Specificity of highly potent miRNA inhibitors

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Introduction

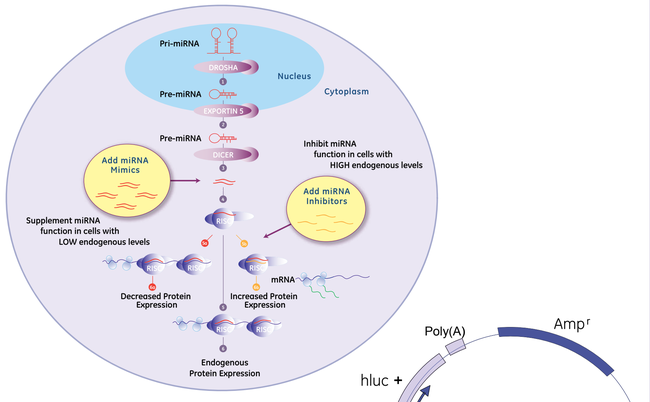
MicroRNAs (miRNAs) have been shown to regulate gene expression through both translational attenuation and cleavage of messenger RNAs. Though these small non-coding RNAs are predicted to play significant roles in development, differentiation, and disease etiology, validation of miRNA targets remains difficult. Highly potent miRNA inhibitors represent invaluable tools for elucidating the roles of miRNAs. However, it is important to be aware that such potent inhibitors may also affect function of other miRNAs, especially miRNA family members. To understand the potential cross-reactivity of miRNA inhibitors, various miRNA inhibitor designs were systematically tested. We demonstrate that mismatches both within and outside the seed region of the miRNA interfere with inhibition. Our findings indicate that certain features important for natural miRNA target recognition also appear to be important for inhibitor specificity. Furthermore, results from specificity experiments in a reporter assay are consistent with results obtained in an endogenous system. Understanding the specificity of inhibitors allows for better interpretation of inhibitor activity in endogenous systems.

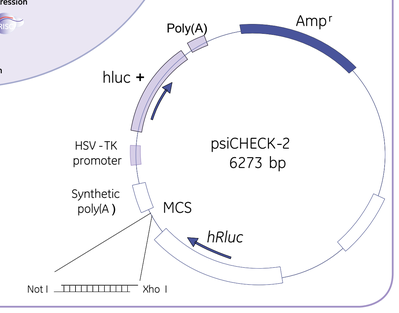
Inhibitors modulate miRNA activity

Two methods were used to test inhibitor function:

1. Dual-luciferase assay (psiCHECK™-2 vector, Promega) that employs both firefly (FLUC) and Renilla (RLUC) luciferase genes. The desired recognition element was cloned into the 3' UTR of RLUC generating a cleavage assay where the mature miRNA was fully complementary to a single site.
2. Determination of ALDOA gene knockdown (a known target for miR-122) using the Panomics bDNA assay (Genospectra).

Cell viability was determined for both assays using the alamarBlue™ assay (Thermo Fisher Scientific) according to manufacturer's instructions. Data used showed less than 25% effect on viability (data not shown).

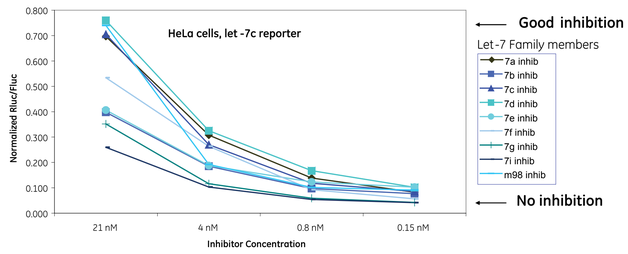




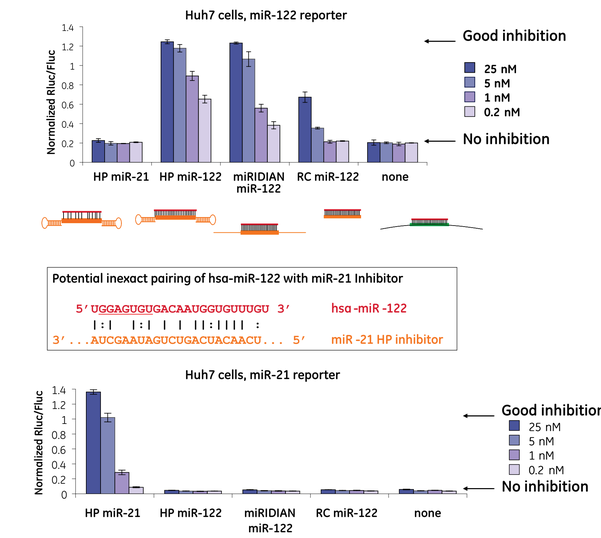
Possible determinants of miRNA/inhibitor specificity

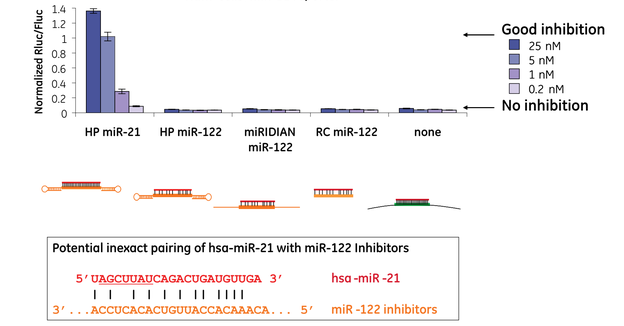
- TargetScan searches UTRs for matches to the mature miRNA at positions 2-8 ("seed"). TargetScan also calculates free energy and additional pairing to nucleotides outside of seed (Lewis *et al.*, 2003).
- Mismatches in the seed region reduce siRNA/miRNA efficacy, and the corresponding change in free energy appears to correlate with function (Doench and Sharp, 2004)
- Number of contiguously paired bases in the seed is important and in some cases sufficient to confer knockdown. Pairing 3' of the miRNA seed to the target site can compensate for "weak" seeds. (Brennecke *et al.*, 2005)
- Seed matches are shown to be an important predictor of siRNA off-targets. (Birmingham *et al.*, 2006)

Inhibitors do cross-react with family members



Inhibitors do not cross-react with closely related non-family members: miR-122 and miR-21

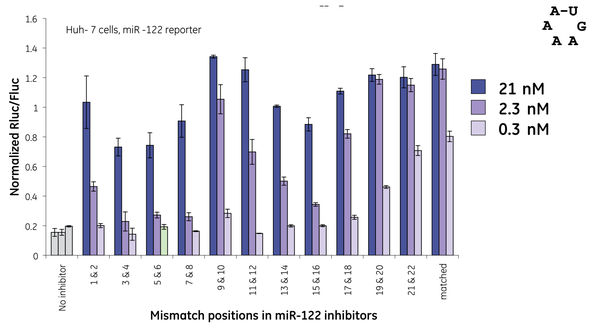




Mismatches within & outside seed region interfere with inhibition

Systematically testing inhibitor specificity:

- Twelve inhibitors were designed against miR-122
- Each inhibitor contained two adjacent mismatches when paired to the mature miRNA:
- Mismatches numbered relative to the 5' end of mature miRNA
- Seed matches are shown to be an important predictor of siRNA off targets. (Birmingham *et al.*, 2006)



miR-122 affects ALDOA mRNA levels *in vivo*

ALDOA is a predicted target of miR-122 and the 3' UTR is conserved at the predicted site

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          1........10........20.........30.........40
Human     GCGGAGGUGUUCCCAGGCUGCCCC--CAACACUCCAGG-CCCUG
Mouse     CCAGAGCUGAACUAAGGCUGCUCCAUCAACACUCCAGGCCCCUG
Rat       CCAGAGCUGAUCUAAGGCUGCUCCAUCGACACUCCAGGCCCCUG
Dog       GCGGAGGUGUUCUAAGGCUGCCCCCUCAACACUCCAGC-CCCCA
                                        miR-122 site
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8 mer miR-122 site in human ALDOA 3 'UTR

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5'...UCCCAGGCUGCC--CCCAACACUCCA...3'
        ||:::| :||       |||||||
   3'UGUUUG-UGGUAACAGUGUGAGGU 5'
```

hsa-miR-122

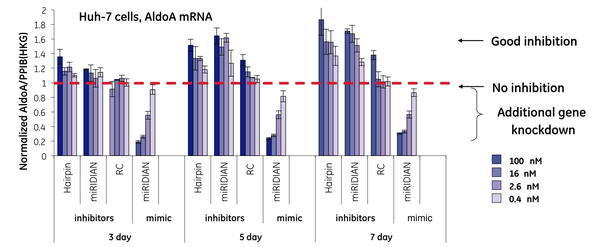
Three miRNA target prediction programs predict the same site for miR-122 in the ALDOA 3' UTR (pictar.bio.nyu.edu, targetscan.org, microrna.sanger.ac.uk/targets/v5).

Knockdown of ALDOA mRNA by miR-122 mimetic has been shown in cell culture (Esau *et. al,* 2006; Fabani *et al.*, 2008).

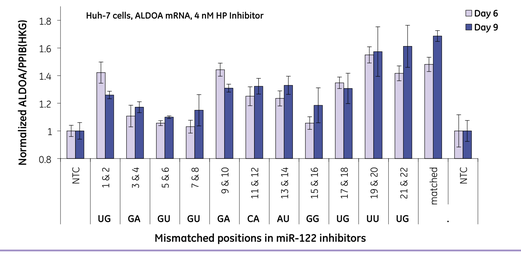
De-repression of ALDOA by miR-122 inhibitors has been demonstrated in mouse liver *in vivo* (Krützfeldt *et al.*, 2005; Esau *et. al,* 2006; Elmén *et al.*, 2008).

Mismatches between the inhibitor and its targeting miRNA were tested for a few positions, effects were dependent on location (Krützfeldt *et al.*, 2007).

miR-122 mimics & inhibitors modulate ALDOA mRNA levels



Mismatched miR-122 inhibitors show similar effects in an endogenous system



Conclusions

- Inhibitors do not cross-react with non-family member miRNAs that share sequence similarity.
- Inhibitors are capable of inhibiting family members miRNAs with imperfect complementarity.
- Position of mismatches affects inhibitor efficacy, as detected by a reporter
- Mismatch positions with greatest to least effect for mir-122 are:
  Seed > region 11-18 > 9 &10 and region 19-22
- This efficacy corresponds to differences in inhibitor functionality as measured on the endogenous target ALDOA.
- Detection of mismatch effects is strongly affected by inhibitor concentration.

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