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Xanthone derivatives as potential inhibitors of miRNA processing by human Dicer: Targeting secondary structures of pre-miRNA by small molecules

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ABSTRACT

In recent years, various biological processes have been found to be regulated by miRNA-mediated gene silencing. A small molecule that modulate the miRNA pathway will provide the biological tool for elucidating mechanisms of miRNA-mediated gene regulation, and can be the drug lead for miRNA related diseases. In this study, we demonstrated that an aminoalkoxy-substituted thioxanthone derivative interferes Dicer-mediated processing of pre-miRNA. Information about the interaction between these xanthone derivatives and pre-miRNAs will enable us to design and develop new small molecule-based inhibitors for miRNA pathway.

MicroRNAs (miRNA) are involved in many biological processes including development, differentiation and carcinogenesis through translational repression by binding to a target mRNA.[1–12] Inhibition of miRNA pathways by altering miRNA expression and/or maturation in cells would modulate gene expression, and enable us to understand miRNA regulatory effects on various biological processes. Anti-miRNA oligonucleotide (antagomir) is the most readily available tool to knock-down the expression of an endogenous miRNA, and thereby perturb miRNA-mediated gene regulation in a sequence specific manner.[13–17] The loss-of-function experiments using antagomirs have so far revealed the important role of miRNAs in various biological processes. In addition to these approaches, small molecules that bind to precursor-miRNA (pre-miRNA) and inhibit Dicer-catalyzed pre-miRNA processing to miRNA will provide other options for modulating miRNA function (Fig. 1). Some aminoglycosides, peptides, and peptoids have been studied as candidates of such small molecule-based inhibitors of pre-miRNA processing.[18–21]

We have studied the synthesis and structure–activity relationships of xanthone and thioxanthone derivatives as the fluorescent indicators for detecting the interactions between RNA and small molecules (Fig. 2).[22,23] Some of the 2,7-disubstituted xanthone and thioxanthone derivatives preferentially bind to certain secondary structures of RNA such as loops and bulges rather than double-stranded regions. Since most pre-miRNAs have such secondary structures, we explored a possibility of inhibitory activity of the xanthone and thioxanthone derivatives against the dicing reaction upon their binding to pre-miRNA. We herein report that an aminoalkoxy-substituted thioxanthone derivative interferes Dicer-mediated processing of pre-miRNA.

We used pre-miR-29a as a substrate for Dicer reaction in this study (Fig. 1). The hairpin secondary structure of pre-miR-29a consisted of a stem loop, a cytosine bulge, a hairpin loop, and double-stranded regions. The chemically synthesized pre-miR-29a was digested by recombinant human Dicer in the presence or absence of the xanthone and thioxanthone derivatives. Putative Dicer cleavage sites to produce a duplex of mature miR-29a strand (miR-29a) and a miR-29a star strand (miR-29a*) were shown by arrows. The products were analyzed by denaturing polyacrylamide gel electrophoresis (Fig. 3). Pre-miR-29a and the mixture of miR-29a and miR-29a* were loaded as controls (lanes 1 and 2, respectively). Recombinant human Dicer digested pre-miR-29a to produce two bands on the gel, which could be detected by Northern blotting using an alkaline phosphatase-labeled DNA probe for mature miR-29a (lane 3). The band of faster mobility is obviously mature miR-29a, and the other is most likely an intermediate, where a nick was made in the 5′ strand of pre-miR-29a (lanes 2–9). While miR-29a* strand cleaved off from the hairpin could not be detected by Northern blotting, the formation of multiple intermediates was detected by the staining of the gel with SYBR Gold (Supplementary Fig. S1).

In the presence of X2S **1** (lane 4), the intensity of three bands remained almost unchanged from that in lane 3, suggesting the little effect of X2S on the dicing reaction of pre-miR-29a. Likewise, X2S $n = 6$ **2** (lane 5), X2S-1′-Me **3** (lane 6), and X2S-*N*,*N*-diMe **4** (lane 7), and X2SS-*N*,*N*-diMe **6** (lane 9) showed no significant effect on the dicing reaction. In contrast, the presence of X2SS **5** effectively suppressed the formation of both the intermediate and mature

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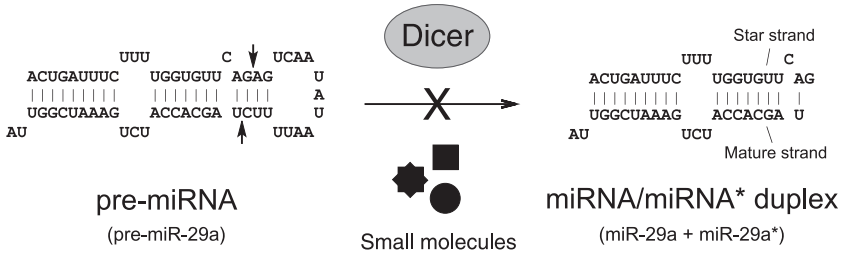

Figure 1. Dicer-mediated processing of pre-miRNA to miRNA duplex and inhibition of the reaction by small molecules.

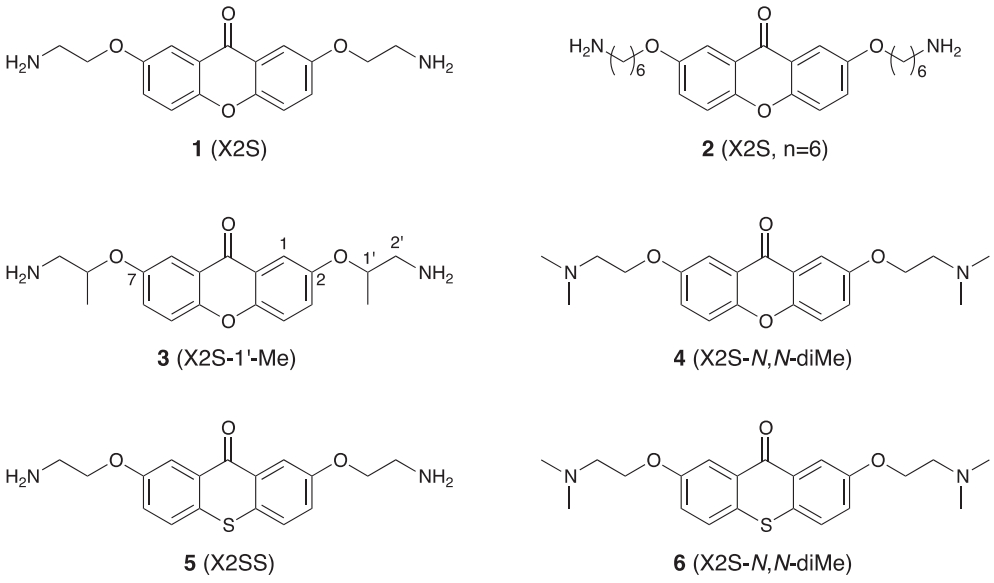

Figure 2. The xanthone and thioxanthone derivatives used in this study.

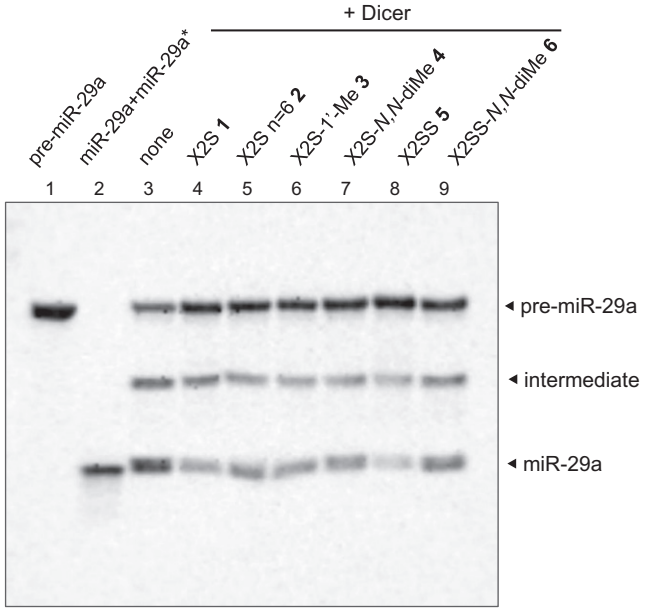

Figure 3. Northern blot analysis of Dicer-catalyzed pre-miR-29a processing in the presence of xanthone and thioxanthone derivatives **1**–**6** (at 200 μM) for 3 h; lane 4, **1**; lane 5, **2**; lane 6, **3**; lane 7, **4**; lane 8, **5**; lane 9, **6**.

miR-29a (lane 8). In our previous studies,[22,23] we have found that the derivatives **1**, **4**, **5**, and **6** had a preference of binding to loop regions over double-stranded regions of RNA, whereas **2** bound to double-stranded region of RNA with high affinity. Derivative **3** did not bind efficiently to both loop and double-stranded regions of RNA due to the steric hindrance of the methyl group on the aminoalkoxy substituent. Our previous data showed that the thioxanthone derivatives are more prominent than the xanthone derivatives for multiple binding. Although we anticipated that such structural preference of the xanthone and thioxanthone derivatives against RNA would influence the inhibitory effect of the derivatives on the dicing reaction, only **5** interfered the dicing reaction of pre-miR-29a.

We next investigated whether or not the xanthone and thioxanthone derivatives interfered the dicing reaction by directly interacting to Dicer. A Long dsRNA was prepared by PCR amplification of a DNA template for DsRed gene and subsequent in vitro transcription by T7 RNA polymerase. This dsRNA was digested with recombinant human Dicer into 21- to 23-mer short RNA in the presence or absence of the xanthone and thioxanthone derivatives, and the resulting digestion products were analyzed by denaturing PAGE (Supplementary Fig. S2). For 3-hour incubation, no significant inhibitory effect of the xanthone and thioxanthone derivatives on Dicer reaction was observed, indicating that the inhibition of the pre-miR-29a processing with **5** was not due to the direct interaction to recombinant human Dicer.

X2SS **5** was selected for further analysis, because it was the most potent inhibitor for dicing reaction of pre-miR-29a. In order to identify X2SS-binding sites of pre-miR-29a responsible for the inhibitory effect of **5** on the dicing reaction, we investigated dicing reaction of two pre-miR-29a mutant as substrates, one without the internal loop (Mutant **A**) and the other without bulge (Mutant **B**).

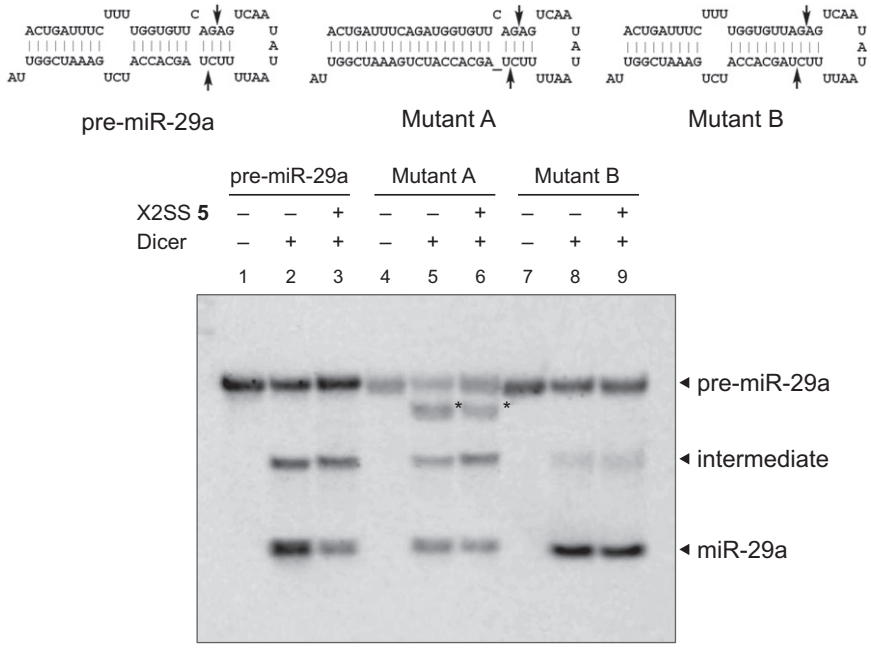

Figure 4. Northern blot analysis of Dicer-catalyzed digestion of pre-miR-29a or its mutants in the absence (lanes 2, 5, and 8) or presence of **5** (lanes 3, 6, and 9). The band with an asterisk in the lanes 5 and 6 represent an alternative intermediate generated by digestion of Mutant **A** by Dicer. Arrows indicate the putative Dicer cleavage sites.

These two mutants were subjected to dicing reaction in the presence of **5**, and the cleavage products were analyzed by Northern blotting (Fig. 4). Note that the total band intensities in lanes 4–6 were significantly lower than those observed in other lanes. This is because of competitive hybridization of cDNA probe to miR-29a with star strand of Mutant **A**; both the probe and star-strand were complementary to miR-29a. When pre-miR-29a was digested by Dicer in the presence of **5**, the band intensity of miR-29a was decreased from 41% (lane 2) to 23% (lane 3) of the total band intensity. In the case of the digestion reaction of Mutant **A**, two extra bands representing alternative intermediates (lanes 5 and 6, asterisk) were observed in addition to miR-29a band, of which intensity was 30% and 22% in the absence and presence of **5**, respectively. The dicing reaction of Mutant **A** was suppressed in the presence of **5** by a factor of 0.7 (22%/30%), showing moderate inhibitory effect of **5** on the reaction when compared to that of pre-miR-29a (0.5, 23%/41%). The formation of two extra bands in addition to the common intermediates may suggest an alternative cleavage reaction. Several reports showed that the structural diversity in pre-miRNA would generate length heterogeneity of miRNA.[24–26] The band intensity of miR-29a produced by dicing reaction of Mutant **B** was almost unchanged in the absence or presence of **5** (51% and 46%, respectively), indicating that the deletion of the bulge mitigated the inhibitory effect of **5** on Dicer reaction. Since the bulge is located near the Dicer cleavage site in pre-miR-29a, it is suggested that the binding of **5** close to a cleavage site is capable of interfering the processing of pre-miR-29a by Dicer.

Among the xanthone and thioxanthone derivatives, X2SS was found to be the most potent inhibitor of pre-miR-29a processing. X2SS has some advantages to be used as the fluorescent indicator, since a ligand that competes for X2SS binding site of pre-miR-29a would be readily used as an inhibitor for pre-miR-29a processing.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.10.108.

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