論 文 の 内 容 の 要 旨

Mechanistic analysis of enhanced RNAi activity by 5'-end modification of the siRNA guide strand

（siRNA ガイド鎖の 5'末端修飾による RNA 干渉活性向上メカニズムの解析）

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General introduction

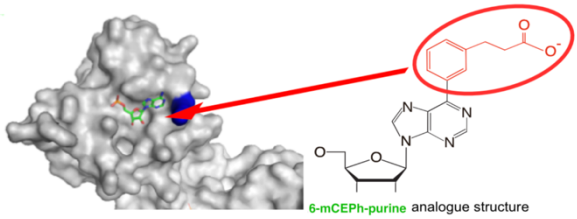
RNA interference (RNAi) is triggered by ~21 nt small interfering RNAs (siRNAs). This regulatory mechanism is conserved from worms to humans. In the cytoplasm, Dicer, an RNaseIII endonuclease, processes long double-stranded RNAs (dsRNAs) into siRNA duplexes. Then, Argonaute protein (Ago) incorporates the siRNA duplex by the assistance of Hsp70/Hsp90 chaperone machinery. This intermediate complex where Ago2 is bound to siRNA duplex is called pre-RNA-induced silencing complex (pre-RISC). Ago then cleaves the passenger strand with its endonucleolytic ("slicer") activity and discards it. The resultant Ago complex with the single-stranded siRNA guide (antisense strand) remaining is called mature RISC or more commonly just RISC. This effector complex is able to slice complementary mRNA targets, inducing their degradation that leads to gene silencing.

Since the discovery that siRNAs can specifically silence target genes in humans, therapeutic application of RNAi has been long awaited and, just recently, the first siRNA-based drug was finally approved. However, siRNA drugs still have rooms for improvement, particularly in their efficacy and stability. A key for such improvement is chemical modifications of siRNAs.

Notably, in a previous study describing the crystal structure of the MID domain of human Ago2, Frank et al. (2010) found that AMP and UMP bind with up to 30-fold higher affinity than either CMP or GMP in the 5'-end nucleotide binding pocket of Ago2. Based on this finding, I hypothesized that chemically modifying the 5' end base of the guide strand could be an effective strategy to increase the affinity of the siRNA duplex in Ago2's pocket and lead to an enhancement of siRNA knockdown potency.

Background

For this purpose, our collaborators at Kyowa Kirin have performed an in-silico screening of modifications at the 5'-end base of the guide strand. This analysis suggested a potential usability of the empty space around the 6th position of the adenine nucleobase **(Fig.1-a)** which seemed to be suitable to be targeted for additional interaction. Based on these notions, "6-(3-(2-carboxyethyl)phenyl)purine (6-mCEPh-purine)", an adenine-derived nucleotide analogue bearing a hydrophobic moiety and an acidic functional group at the position 6 was designed in order to fill in the empty space. **(Fig.1-b)**.



(Fig.1-a)　(Fig.1-b)

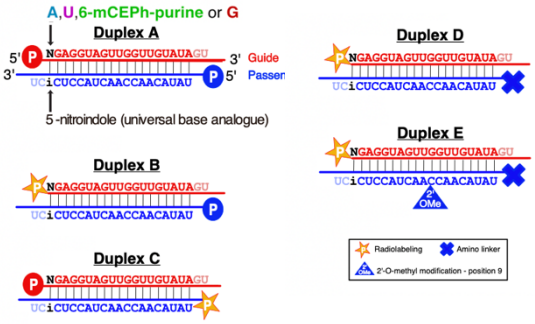
Strikingly, our collaborators have found that the "6-mCEPh-purine" compound can enhance the RNAi activity by ~3 fold in cultured human cells as well as in vivo mouse models.

My research presented below aims at deciphering which mechanistic step in the RNAi pathway this chemical modification enhances the siRNA potency, which will complement the cell-based and in vivo studies by Kyowa Kirin.

Material and methods

To dissect the underlying molecular mechanism behind the enhanced activity of 5'-modified siRNA duplexes, I used a series of biochemical approaches including an in vitro target cleavage assay and a native gel analysis of siRNA-Ago2 complex formation to quantitatively evaluate the effect of the modification at each step in RISC assembly and function.

To monitor Ago2–RISC formation I used the derivatives of siRNA duplexes bearing U, A, G or "6-mCEPh-purine" at the 5' end of the guide strand **(Fig.2)**. Changing the 5' end nucleotide alters the base pairing status at this position, disturbing the thermodynamic asymmetry of the duplexes and guide strand selection. To avoid this problem, all of siRNA duplexes were designed to hold the universal base 5-nitroindole at the position 19 of the passenger strand, which hybridizes equally with all four nucleotides (Liu et al, 2004). For our in vitro study, lysate from HEK293T cells overexpressing Ago2 was prepared according the method described by Yoda et al. (2010).

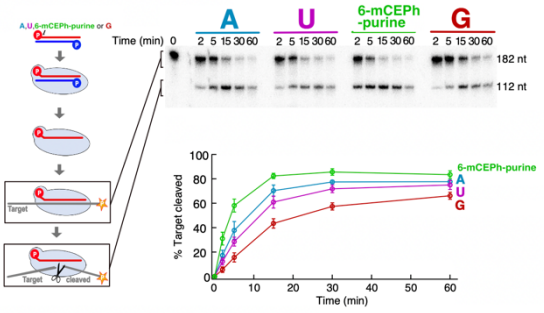


(Fig.2)

Results

I first asked whether the previous results found in cells and in mice can be reproduced in vitro. For this purpose, I used a well-established technique called "cleavage assay" which allow to monitor the target cleavage activity amongst the different duplexes bearing the different nucleotides (A, U, G, "6-mCEPh-purine") using a target RNA perfectly complementary to the guide strand. The cleavage products were separated by denaturing PAGE and I confirmed that the target cleavage activity was significantly enhanced by the "6-mCEPh-purine" modification at the guide 5'-end compared to the other nucleotides **(Fig.3).**

I next investigated what exactly happens during the intermediate steps from the siRNA duplex loading to mature RISC formation. On this goal, to carefully look into the human RISC-assembly, we decided to use a series of native agarose gel. This method developed by Kawamata et al. (2009) permits to biochemically discriminate pre-RISC and mature RISC on a vertical agarose gel and follow the kinetics of the RISC assembly.
It is important to note that, in this native agarose gel assay, the 2′-*O*-methyl target complementary to the guide strand was included in the reaction mix to avoid any binding of RISC to endogenous mRNA present in the lysate, which enables the detection of mature RISC as a sharp signal.



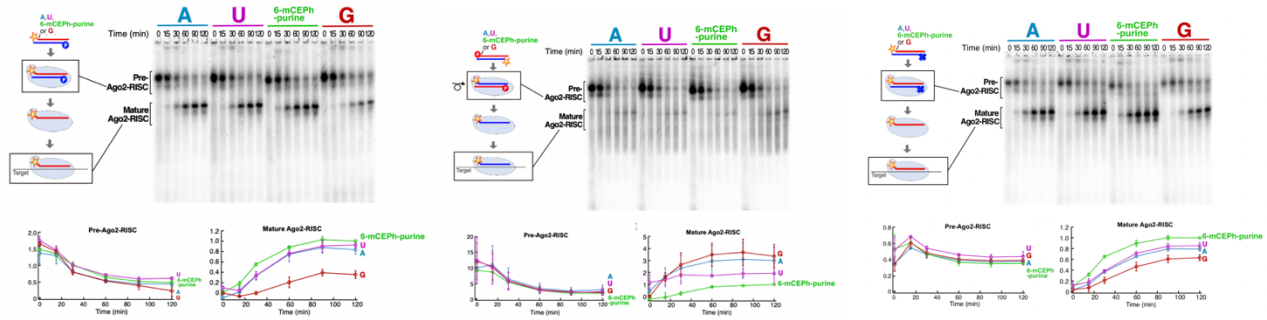
(Fig.3)

Remarkably, the results showed that 6-mCEPh-purine improved the formation of mature RISC **(Fig.4),** confirming the target cleavage assay **(Fig. 3)**. However, it remained unclear which step during RISC assembly was enhanced by this modification. There can be two explanations:

1) 6-mCEPh-purine helped the guide strand selection upon duplex loading.
2) 6-mCEPh-purine enhanced the stability/anchoring of the siRNA in mature RISC.

To investigate the hypothesis (1), I decided to radiolabel the passenger strand, and found that the mature RISC formed for 6-mCEPh-purine was slightly lower than for A, U and G. This data indicates 6-mCEPh-purine can ameliorate the guide strand selection by fixing the orientation of the duplex loading **(Fig.5).** Nevertheless, the effect observed here did not fully explain the degree of enhancement of mature RISC formation observed in **(Fig.4).**

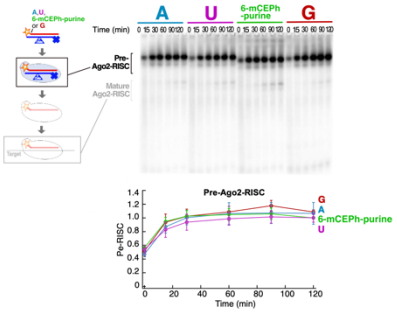
To test the hypothesis (2), I needed to check whether 6-mCEPh-purine enhances the mature RISC formation, independently of the guide strand selection. For this purpose, I used an amino-linker placed at the 5' end of the passenger, which completely blocks Ago-loading of this strand. Accordingly, the duplex was not allowed to "flip-flop" and the loading orientation was fixed in such a way that the radiolabel strand is always chosen as the guide. Still, I observed significantly enhanced formation of mature RISC with 6-mCEPh-purine, indicating that this modification enhances mature RISC formation independently of the guide strand selection **(Fig.6).**



(Fig.4) (Fig.5) (Fig.6)

This data **(Fig.6)** demonstrates that 6-mCEPh-purine modification enhances the stability/anchoring of the guide strand, at least in mature RISC. However, because formation of pre-RISC is always only transient, it still remained unclear if the 6-mCEPh-purine modification has any effect on the efficiency of duplex loading.

To address this question, the siRNA duplex was modified by a 2'-O-methyl group at the position 9 of the passenger strand. This modification blocks the expulsion of the passenger strand, allowing us to check the efficiency of the duplex loading by purely monitoring the formation of pre-RISC.
Interestingly, this data **(Fig.7)** showed that the loading of the different duplexes was virtually equal amongst them. Taken all together, our data demonstrated that 6-mCEPh-purine increases mature RISC formation and stability upon or after passenger strand ejection from Ago2, but not when siRNA duplexes are first loaded into Ago2.

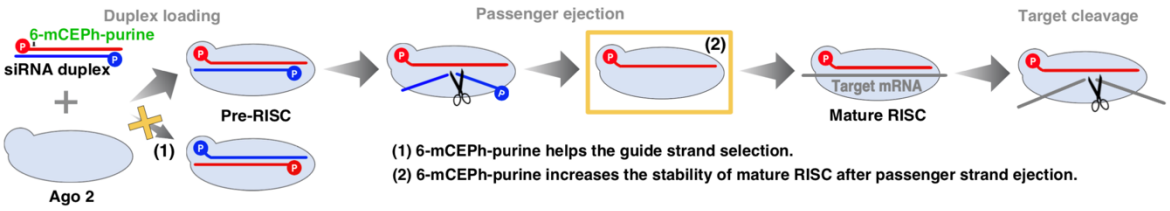


(Fig.7)

Conclusion

I found that the modification improves the formation of mature RISC in multiple ways, (1) slightly with the fixation of the loading orientation of siRNA duplexes and (2) mainly with the increased stability of mature RISC upon or after passenger strand ejection **(Fig.8)**.

My data will provide a molecular platform for further development of siRNA therapeutics.



(Fig.8)