Specificity and functionality of microRNA inhibitors

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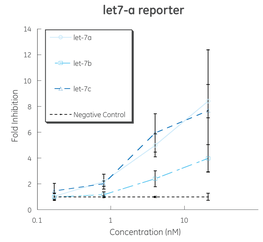
Introduction

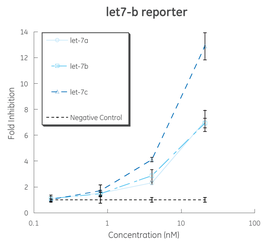
Highly potent microRNA (miRNA) inhibitors are valuable tools for elucidating the roles of miRNAs and their targets. Although it is known that pairings between endogenous miRNAs and their natural targets in animals generally involve base pair mismatches, studies of how mismatches between endogenous miRNAs and artificial inhibitor targets might affect inhibitor specificity and functionality have been very limited. Using a Luciferase reporter system we have investigated the specicity of miRNA inhibitors. We first confirmed significant levels of cross-reactivity among the closely related let-7 family members. Subsequently, a systematic study of mismatches incorporated into inhibitors of single-family-member miRNAs identified two regions that are important for overall inhibitor functionality. Our findings indicate that features important for natural miRNA target recognition also appear to be important for inhibitor specificity. Understanding the specificity of inhibitors allows for better interpretation of inhibitor activity in endogenous systems.

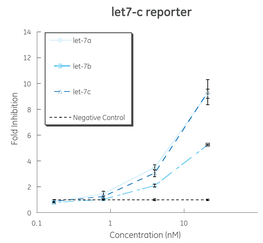
Cross-reactivity is evident among let-7 microRNA family member inhibitors

Name	Mature Sequence 5'-3'
hsa-let-7a	U*GAGGUA*GUAGGUUGUAUAGUU
hsa-let-7b	U*GAGGUA*GUAGGUUGUGUGGUU
hsa-let-7c	U*GAGGUA*GUAGGUUGUAUGGUU
hsa-let-7d	A*GAGGUA*GUAGGUUGCAUAGUU
hsa-let-7e	U*GAGGUA*GGAGGUUGUAUAGUU
hsa-let-7f	U*GAGGUA*GUAGAUUGUAUAGUU
hsa-let-7g	U*GAGGUA*GUAGUUUGUACAGUU
hsa-let-7i	U*GAGGUA*GUAGUUUGUGCUGUU
hsa-miR-98	U*GAGGUA*GUAAGUUGUAUUGUU

Sequences from miRBase, seed is in italics. Nucleotides at which other family members differ from let-7a are underlined.







Inhibitors of all let-7 family members cross-react.

No consistent ranking of interaction is found across reporter assays for different family members.

Sequence similarity between miR-21 and miR-122 does not cause cross-reactivity between inhibitors

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hsa-miR-122a    5' UGGAGUGUGACAAUGGUGUUUGU 3'
                   |:|  |:|  |  |  ||:|||| :
miR-21          3' AUCGAAUAGUCUGACUACAACU  5'
inhibitor

hsa-miR-21      5' UAGCUUAUCAGACUGAUGUUGA  3'
                    |  |  |  |  |  ||  ||||
miR-122a        3' ACCUCACACUGUUACCACAAACA 5'
Inhibitor
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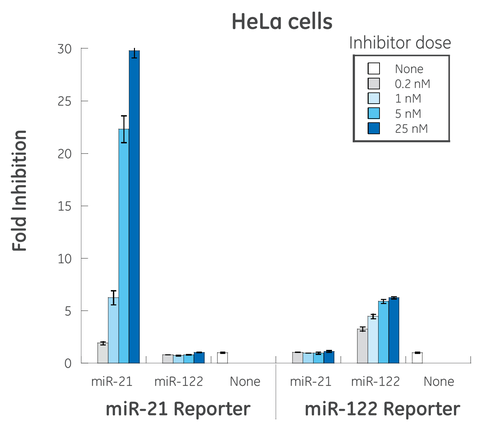
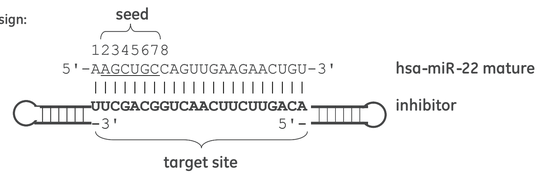


Inhibitors of non-family members with partial sequence similarity do not cross react.

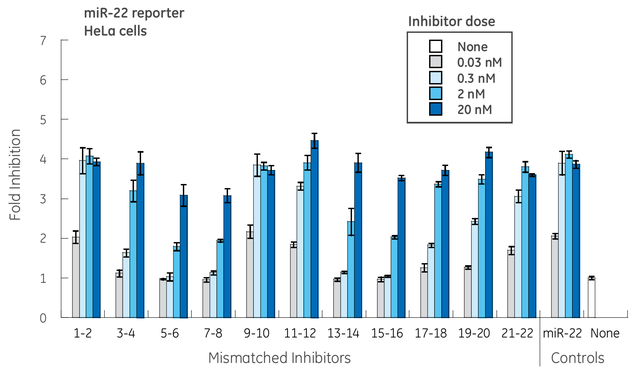
Systematic testing of mismatched inhibitors

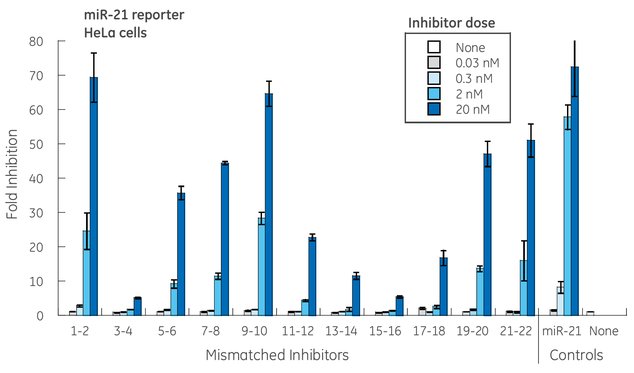


Inhibitor target site mismatch design:

Mismatch Position	Target site sequence (5' to 3')
None	ACAGUUCUUCAACUGGCAGCUU
1-2	ACAGUUCUUCAACUGGCAGCAA
3-4	ACAGUUCUUCAACUGGCACGUU
5-6	ACAGUUCUUCAACUGGGUGCUU
7-8	ACAGUUCUUCAACUCCCAGCUU
9-10	ACAGUUCUUCAAGAGGCAGCUU
:	:
17-18	ACAGAACUUCAACUGGCAGCUU
19-20	ACUCUUCUUCAACUGGCAGCUU
21-22	UGAGUUCUUCAACUGGCAGCUU

Mismatched inhibitor target sites show that position of mismatch affects functionality

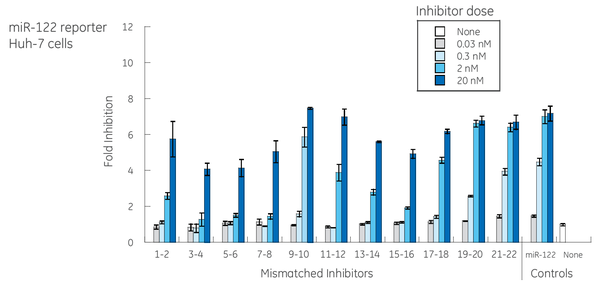


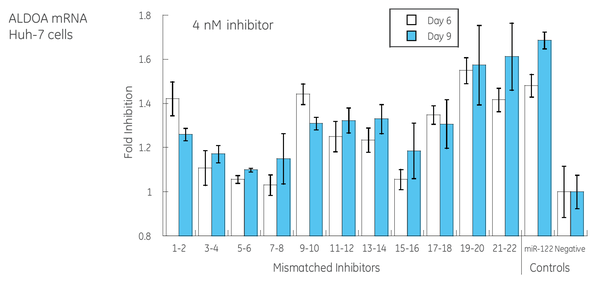


Methods

1. All inhibitors are fully 2'-O-methylated molecules synthesized at at Dharmacon according to the miRIDIAN Hairpin inhibitor design.
2. Dual-luciferase reporters were derived from the psiCHECK-2™ vector (Promega) that contains both firefly (FLUC) and Renilla (RLUC) luciferase genes. The desired microRNA target was cloned into the 3' UTR of the RLUC gene generating a cleavage assay where the mature miRNA is fully complementary to a single site. Results shown are averages from triplicate wells, normalized to corresponding controls, then expressed as fold-inhibition relative to negative control. Error bars are +/- one sample standard deviation of the original triplicate data, scaled for all subsequent calculations.
3. Levels of aldolase A (ALDOA) messenger RNA (mRNA) (a known target for miR-122) were determined using the QuantiGene Assay (branched DNA; Affymetrix, Panomics products).

Mismatches affect expression of an endogenous target with similar positional dependence





Examples of variation in sequence identity among microRNA family members and a non-family member with matching seeds

miRNA name	Mature sequence from 5' to 3' end
Family members with sequence identity in both the seed region and the 3' region	
hsa-miR-15a[1]	UAGCAGCACAUA**AUGGUU**UGUG
hsa-miR-15b[1]	UAGCAGCACAUC**AUGGUU**UACA
Family members with sequence identity in the seed region but very limited identity in the 3' region	
hsa-miR-15a[1]	UAGCAGCACAUA**AUGGUU**UGUG
hsa-miR-16[1]	UAGCAGCACGUA**AAUAUU**GGCG
Non-family members with sequence identity both in the seed region and in the 3' region	
hsa-miR-15a[1]	UAGCAGCACAUA**AUGGUU**UGUG
hsa-miR-497[2]	CAGCAGCACACU**GUGGUUU**GU

Notes: The seed region is in italics, the 3' region (position 13-18) is bold
[1]Member of the miR-15 family
[2]Member of the miR-497 family

Conclusion

Mismatch position in the target region of the inhibitor affects inhibitor efficacy.

The seed region (position 3-8) and a 3' region (positions 13-18) appear to be equally important for inhibitor functionality and specificity.

Results of the mismatch study can help identify potential cross-reacting microRNAs.

Future application of these results may help design more effective inhibitor experiments:

For miRNAs whose inhibitors are expected to cross-react, use of a pool of inhibitors may give more consistent results.

For miRNA family members whose inhibitors may not all be expected to cross-react, use of a pool of inhibitors may reveal a phenotype otherwise obscured by redundancy of miRNA function.

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