

# Mechanisms of Action for RNAi with Chemically Modified Duplexes

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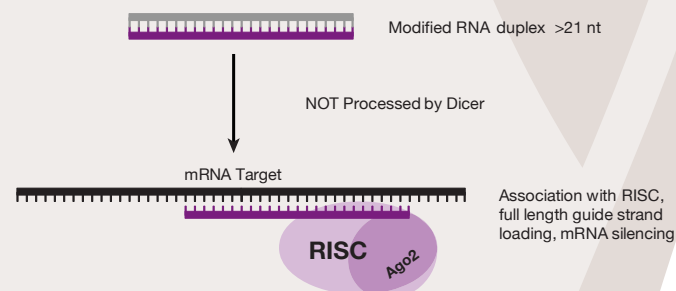


RXi Pharmaceuticals  
Next Generation in RNAi

## Abstract

It has been demonstrated that synthetic RNAi compounds longer than ~25 nucleotides (nt) are processed by Dicer proteins within the cell to create classic 21 nt siRNAs with 2 nt overhangs. It has generally been assumed that Dicer processing is necessary for loading into RISC. Furthermore, it has also been proposed that Dicer processing might actually enhance loading into RISC resulting in improved silencing activity. We have examined Dicer processing, RISC loading, and target mRNA cleavage of RNA duplexes  $\geq$  25 nt in length with a collection of chemically modified, short RNAs. We demonstrate that certain of these RNA configurations are not processed by Dicer in vitro with purified proteins or in cells. These longer duplexes have activity that is equal to or improved over that of a "standard" siRNA, despite the fact that they are not Dicer processed. We conclude that very efficient silencing activity can be demonstrated without Dicer processing of the RNA duplex.

**Figure 1: New Model for RNAi With RNA Duplexes >21 nt in Length**



Chemically modified duplexes >21 nt long interact with the RISC and the passenger (sense) strand is cleaved leaving the guide (antisense) strand in the protein complex. The guide strand is able to direct RNAi similar to 'siRNAs' where mRNA cleavage takes place between nucleotide 10 and 11 on this strand.

## Methods

### Transfection, Preparation of Cell Lysates and IP of Myc-Ago2, and Measuring Gene Silencing Activity

293S cells selectively expressing myc-Ago2 (obtained from G. Hannon, Cold Spring Harbor Lab, NY) were transfected with 25 nM RNA duplexes using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's recommendations. An aliquot of the cell lysate was taken for the QuantiGene bDNA assay (Panomics) to measure silencing activity. The cells were prepared and IP reactions were set up as previously described by Liu et al Science 305, 1437 (2004).

### mRNA Cleavage Assay and Northern Blot for Antisense Strand of Transfected Duplexes in RISC

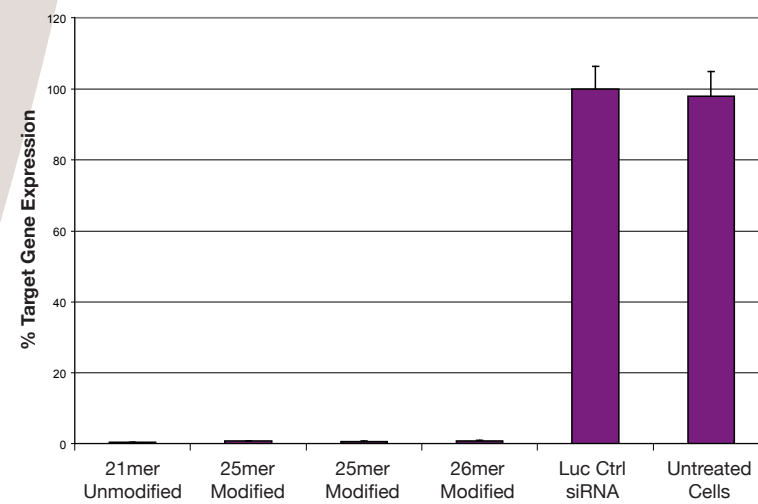
After overnight incubation with anti-myc-beads the beads were washed and the RNA was disassociated from the Ago2/RISC and beads through phenol-chloroform extraction (Trizol, Invitrogen) for the Northern Blot. Beads were washed and re-constituted in buffer for the mRNA cleavage assay. Northern conditions and incubations conditions are previously described by Liu et al Science 305, 1437 (2004).

### Dicer Enzyme Processing Assay

RNA duplexes were incubated with 0.5 units of human recombinant dicer enzyme (Genlantis) for 8 or 16 hours. The reaction and gel loading conditions were previously described by Genlantis and Kim et al Nature Biotechnology 23, 222 (2005).

## Results

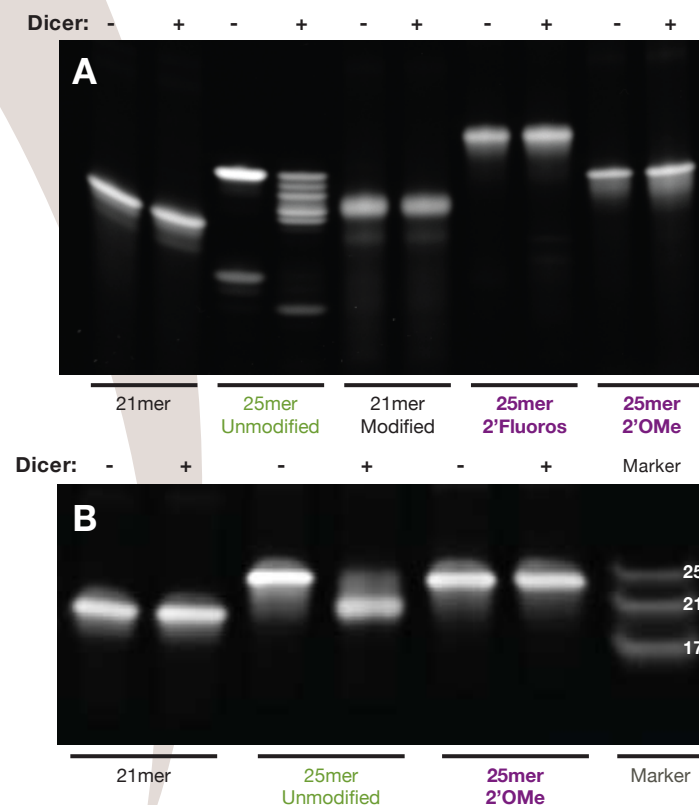
**Figure 2: Chemically Modified Duplexes are Potent for RNAi**



### Gene Silencing Activity of Unmodified and Modified Small RNA duplexes

bDNA assay of total RNA from 293S cells 48 hours after transfection with 25 nM RNA duplexes. Samples were normalized to the cyclophilin b gene and % expression was adjusted to that of a control, non-targeting duplex set to 100%. EC50 values for these RNA duplexes against the target gene are between 100-300 pM.

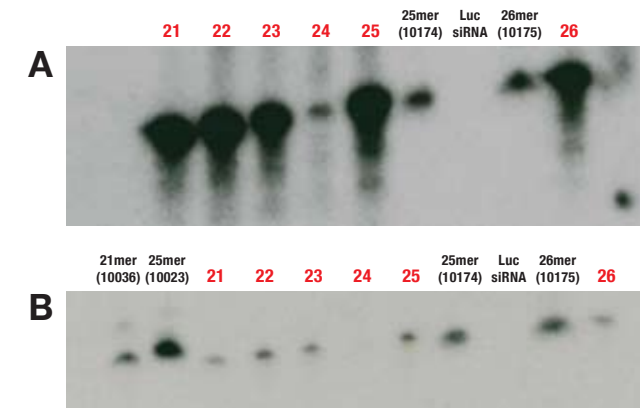
**Figure 3: Chemically Modified Duplexes are NOT Processed**



### Dicer Enzyme Processing Assay

RNA duplexes were incubated with or without 0.5 units of recombinant human dicer enzyme for 8 (Panel A) or 16 (Panel B) hours. After incubations, reactions were stopped by addition of loading buffer and snap freezing. Samples are shown on a 20% TBE-polyacrylamide gel after staining with SYBR Green.

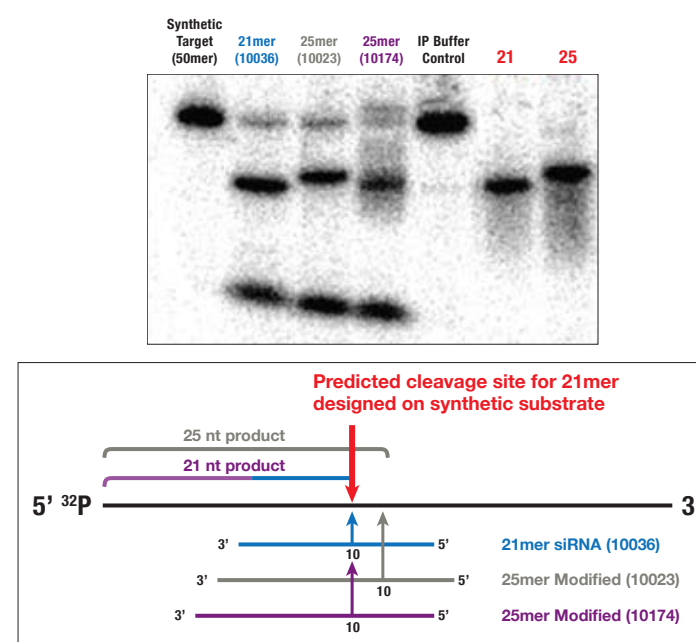
**Figure 4: Intact Modified Duplexes >21 nt will Load into RNA Silencing Complex for RNAi**



### IP-Northern Blot of Transfected RNA Complexed with Ago2 from Ago2 Immunoprecipitates

RNA Duplexes were transfected into 293S cells expressing myc-Ago2. Ago2 was immunoprecipitated from cell lysates and RNA in the IP fraction was extracted. Samples and <sup>32</sup>P-labeled size markers (shown in red) were run on a 15% TBE-Urea denaturing polyacrylamide gel, transferred to a nylon membrane, and blotted overnight. Panel A is probed with a <sup>32</sup>P-labeled LNA probe specific to antisense strands in 10174 and 10175. The <sup>32</sup>P-labeled LNA probe in panel B is complimentary to all antisense strands except that of the Luc siRNA.

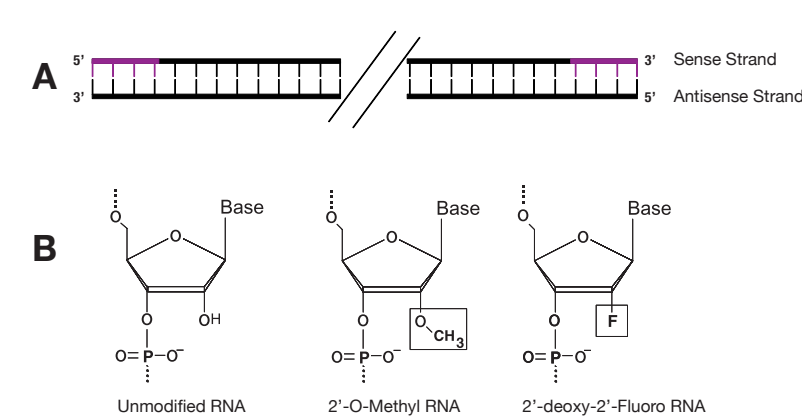
**Figure 5: Chemically Modified 25 nt Duplexes Cleave mRNA at Nucleotide Position 10**



### Cleavage Assay Using Transfected Small RNA Duplexes in an Immunoprecipitated RISC/Ago2

After transfection with the duplexes indicated above, Ago2 was immunoprecipitated from cell lysates and re-constituted in buffer. IP Ago2/RISC/RNA samples were then incubated with a <sup>32</sup>P-end labeled synthetic substrate for 2 hours at 37°C. The reaction was stopped by addition of loading buffer and loaded onto a 15% TBE-polyacrylamide gel along with <sup>32</sup>P-labeled size markers. The gel was visualized using phosphor film and phosphor imager. The substrate was consistently cleaved across from nucleotide 10 of the antisense strand of the original duplex.

**Figure 6: Example of a Modification Pattern that is NOT Dicer Processed**



The illustration shown in panel A is of a RNA duplex with a length of at least 25 bases and no overhangs. The sense strand has 2' modifications (e.g. 2'F, 2'OMe) on the nucleotides shown in purple and unmodified ribonucleotides shown in black (OH in 2' position). In this particular model the modifications are present on only the sense strand and the antisense strand is completely unmodified. Panel B shows the structure of RNA nucleotides with or without 2' position modifications.

## Discussion

These data demonstrate that certain modified RNAi compounds >21 nt are not processed by dicer, yet are very efficient triggers of RNAi. Cleavage of the target strand is detected solely at a position between nucleotides 10 and 11 of the guide strand. It has been suggested that dicer processing is necessary for RNAi activity and that only processed siRNA is active in the RISC complex. These results demonstrate that this is not the case. Furthermore, these RNAi compounds (that are not dicer substrates) maintain their potency in cell assays. The compounds exemplified in this report have EC50 values in the 100-300 pM range. We have identified modified RNAi compounds with EC50 values as low as 11 pM (W. Salomon, RXi, data not shown). These data show that dicer processing is not required for the generation of extremely potent RNAi compounds.

## Acknowledgements

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