonradiolabeled, uncleavable target oligonucleotide complementary to the passenger strand was added. (C) A representative result of the native agarose gel assay. (D,E) Quantification of pre-Ago2-RISC (D) and mature Ago2-RISC (E) formation. The quantified signals were normalized to the mature Ago2-RISC value of 6-mCEPh-purine at 120 min. The graphs show the average ± SD from three independent experiments using the same set of reagents. The ANOVA P-values are given in insets under the graphs. The results of pairwise comparison on the effect of 6-mCEPh-purine compared to natural nucleotides are indicated by asterisks (*) P < 0.05, (**) P < 0.005, (***) P < 0.0005. The detailed results of statistical analyses are summarized in Supplementary Table 1.

However, because pre-RISC is continuously converted into mature RISC during RISC assembly, it still remained unclear if the 6-mCEPh-purine modification has any direct effect on the efficiency of duplex loading per se. To precisely address this point, a 2′-O-methyl modification was introduced at the position 9 of the passenger strand, which blocks slicing and ejection of the passenger strand (Fig. 5A,B; Miyoshi et al., 2005, Leuschner et al., 2006) and allows us to purely monitor the efficiency of duplex loading. Our data showed that pre-Ago2-RISC formation was virtually equal among the four different siRNA duplexes (Fig. 5C,D). Taken all together, our data suggest that the 6-mCEPh-purine modification does not affect the efficiency of siRNA duplex loading into Ago2 to form pre-RISC, but increases mature RISC stability after the passenger strand is ejected.
Figure 4: 6-mC Eph-purine enhances mature RISC formation independently of the guide strand selection. (A) Four siRNA duplexes used in this set of experiments. Each of them bore different nucleotides (A, U, 6-mC Eph-purine or G) at the 5’ end of the guide strand and 5-nitroindole at position 19 of the passenger strand. The guide strand was radiolabeled at the 5’ monophosphate, whereas the passenger strand held a 5’ amino linker that fixes the loading orientation by blocking this strand from being anchored in the 5’ nucleotide-binding pocket of Ago2. (B) Scheme of RISC assembly. A nonradiolabeled, uncleavable target oligonucleotide was added. (C) A representative result of the native agarose gel assay. (D,E) Quantification of pre-Ago2-RISC (D) and mature Ago2-RISC (E) formation. The quantified signals were normalized to the mature Ago2-RISC value of 6-mC Eph-purine at 120 min. The graphs show the average ± SD from three independent experiments using the same set of reagents. The ANOVA P-values are given in insets under the graphs. The results of pairwise comparison on the effect of 6-mC Eph-purine compared to natural nucleotides are indicated by asterisks (* P < 0.05, ** P < 0.005, *** P < 0.0005). The detailed results of statistical analyses are summarized in Supplementary Table 1.

The target does not influence enhanced mature RISC formation by 6-mC Eph-purine

As described above, we routinely include a complementary nonradiolabeled 30-nt 2’-O-methyl oligonucleotide in our native agarose gel assay, which enables detection of trapped mature RISC as a sharp signal on the native agarose gel by avoiding its binding to heterogenous mRNAs present in the lysate; in the absence of the target, the signal of mature Ago2-RISC is detected as a smeared signal on the gel (Suppl. Fig. 5). However, it has been reported that interactions between mature RISC and its targets can promote destabilization and/or “unloading” of the guide strand from human Ago2-RISC [Ameres et al., 2010, Cazalla et al., 2010, De et al., 2013, Park et al., 2017a]. To evaluate the effect of the 6-mC Eph-purine modification on RISC assembly in the absence of targets, we treated the Ago2-overexpressing HEK293T lysate with micrococcal nuclease (MNase) to digest and deplete endogenous mRNAs [Svitkin and Sonenberg, 2004]. After quenching the MNase activity by EGTA, we then performed the native agarose gel assay without adding a 2’-O-
Figure 5: The efficiency of duplex loading is not affected by different 5’-end nucleobases. (A) Four siRNA duplexes used in this set of experiments. Each of them bore different nucleotides (A, U, 6-mCeph-purine or G) at the 5’ end of the guide strand and 5-nitroindole at position 19 of the passenger strand. The guide strand was radiolabeled at the 5’ monophosphate, whereas the passenger strand held a 5’ amino linker that fixes the loading orientation and a 2’-O-methyl modification that blocks passenger ejection. (B) Scheme of RISC assembly. A nonradiolabeled, uncleavable target oligonucleotide was added. (C) A representative result of the native agarose gel assay. (D) Quantification of pre-Ago2-RISC formation. The quantified signals were normalized to the pre-Ago2-RISC value of 6-mCeph-purine at 120 min. The graph shows the average ± SD from three independent experiments using the same set of reagents. The ANOVA P-values are given in an inset under the graphs. The results of pairwise comparison on the effect of 6-mCeph-purine compared to natural nucleotides are indicated by asterisks (*) P < 0.05, (**) P < 0.005, (***) P < 0.0005. The detailed results of statistical analyses are summarized in Supplementary Table 1.

methyl target oligonucleotide (Fig. 6A,B). In this condition, mature Ago2-RISC can be detected as a discrete signal from pre-Ago2-RISC even without the target RNAs (Suppl. Fig. 3). Still, we observed significantly enhanced formation of mature Ago2-RISC with 6-mCeph-purine, as well as comparable formation of pre-Ago2-RISC among the different duplexes (Fig. 6C–E). We concluded that the observed enhancement of mature RISC formation by 6-mCeph-purine is independent of target-mediated unloading or destabilization of the guide strand.

Passenger strand cleavage is not affected by the 6-mCeph-purine modification

It is known that human Ago2 can cleave the passenger strand within pre-RISC, which facilitates the passenger strand ejection and mature RISC formation [Matranga et al., 2005; Miyoshi et al., 2005; Rand et al., 2005; Leuschner et al., 2006; Park and Shin, 2015]. Therefore, the efficiency of passenger strand ejection can be estimated by monitoring the production of the cleavage fragment of the passenger strand, which is rapidly degraded in normal conditions but can be trapped for detec-
Figure 6: The target does not influence enhanced mature RISC formation by 6-mCEPh-purine. (A) Four siRNA duplexes used in this set of experiments. Each of them bore different nucleotides (A, U, 6-mCEPh-purine or G) at the 5′ end of the guide strand and 5-nitroindole at position 19 of the passenger strand. The guide strand was radiolabeled at the 5′ monophosphate, whereas the passenger strand held a 5′ amino linker that fixes the loading orientation. (B) Scheme of RISC assembly. The lysate was treated with MNase for the depletion of endogenous mRNAs, and no target was added. (C) A representative result of the native agarose gel assay. (D,E) Quantification of pre-Ago2-RISC (D) and mature Ago2-RISC (E) formation. The quantified signals were normalized to the mature Ago2-RISC value of 6-mCEPh-purine at 120 min. The graphs show the average ± SD from three independent experiments using the same set of reagents. The ANOVA P-values are given in insets under the graphs. The results of pairwise comparison on the effect of 6-mCEPh-purine compared to natural nucleotides are indicated by asterisks (*) P < 0.05, (**) P < 0.005, (***) P < 0.0005. The detailed results of statistical analyses are summarized in Supplementary Table 1.

Discussion

The effects of siRNA chemical modifications on the RNAi activity have been analyzed in many studies [Khvorova and Watts, 2017; Egli and Manoharan, 2019; Setten et al., 2019]. However, most
Figure 7: The 6-mCEPh-purine modification does not impact the passenger cleavage. (A) Four siRNA duplexes used in this set of experiments. They bore different nucleotides (A, U, 6-mCEPh-purine or G) at the 5′ end of the guide strand and 5-nitroindole at position 19 of the passenger strand. (B) Scheme of RISC assembly and passenger cleavage. The passenger was 3′ radiolabeled. (C) A representative result of the passenger cleavage assay. The upper and lower bands correspond to the full-length and cleaved passenger strand, respectively. (D) Quantification of the target cleavage assay. The graph shows the average ± SD from three independent experiments using the same set of reagents. The ANOVA P-values are given in an inset under the graph. The results of pairwise comparison on the effect of 6-mCEPh-purine compared to natural nucleotides are indicated by asterisks (*) P < 0.05, (**) P < 0.005, (***) P < 0.0005. The detailed results of statistical analyses are summarized in Supplementary Table 1.

of the previous studies have relied on luciferase reporter assays in cultured cells, which can only evaluate the final outcome of target silencing. In this study, we used a series of biochemical approaches including native agarose gel electrophoresis and analyzed the mechanistic effect of the 6-mCEPh-purine modification at the 5′ end of the guide strand at each step during Ago2-RISC assembly. We found that 6-mCEPh-purine improves the formation of mature RISC at least in two different ways (Fig. 8): (1) 6-mCEPh-purine enhances the guide strand selection by fixing the loading orientation of siRNA duplexes (Fig. 3), and more importantly, (2) 6-mCEPh-purine increases the stability of mature RISC after passenger strand ejection (Figs. 4–6). On the other hand, no apparent difference was observed for the amount of pre-Ago2-RISC formed (Fig. 5). Thus, 6-mCEPh-purine does not quantitatively affect the efficiency of duplex loading, even though it can qualitatively change the orientation of the duplex in pre-Ago2-RISC (Fig. 8).

It has been suggested that anchoring of the guide 5′ monophosphate in the 5′ nucleotide-binding pocket is the first critical event in the loading of siRNA duplexes into Ago [Elkayam et al., 2012; Nakanishi et al., 2012; Schirle and MacRae, 2012; Iwasaki et al., 2015]. It is therefore reasonable to speculate that the guide 5′ nucleobase, adjacent to the 5′ monophosphate, is also anchored in the pocket at the step of duplex loading. However, enhancement by 6-mCEPh-purine was undetectable in the efficiency of pre-Ago2-RISC formation and became apparent only at the step of
Figure 8: A model for the molecular mechanism by which 6-mCepH-purine enhances the RNAi activity. 6-mCepH-purine modification improves the formation of mature RISC in two different ways: (1) 6-mCepH-purine helps the guide strand selection. (2) 6-mCepH-purine increases the stability of mature RISC after passenger strand ejection.

mature Ago2-RISC formation. There are two possible explanations for this counterintuitive observation. First, the “double-stranded state” of siRNA duplexes in pre-Ago2-RISC may act as an additional anchor point for Ago2, masking the effect of the 5’ nucleobase anchoring; this internal anchor point is lost after passenger ejection, making the 5’ nucleobase anchoring more important (Suppl. Fig. 4, Model 1). This is reminiscent of what we have previously proposed for Drosophila Ago1, where the enhancement effect of preferred nucleobases (A and U) was more prominent in mature Ago1-RISC than in pre-Ago1-RISC [Kawamata et al., 2011]. Alternatively, but not mutually exclusively, the 5’ nucleobase may not be fully anchored in the 5’ nucleotide-binding pocket immediately upon duplex loading due to some structural constraints, and may become fixed in there only after ejection of the passenger strand (Suppl. Fig. 4, Model 2). In either case, the 6-mCepH-purine modification at the guide 5’ nucleobase leads to the formation of more mature Ago2-RISC (Fig. 4), which explains higher RNAi efficiency observed in vitro (Fig. 1), in cells and in vivo [Shinohara et al., 2021].

Although siRNA therapeutics have a great potential, there is still room for improvement in their efficacy while reducing their dosage and cost [Khvorova and Watts, 2017, Egli and Manoharan, 2019, Setten et al., 2019]. Our current study will help to provide a molecular platform for mechanistic understanding of chemically modified siRNAs, which will contribute to further development of RNAi drugs. Notably, 5’-(E)-vinylphosphonate (5’-E-VP), which mimics the 5’ phosphate of the siRNA guide strand, is known to enhance not only the metabolic stability and also the binding affinity to Ago2, leading to better silencing in vitro and in vivo [Lima et al., 2012, Prakash et al., 2016, Elkayam et al., 2017, Haraszti et al., 2017]. Given that 6-mCepH-purine is a 5’ nucleobase modification that is theoretically compatible with the 5’-E-VP modification, it would be interesting to analyze in the future if and how these two adjacent chemical modifications synergize to enhance the siRNA efficiency.

Materials and Methods

Cell culture

HEK 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Sigma) at 37°C in 5% CO2.

Overexpression of FLAG-tagged hAgo2 protein in HEK 293T cells

HEK293T cells at ~80% confluence were transfected with 10 µg/10-cm dish of pRESneo-FLAG-HA-Ago2 [Meister et al., 2004] by using Lipofectamine 3000 (Thermo Fisher). The cells were harvested after 48 h.
Cell lysate preparation

HEK293T cells were washed three times with cold PBS (pH 7.4) and collected by centrifugation at 1000g for 3 min at 4°C. The cell pellet was resuspended in two packed-cell volume of lysis buffer (30 mM HEPES-KOH [pH 7.4], 100 mM potassium acetate 2 mM magnesium acetate) containing 5 mM DTT and 1× EDTA-free Complete Protease Inhibitor tablets (Roche) and subjected to Dounce homogenization. Subsequently, the lysate was clarified by centrifugation at 17,000g for 30 min at 4°C. The supernatant was flash frozen in liquid nitrogen and immediately stored at -80°C in single-use aliquots.

Preparation of siRNA duplexes

The sequences, chemical modifications, and radiolabeling of the siRNA duplexes are shown in respective figures. The guide and passenger strands were heat-annealed in lysis buffer as previously described [Haley et al., 2003].

Preparation of the target mRNA for cleavage assay

The 182-nt target mRNA for the cleavage assay was in vitro transcribed using T7-Scribe Standard RNA IVT Kit (Cellscript) from the PCR product amplified from pGL3-basic vector (Promega) as previously described [Haley et al., 2003, Naruse et al., 2018]. The target mRNA was gel purified and radiolabeled at the 5’ cap using the ScriptCap m7G Capping System (Cellscript) and [α-32P] GTP (PerkinElmer) according to the manufacturer’s instructions.

Target cleavage assay

Target mRNA cleavage assays were performed in 20 µL reactions mixtures as described [Haley et al., 2003] with the following modifications: 2 µL of 200 nM 5’-phosphorylated siRNA duplexes were incubated at 25°C with 10 µL of lysate from HEK293T cells overexpressing FLAG-tagged Ago2 and 6 µL of 40× reaction mix (containing ATP, the ATP regeneration system and the RNase inhibitor; described in detail in [Haley et al., 2003]). Then, 2 µL of ~10 nM cap-radiolabeled target mRNA was added. An amount of 2 µL of the reaction mixture was taken at each time point, mixed with 8 µL of low-salt PK solution (0.125% SDS, 12.5 mM EDTA, 12.5 mM HEPES-KOH (pH 7.4), 12.5% Proteinase K), and incubated at 55°C for 10 min. An equal volume of 2× formamide dye (containing ATP, the ATP regeneration system and the RNase inhibitor; described in detail in [Haley et al., 2003]) was then added and incubated at 68°C for 5 min. The cleavage products were analyzed on an 8% denaturing polyacrylamide gel. Gels were dried and imaged by Typhoon FLA 7000 (GE Healthcare Life Sciences) and quantified using MultiGauge software (Fujifilm Life Sciences). Graphs were prepared using IgorPro (WaveMetrics).

Native agarose gel analysis

Native agarose gel analysis was performed essentially as previously described [Kawamata et al., 2009]. Briefly, the 5’ end of the guide strands was radiolabeled by [γ-32P] ATP (PerkinElmer) and T4 Polynucleotide Kinase (Takara). The radiolabeling efficiency was comparable among A, U, 6-mCpG-purine and G (~1.05–1.27-fold, depending on the experiments) and the specific radioactivity was further normalized before being annealed to a 1.5-fold excess amount of the nonradiolabeled passenger strand. 1.4% (w/v) agarose gels (Low Range Ultra Agarose, Bio-Rad Laboratories) were cast vertically between glass plates with 1.5–2 mm-thick side spacers and a 0.5 mm-thick bottom spacer (16 cm × 16 cm). The reaction mixture containing 10 µL Ago2-expressing HEK293T cell lysate, 6 µL 40× reaction mix, 2 µL 15% (w/v) Ficoll 400 in lysis buffer, 2 µL of ~50 nM siRNA duplexes with the 5’ monophosphate of the guide strand radiolabeled, and 2 µL of 50 nM 2’-O-methyl target oligonucleotide (5’-mUmCmUmUmCmAmCmAmUmAmCmAmCmAmCmAmCmAmCmAmCmAmCmAmCmAmCmAmCmAmCmAmCmAmCmAmCmAmCmAmCmAmCmAmCmCmAmCmCmCmC/5-nitroindole/mAmCmCmCmU-3’) was incubated at 25°C. This target oligonucleotide was complementary to the siRNA guide strand and contained a 5-nitroindole at the
position opposite the 5′-end nucleotide of the guide strand. In Figure 3, the passenger strand instead of the guide strand was radiolabeled and the corresponding target oligonucleotide (5′-mUmCmUmUmAmGmUmGmAmGmGmUmAmGmUmAmGmGmUmAmU-3′) was used. After incubation, the complexes were resolved by native agarose gel electrophoresis with 0.5× TBE buffer at 300 V at 4°C for 1.5 h. Complexes were detected by PhosphorImager (FLA-7000 image analyzer, Fujifilm) and quantified using MultiGauge software (Fujifilm). The background signals were appropriately subtracted by using an empty space for each lane on the gel as the “0” value and the graphs were prepared using IgorPro (WaveMetrics).

**MNase treatment for digestion of endogenous mRNAs**

First, naïve HEK293T cell lysate was fivefold diluted in lysis buffer. Then, MNase (2,000,000 gel unit/mL; NEB) was 125-fold diluted in the diluted native cell lysate. Finally, 100 µL of Ago2-expressing HEK293T cell lysate was incubated with 4 µL of the diluted MNase solution in the presence of 1 mM Ca(OAc)2 at 25°C for 20 min. 4 mM EGTA (final concentration) was added to stop the reaction. The native gel assay using MNase-treated lysate was performed in the absence of target oligonucleotide (Fig. 6; Suppl. Fig. 3).

**Detection of passenger strand cleavage**

Detection of passenger strand cleavage was performed as described [Matranga et al., 2005] with the following modifications: The 3′ end of the passenger strand was radiolabeled by [α-32P]cordycepin-5′-triphosphate (PerkinElmer) and yeast poly(A) polymerase (Thermo Fisher Scientific), while the 5′ end of the guide strand was nonradioactively monophosphorylated. Typically, in 20 µL reactions, 2 µL of siRNA duplexes were incubated at 25°C with 10 µL of lysate from HEK293T cells overexpressing FLAG-tagged Ago2, 2 µL of 1 µM 2′-O-methyl oligonucleotide complementary to the passenger strand, and 6 µL of 40× reaction mix. An amount of 2 µL of the reaction mixture was taken at each time point, mixed with 8 µL of low-salt PK solution, and incubated at 55°C for 10 min. An equal volume of 2× formamide dye was then added and incubated at 68°C for 5 min. The 3′ cleavage fragments of the passenger strand were analyzed on an 15% denaturing polyacrylamide gel. Gels were dried and imaged by Typhoon FLA 7000 (GE Healthcare Life Sciences) and quantified using MultiGauge software (Fujifilm Life Sciences). Graphs were prepared using IgorPro (WaveMetrics).

**Statistical analyses**

Significance of the observed differences was assessed by an analysis-of-variance (ANOVA) on a model with two explanatory variables: the 5′ nucleotide identity and a variable related to time by a bijective function (for mature RISC accumulation: variable = 1–2–t/τ; for pre-RISC decrease: variable = 2–t/τ with t indicating time and τ indicating an estimated doubling or halving time [10 min for Figs. 1, 5 and 7, 50 min for Figs. 2, 4 and 6]). These log-transforming functions were chosen because the abundance of mature RISC and pre-RISC should vary linearly to these variables in a simplified kinetics model. In every analysis throughout this study, ANOVA always found a significant effect (P < 0.05) of this time-dependent variable. Whenever a significant effect was also found for the 5′ nucleotide identity, the significance of the effect of an interaction between these two variables was also assessed. Whenever 5′ nucleotide identity, or its interaction with the time-dependent variable, was found to have a significant effect (P < 0.05), pairwise comparisons with false discovery rate correction were performed to compare the effect of 6-mCEPh-purine to natural nucleotides. Such pairwise comparisons were also performed by an ANOVA model with the same two explanatory variables. The results of statistical analyses are summarized in Supplementary Table 1. The results of pairwise comparison for the 5′ nucleotide identity (the effect of 6-mCEPh-purine compared to natural nucleotides) are indicated as asterisks in the figures (* P < 0.05; ** P < 0.005; *** P < 0.0005). Conditions for applicability of the ANOVA were verified by Levene’s test (for variance homogeneity) and Shapiro Wilk’s test (for residual
normality); whenever conditions were not met, ANOVA was performed on log(values) instead of values themselves. Log-transformation was always sufficient to make the data suitable for ANOVA, except for pre-RISC abundance in Figure 2 (where the distribution of residuals was slightly skewed toward higher values, making the estimation of P-value by ANOVA imprecise for that analysis).

Scripts and data files are available at [https://github.com/HkeyHKey/Brechin_et_al_2020](https://github.com/HkeyHKey/Brechin_et_al_2020).

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**Author contributions**

V.B. performed the experiments. F.S. and J.S. provided the 6-mCEPh-purine-modified siRNAs. V.B. and Y.T. performed data analysis and interpretation. H.S. performed the statistical analyses. V.B. and Y.T. wrote the manuscript. Y.T. supervised the study. Competing interest statement: F.S. and J.I.S. are current employees of Kyowa Kirin Co., Ltd.

**References**

[Adams et al., 2018] Adams, D., Gonzalez-Duarte, A., O’Riordan, W. D., Yang, C.-C., Ueda, M., Kristen, A. V., Tourne, I., Schmidt, H. H., Coelho, T., Berk, J. L., Lin, K.-P., Vita, G., Attarian, S., Planté-Bordeneuve, V., Mezei, M. M., Campistol, J. M., Buades, J., Brannagan, T. H. r., Kim, B. J., Oh, J., Parman, Y., Sekijima, Y., Hawkins, P. N., Solomon, S. D., Polydefkis, M., Dyck, P. J., Gandhi, P. J., Goyal, S., Chen, J., Strahs, A. L., Nochur, S. V., Sweetser, M. T., Garg, P. P., Vaiishnaw, A. K., Gollob, J. A., and Suhr, O. B. (2018). Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis. *N Engl J Med*, 379(1):11–21.

[Alexopoulou et al., 2001] Alexopoulou, L., Holt, A., Medzhitov, R., and Flavell, R. (2001). Recognition of double-stranded RNA and activation of NF-kappa B by Toll-like receptor 3. *Nature*, 413(6857):732–738.

[Ameres et al., 2010] Ameres, S. L., Horwich, M. D., Hung, J. H., Xu, J., Ghildiyal, M., Weng, Z., and Zamore, P. D. (2010). Target RNA-directed trimming and tailing of small silencing RNAs. *Science*, 328(5985):1534–1539.

[Barber, 2005] Barber, G. (2005). The dsRNA-dependent protein kinase, PKR and cell death. *Cell Death and Differentiation*, 12(6):563–570.

[Czauderna et al., 2003] Czauderna, F., Fechtner, M., Dames, S., Aygün, H., Klippel, A., Pronk, G. J., Giese, K., and Kaufmann, J. (2003). Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. *Nucleic Acids Research*, 31(11):2705–2716.
De et al., 2013] De, N., Young, L., Lau, P.-W., Meisner, N.-C., Morrissey, D., and MacRae, I. (2013). Highly complementary target RNAs promote release of guide RNAs from human Argonaute2. *Molecular Cell*, 50(3):344–355.

Egli and Manoharan, 2019] Egli, M. and Manoharan, M. (2019). Re-engineering RNA molecules into therapeutic agents. *Accounts of Chemical Research*, 52(4):1036–1047.

Elbashir et al., 2001] Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*, 411(6836):494–498.

Elkayam et al., 2012] Elkayam, E., Kuhn, C.-D., Tocilj, A., Haase, A., Greene, E., Hannon, G., and Joshua-Tor, L. (2012). The structure of human argonaute-2 in complex with mir-20a. *Cell*, 150(1):100–110.

Elkayam et al., 2017] Elkayam, E., Parmar, R., Brown, C. R., Willoughby, J. L., Theile, C. S., Manoharan, M., and Joshua-Tor, L. (2017). siRNA carrying an (E)-vinylphosphonate moiety at the 5’ end of the guide strand augments gene silencing by enhanced binding to human Argonaute-2. *Nucleic Acids Research*, 45(6):3528–3536.

Estornes et al., 2012] Estornes, Y., Toscano, F., Virard, F., Jacquemin, G., Pierrot, A., Vanbervliet, B., Bonnin, M., Lahaoui, N., Mercier-Gouy, P., Pacheco, Y., Salaun, B., Remo, T., Micheau, O., and Lebecque, S. (2012). dsRNA induces apoptosis through an atypical death complex associating TLR3 to caspase-8. *Cell Death Differ*, 19(9):1482–1494.

Frank et al., 2010] Frank, F., Sonenberg, N., and Nagar, B. (2010). Structural basis for 5’-nucleotide base-specific recognition of guide RNA by human AGO2. *Nature*, 465(7299):818–822.

Gantier and Williams, 2007] Gantier, M. P. and Williams, B. R. (2007). The response of mammalian cells to double-stranded rna. *Cytokine and Growth Factor Reviews*, 18(5):363–371.

Haley et al., 2003] Haley, B., Tang, G., and Zamore, P. D. (2003). In vitro analysis of rna interference in *Drosophila melanogaster*. *Methods*, 30(4):330–336.

Haraszti et al., 2017] Haraszti, R. A., Roux, L., Coles, A. H., Turanov, A. A., Alterman, J. F., Echeverria, D., Godinho, B. M., Aronin, N., and Khvorova, A. (2017). 5’-Vinylphosphonate improves tissue accumulation and efficacy of conjugated siRNAs in vivo. *Nucleic Acids Research*, 45(13):7581–7592.

Huntzinger and Izaurralde, 2011] Huntzinger, E. and Izaurralde, E. (2011). Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat Rev Genet*, 12(2):99–110.

Iki et al., 2010] Iki, T., Yoshikawa, M., Nishikiori, M., Jaudal, M. C., Matsumoto-Yokoyama, E., Mitsuhara, I., Meshi, T., and Ishikawa, M. (2010). In vitro assembly of plant rna-induced silencing complexes facilitated by molecular chaperone HSP90. *Molecular Cell*, 39(2):282–291.

Iwakawa and Tomari, 2015] Iwakawa, H.-o. and Tomari, Y. (2015). The functions of micrornas: mRNA decay and translational repression. *Trends in Cell Biology*, 25(11):651–665.

Iwasaki et al., 2010] Iwasaki, S., Kobayashi, M., Yoda, M., Sakaguchi, Y., Katsuma, S., Suzuki, T., and Tomari, Y. (2010). Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. *Molecular Cell*, 39(2):292–299.

Iwasaki et al., 2015] Iwasaki, S., Sasaki, H. M., Sakaguchi, Y., Suzuki, T., Tadakuma, H., and Tomari, Y. (2015). Defining fundamental steps in the assembly of the *Drosophila* RNAi enzyme complex. *Nature*, 521(7553):533–536.
[Johnston et al., 2010] Johnston, M., Geoffroy, M.-C., Sobala, A., Hay, R., and Hutvagner, G. (2010). HSP90 protein stabilizes unloaded Argonaute complexes and microscopic P-bodies in human cells. *Molecular Biology of the Cell*, 21(9):1462–1469. PMID: 20237157.

[Kawamata et al., 2009] Kawamata, T., Seitz, H., and Tomari, Y. (2009). Structural determinants of miRNAs for RISC loading and slicer-independent unwinding. *Nat Struct Mol Biol*, 16(9):953–960.

[Kawamata and Tomari, 2011] Kawamata, T. and Tomari, Y. (2011). Native gel analysis for RISC assembly. *Methods Mol Biol.*, 725:91–105.

[Kawamata et al., 2011] Kawamata, T., Yoda, M., and Tomari, Y. (2011). Multilayer checkpoints for microRNA authenticity during RISC assembly. *EMBO Rep.*, 12(9):944–949.

[Khvorova and Watts, 2017] Khvorova, A. and Watts, J. K. (2017). The chemical evolution of oligonucleotide therapies of clinical utility. *Nat Biotecnol*, 35(3):238–248.

[Kobayashi and Tomari, 2016] Kobayashi, H. and Tomari, Y. (2016). Risc assembly: coordination between small RNAs and argonaute proteins. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, 1859(1):71–81. SI: Clues to long noncoding RNA taxonomy.

[Kwak and Tomari, 2012] Kwak, P. B. and Tomari, Y. (2012). The N domain of Argonaute drives duplex unwinding during RISC assembly. *Nat Struct Mol Biol*, 19(2):145–151.

[Leuschner et al., 2006] Leuschner, P. J. F., Ameres, S. L., Kueng, S., and Martinez, J. (2006). Cleavage of the siRNA passenger strand during RISC assembly in human cells. *EMBO Rep.*, 7(3):314–320.

[Lima et al., 2012] Lima, W. F., Prakash, T. P., Murray, H. M., Kinberger, G. A., Li, W., Chappell, A. E., Li, C. S., Murray, S. F., Gaus, H., Seth, P. P., Swayze, E. E., and Crooke, S. T. (2012). Single-stranded siRNAs activate RNAi in animals. *Cell*, 150(5):883–894.

[Liu et al., 2004] Liu, J., Carmell, M. A., Rivas, F. V., Marsden, C. G., Thomson, J. M., Song, J. J., Hammond, S. M., Joshua-Tor, L., and Hannon, G. J. (2004). Argonaute2 is the catalytic engine of mammalian RNAi. *Science*, 305(5689):1437–1441.

[Loakes and Brown, 1994] Loakes, D. and Brown, D. M. (1994). 5-Nitroindole as an universal base analogue. *Nucleic Acids Res.*, 22(20):4039–4043.

[Marques and Williams, 2005] Marques, J. T. and Williams, B. R. G. (2005). Activation of the mammalian immune system by siRNAs. *Nat Biotechnol*, 23(11):1399–1405.

[Matranga et al., 2005] Matranga, C., Tomari, Y., Shin, C., Bartel, D. P., and Zamore, P. D. (2005). Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell*, 123(4):607–620.

[Meister et al., 2004] Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G., and Tuschl, T. (2004). Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol Cell*, 15(2):185–197.

[Miyoshi et al., 2005] Miyoshi, K., Tsukumo, H., Nagami, T., Siomi, H., and Siomi, M. C. (2005). Slicer function of *Drosophila* Argonautes and its involvement in RISC formation. *Genes Dev.*, 19(23):2837–2848.

[Miyoshi et al., 2010] Miyoshi, T., Takeuchi, A., Siomi, H., and Siomi, M. C. (2010). A direct role for Hsp90 in pre-RISC formation in *Drosophila*. *Nat Struct Mol Biol*, 17(8):1024–1026.

[Nakanishi et al., 2012] Nakanishi, K., Weinberg, D. E., Bartel, D. P., and Patel, D. J. (2012). Structure of yeast Argonaute with guide RNA. *Nature*, 486(7403):368–374.
[Naruse et al., 2018] Naruse, K., Matsuura-Suzuki, E., Watanabe, M., Iwasaki, S., and Tomari, Y. (2018). In vitro reconstitution of chaperone-mediated human RISC assembly. *RNA, 24*(1):6–11.

[Park and Shin, 2015] Park, J. H. and Shin, C. (2015). Slicer-independent mechanism drives small-RNA strand separation during human RISC assembly. *Nucleic Acids Res*, 43(19):9418–9433.

[Park et al., 2017a] Park, J. H., Shin, S.-Y., and Shin, C. (2017a). Non-canonical targets destabilize microRNAs in human Argonautes. *Nucleic Acids Res*, 45(4):1569–1583.

[Park et al., 2017b] Park, M. S., Phan, H.-D., Busch, F., Hinckley, S. H., Brackbill, J. A., Wysocki, V. H., and Nakanishi, K. (2017b). Human Argonaute3 has slicer activity. *Nucleic Acids Res*, 45(20):11867–11877.

[Prakash et al., 2016] Prakash, T. P., Kinberger, G. A., Murray, H. M., Chappell, A., Riney, S., Graham, M. J., Lima, W. F., Swayze, E. E., and Seth, P. P. (2016). Synergistic effect of phosphorothioate, 5‘-vinylphosphonate and GalNAc modifications for enhancing activity of synthetic siRNA. *Bioorg Med Chem Lett*, 26(12):2817–2820.

[Rand et al., 2005] Rand, T. A., Petersen, S., Du, F., and Wang, X. (2005). Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell, 123*(4):621–629.

[Schirle and MacRae, 2012] Schirle, N. T. and MacRae, I. J. (2012). The crystal structure of human Argonaute2. *Science, 336*(6084):1037–1040.

[Schirle et al., 2015] Schirle, N. T., Sheu-Gruttadauria, J., Chandradoss, S. D., Joo, C., and MacRae, I. J. (2015). Water-mediated recognition of t1-adenosine anchors Argonaute2 to microRNA targets. *Elife, 4*:e07646.

[Schwarz et al., 2003] Schwarz, D. S., Hutvágner, G., Du, T., Xu, Z., Aronin, N., and Zamore, P. D. (2003). Asymmetry in the assembly of the RNAi enzyme complex. *Cell, 115*(2):199–208.

[Setten et al., 2019] Setten, R. L., Rossi, J. J., and Han, S. P. (2019). The current state and future directions of RNAi-based therapeutics. *Nat Rev Drug Discov*, 18(6):421–446.

[Shinohara et al., 2021] Shinohara, F., Oashi, T., Harumoto, T., Nishikawa, T., Takayama, Y., Miyagi, H., Takahashi, Y., Nakajima, T., Sawada, T., Koda, Y., Makino, A., Sato, A., Hamaguchi, K., Suzuki, M., Yamamoto, J., Tomari, Y., and Saito, J.-I. (2021). siRNA potency enhancement via chemical modifications of nucleotide bases at the 5′-end of the siRNA guide strand. *RNA, 27*(2):163–173.

[Song et al., 2004] Song, J. J., Smith, S. K., Hannon, G. J., and Joshua-Tor, L. (2004). Crystal structure of Argonaute and its implications for RISC Slicer activity. *Science, 305*(5689):1434–1437.

[Svitkin and Sonenberg, 2004] Svitkin, Y. V. and Sonenberg, N. (2004). An efficient system for cap- and poly(A)-dependent translation in vitro. *Methods Mol Biol, 257*:155–170.

[Tsuboyama et al., 2018] Tsuboyama, K., Tadakuma, H., and Tomari, Y. (2018). Conformational activation of Argonaute by distinct yet coordinated actions of the Hsp70 and Hsp90 chaperone systems. *Mol Cell, 70*(4):722–729.

[Wang et al., 2008] Wang, Y., Sheng, G., Juranek, S., Tuschl, T., and Patel, D. J. (2008). Structure of the guide-strand-containing Argonaute silencing complex. *Nature, 456*(7219):209–213.

[Yoda et al., 2010] Yoda, M., Kawamata, T., Paroo, Z., Ye, X., Iwasaki, S., Liu, Q., and Tomari, Y. (2010). ATP-dependent human RISC assembly pathways. *Nat Struct Mol Biol, 17*(1):17–23.
[Yuan et al., 2005] Yuan, Y.-R., Pei, Y., Ma, J.-B., Kuryavyi, V., Zhadina, M., Meister, G., Chen, H.-Y., Dauter, Z., Tuschl, T., and Patel, D. J. (2005). Crystal structure of A. aeolicus argonaute, a site-specific DNA-guided endoribonuclease, provides insights into RISC-mediated mRNA cleavage. *Mol Cell*, 19(3):405–419.

Supplemental material

Supplementary Figure 1: **Chemical structure of 5-nitroindole.**
Supplementary Figure 2: **Native agarose gel analysis of a luciferase-derived siRNA sequence.** RISC assembly of an siRNA sequence derived from firefly luciferase (Luc) was analyzed on the native agarose gel. Stronger signals for mature RISC-like bands (asterisk) were observed with 6-mCEPh-purine compared to A. Please note that the identities of the bands for the let-7 siRNA sequence (Figures 2–6) have been previously determined in a rigorous manner [Yoda et al., 2010], but those for the Luc sequence remain to be validated.
Supplementary Figure 3: The effect of MNase treatment on the native agarose gel assay. Digestion of endogenous mRNAs by the MNase treatment allowed the detection of mature Ago2-RISC as a discrete signal on the native agarose gel in the absence of targets.
Supplementary Figure 4: Two models to explain no apparent change in pre-RISC formation by 6-mC EP h-purine. Model 1: The “double-stranded state” of siRNA duplexes acts as an additional anchor point for Ago2 (cyan flag with oval), masking the effect of the 5’ nucleobase anchoring (green flag with circle) at the step of pre-Ago2-RISC formation. Model 2: Some structural constraints hinder the 5’ nucleobase to be anchored in the 5’ nucleotide-binding pocket at the step of pre-Ago2-RISC formation.
Figure 1D and E: Percentage target cleaved (3 independent experiments) (untransformed data):

| ANOVA on the effect of time and nt identity (no interaction) | ANOVA on the effect of time and nt identity, and their interaction | Pairwise comparisons | Verification of ANOVA applicability |
|-------------------------------------------------------------|---------------------------------------------------------------|---------------------|-----------------------------------|
| p-value for nt identity                                   | p-value for interaction between nt identity and time         | Comparison          | FDR-adjusted p-value for pairwise comparison (effect of nt identity) | FDR-adjusted p-value for pairwise comparison (interaction between nt identity and time) | Test           | p-value |
| 8.48 × 10 −12                                             | 1.33 × 10 −11                                               | A vs U              | 0.0023                           | (not applicable)                       | Levene         | 0.045     |
|                                                           |                                                               | A vs 6-mCEPh-purine | 0.0012                           | (not applicable)                       | Shapiro-Wilk   | 0.905     |
|                                                           |                                                               | U vs G              | 6 × 10 −5                 | (not applicable)                       | on residuals   |           |
|                                                           |                                                               | U vs 6-mCEPh-purine | 0.9642                          | (not applicable)                       |               |           |
|                                                           |                                                               | 6-mCEPh-purine vs G | 5.49 × 10 −8                 | (not applicable)                       |               |           |

(data and scripts for the statistical analyses in Figure [1](https://github.com/HKeyHKey/Brechin_et_al_2020/tree/master/Figure_1))

Figure 2C, D and E: Pre-RISC abundance (3 independent experiments) (log-transformed data):

| ANOVA on the effect of time and nt identity (no interaction) | ANOVA on the effect of time and nt identity, and their interaction | Pairwise comparisons | Verification of ANOVA applicability |
|-------------------------------------------------------------|---------------------------------------------------------------|---------------------|-----------------------------------|
| p-value for nt identity                                   | p-value for interaction between nt identity and time         | Comparison          | FDR-adjusted p-value for pairwise comparison (effect of nt identity) | FDR-adjusted p-value for pairwise comparison (interaction between nt identity and time) | Test           | p-value |
| 0.0043 (imprecisely determined: residuals are not really normally distributed) | 0.00012 (imprecisely determined: residuals are not really normally distributed) | A vs U              | 0.1020                           | 0.8324                           | Levene         | 0.905     |
|                                                           |                                                               | A vs 6-mCEPh-purine | 0.8586                           | 0.998                           | Shapiro-Wilk   | 0.00643   |
|                                                           |                                                               | A vs G              | 0.957                           | 0.518                           | on residuals   |           |
|                                                           |                                                               | U vs G              | 0.228                           | 0.918                           |               |           |
|                                                           |                                                               | 6-mCEPh-purine vs G | 0.3048                          | 0.00274                         | (log-transf.)  |           |

Mature RISC abundance (3 independent experiments) (untransformed data):

| ANOVA on the effect of time and nt identity (no interaction) | ANOVA on the effect of time and nt identity, and their interaction | Pairwise comparisons | Verification of ANOVA applicability |
|-------------------------------------------------------------|---------------------------------------------------------------|---------------------|-----------------------------------|
| p-value for nt identity                                   | p-value for interaction between nt identity and time         | Comparison          | FDR-adjusted p-value for pairwise comparison (effect of nt identity) | FDR-adjusted p-value for pairwise comparison (interaction between nt identity and time) | Test           | p-value |
| 9.91 × 10 −14                                             | < 2 × 10 −16                                                | A vs U              | 0.0366                           | 0.8324                           | Levene         | 0.983     |
|                                                           |                                                               | A vs 6-mCEPh-purine | 0.00096                          | 4.96 × 10 ^-8                   | Shapiro-Wilk   | 0.918     |
|                                                           |                                                               | A vs G              | 1.98 × 10 ^-11                  | 4.53 × 10 ^-8                   | on residuals   |           |
|                                                           |                                                               | U vs 6-mCEPh-purine | 0.497                           | 0.964                           |               |           |
|                                                           |                                                               | U vs G              | 5.49 × 10 ^-11                  | 2.18 × 10 ^-8                   |               |           |
|                                                           |                                                               | 6-mCEPh-purine vs G | 6 × 10 ^-10                     | 1.28 × 10 ^-8                   | (log-transf.)  |           |

(data and scripts for the statistical analyses in Figure [2](https://github.com/HKeyHKey/Brechin_et_al_2020/tree/master/Figure_2))

Figure 3C, D and E: Pre-RISC abundance (3 independent experiments) (log-transformed data):

| ANOVA on the effect of time and nt identity (no interaction) | ANOVA on the effect of time and nt identity, and their interaction | Pairwise comparisons | Verification of ANOVA applicability |
|-------------------------------------------------------------|---------------------------------------------------------------|---------------------|-----------------------------------|
| p-value for nt identity                                   | p-value for interaction between nt identity and time         | Comparison          | FDR-adjusted p-value for pairwise comparison (effect of nt identity) | FDR-adjusted p-value for pairwise comparison (interaction between nt identity and time) | Test           | p-value |
| 0.0964 (not applicable)                                   | (not applicable)                                             | (not applicable)    | (not applicable)                   | (not applicable)                   | Shapiro-Wilk   | 0.00274   |
|                                                           |                                                               | Levene              | 0.902                            | (log-transf.)                      |               |           |
|                                                           |                                                               | (log-transf.)       |                                 |                                 |               |           |

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Supplementary Table 1: Summary of statistical analyses. p-values indicated in Figures are shown in red. See Materials and Methods for the details of the analyses.
(continued from previous page)

**Mature RISC abundance (3 independent experiments) (untransformed data):**

| ANOVA on the effect of time and nt identity (no interaction) | ANOVA on the effect of time and nt identity, and their interaction | Pairwise comparisons | Verification of ANOVA applicability |
|------------------------------------------------------------|---------------------------------------------------------------|---------------------|----------------------------------|
| p-value for nt identity | p-value for interaction between nt identity and time | p-value for pairwise comparison (effect of nt identity) | FDR-adjusted p-value for pairwise comparison (between nt identity and time) |
|----------------------------|---------------------------------------------------------------|---------------------|----------------------------------|
| $4.51 \times 10^{-15}$ | $< 2 \times 10^{-16}$ | $3.13 \times 10^{-7}$ | A vs U: $0.001572$ | Levene: $0.8$ |
|                            |                                                               |                     | A vs G: $0.157$ | Shapiro-Wilk on residuals: $0.341$ |
|                            |                                                               |                     | U vs 6-mC5Ep-purine: $6.90 \times 10^{-14}$ |                     |
|                            |                                                               |                     | U vs G: $2.19 \times 10^{-6}$ |                     |
|                            |                                                               |                     | 6-mC5Ep-purine vs G: $6.72 \times 10^{-14}$ |                     |

(data and scripts for the statistical analyses in Figure 3: [https://github.com/HKey/HKey/Brechin_et_al_2020/tree/master/Figure_3](https://github.com/HKey/HKey/Brechin_et_al_2020/tree/master/Figure_3))

**Figure 4C, D and E:**
Pre-RISC abundance (3 independent experiments) (untransformed data):

| ANOVA on the effect of time and nt identity (no interaction) | ANOVA on the effect of time and nt identity, and their interaction | Pairwise comparisons | Verification of ANOVA applicability |
|------------------------------------------------------------|---------------------------------------------------------------|---------------------|----------------------------------|
| p-value for nt identity | p-value for interaction between nt identity and time | p-value for pairwise comparison (effect of nt identity) | FDR-adjusted p-value for pairwise comparison (between nt identity and time) |
|----------------------------|---------------------------------------------------------------|---------------------|----------------------------------|
| $2.25 \times 10^{-5}$ | $3.73 \times 10^{-6}$ | $0.00206$ | A vs U: $1.76 \times 10^{-7}$ | Levene: $0.876$ |
|                            |                                                               |                     | A vs 6-mC5Ep-purine: $0.022$ | Shapiro-Wilk on residuals: $0.594$ |
|                            |                                                               |                     | A vs G: $0.423$ |                     |
|                            |                                                               |                     | U vs 6-mC5Ep-purine: $1.76 \times 10^{-7}$ |                     |
|                            |                                                               |                     | U vs G: $0.00218$ |                     |
|                            |                                                               |                     | 6-mC5Ep-purine vs G: $0.062$ |                     |

(data and scripts for the statistical analyses in Figure 3: [https://github.com/HKey/HKey/Brechin_et_al_2020/tree/master/Figure_3](https://github.com/HKey/HKey/Brechin_et_al_2020/tree/master/Figure_3))

**Mature RISC abundance (3 independent experiments) (untransformed data):**

| ANOVA on the effect of time and nt identity (no interaction) | ANOVA on the effect of time and nt identity, and their interaction | Pairwise comparisons | Verification of ANOVA applicability |
|------------------------------------------------------------|---------------------------------------------------------------|---------------------|----------------------------------|
| p-value for nt identity | p-value for interaction between nt identity and time | p-value for pairwise comparison (effect of nt identity) | FDR-adjusted p-value for pairwise comparison (between nt identity and time) |
|----------------------------|---------------------------------------------------------------|---------------------|----------------------------------|
| $< 2 \times 10^{-16}$ | $< 2 \times 10^{-16}$ | $0.00241$ | A vs U: $0.00384$ | Levene: $0.918$ |
|                            |                                                               |                     | A vs 6-mC5Ep-purine: $1.87 \times 10^{-7}$ | Shapiro-Wilk on residuals: $0.0621$ |
|                            |                                                               |                     | A vs G: $8.34 \times 10^{-7}$ |                     |
|                            |                                                               |                     | U vs 6-mC5Ep-purine: $0.00319$ | Shapiro-Wilk on residuals: $0.0543$ |
|                            |                                                               |                     | U vs G: $3.96 \times 10^{-9}$ |                     |
|                            |                                                               |                     | 6-mC5Ep-purine vs G: $2.09 \times 10^{-9}$ |                     |

(data and scripts for the statistical analyses in Figure 4: [https://github.com/HKey/HKey/Brechin_et_al_2020/tree/master/Figure_4](https://github.com/HKey/HKey/Brechin_et_al_2020/tree/master/Figure_4))

**Figure 5C and D:**
Pre-RISC abundance (3 independent experiments) (log-transformed data):

| ANOVA on the effect of time and nt identity (no interaction) | ANOVA on the effect of time and nt identity, and their interaction | Pairwise comparisons | Verification of ANOVA applicability |
|------------------------------------------------------------|---------------------------------------------------------------|---------------------|----------------------------------|
| p-value for nt identity | p-value for interaction between nt identity and time | p-value for pairwise comparison (effect of nt identity) | FDR-adjusted p-value for pairwise comparison (between nt identity and time) |
|----------------------------|---------------------------------------------------------------|---------------------|----------------------------------|
| $0.0768$ | (not applicable) | (not applicable) | Levene: $0.592$ |
|                            |                                                               |                     | Shapiro-Wilk on residuals: $0.0052$ |
|                            |                                                               |                     | Levene (log-transf.): $0.997$ |
|                            |                                                               |                     | Shapiro-Wilk on residuals (log-transf.): $0.140$ |

(data and scripts for the statistical analyses in Figure 5: [https://github.com/HKey/HKey/Brechin_et_al_2020/tree/master/Figure_5](https://github.com/HKey/HKey/Brechin_et_al_2020/tree/master/Figure_5))

(to be continued next page)
Figure 6C, D and E:
Pre-RISC abundance (3 independent experiments) (untransformed data):

| p-value for nt identity | p-value for nt identity | p-value for interaction between nt identity and time | Comparison | FDR-adjusted p-value for pairwise comparison (effect of nt identity) | FDR-adjusted p-value for pairwise comparison (interaction between nt identity and time) | Test | p-value |
|-------------------------|-------------------------|-----------------------------------------------|------------|-----------------------------------------------------------------|-----------------------------------------------------------------|------|---------|
| 0.7648                  | (not applicable)        | (not applicable)                              |            |                                                                 |                                                                 |      |         |

Mature RISC abundance (3 independent experiments) (untransformed data):

| p-value for nt identity | p-value for nt identity | p-value for interaction between nt identity and time | Comparison | FDR-adjusted p-value for pairwise comparison (effect of nt identity) | FDR-adjusted p-value for pairwise comparison (interaction between nt identity and time) | Test | p-value |
|-------------------------|-------------------------|-----------------------------------------------|------------|-----------------------------------------------------------------|-----------------------------------------------------------------|------|---------|
| 1.07 × 10^{-12}         | 1.28 × 10^{-13}         | 0.00923                                       |            |                                                                 |                                                                 |      |         |

(data and scripts for the statistical analyses in Figure 6: [https://github.com/HKeyHKey/Brechin_et_al_2020/tree/master/Figure_6](https://github.com/HKeyHKey/Brechin_et_al_2020/tree/master/Figure_6))

Figure 7C and D:
Percentage passenger cleaved (3 independent experiments) (untransformed data):

| p-value for nt identity | p-value for nt identity | p-value for interaction between nt identity and time | Comparison | FDR-adjusted p-value for pairwise comparison (effect of nt identity) | FDR-adjusted p-value for pairwise comparison (interaction between nt identity and time) | Test | p-value |
|-------------------------|-------------------------|-----------------------------------------------|------------|-----------------------------------------------------------------|-----------------------------------------------------------------|------|---------|
| 0.352                   | (not applicable)        | (not applicable)                              |            |                                                                 |                                                                 |      |         |

(data and scripts for the statistical analyses in Figure 7: [https://github.com/HKeyHKey/Brechin_et_al_2020/tree/master/Figure_7](https://github.com/HKeyHKey/Brechin_et_al_2020/tree/master/Figure_7))