Rank order TRiM™ activity correlates with RISC Loading and can be enhanced by cPrp modification

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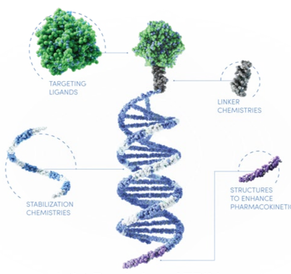
TRiM™ Platform



Artist Rendering of TRIM

- Rules and algorithms allow selection of optimized RNAi trigger sequences
- Limit cross-reactivity with off-target genes
- Maximize innate stability
- Rational use and placement of modifying chemistries
- Targeting moiety facilitates cellular uptake and endocytosis of triggers
- Active endosomal escape chemistries not required

Rationale

Despite advances in rational TRiM™ design, activity in vivo varies greatly

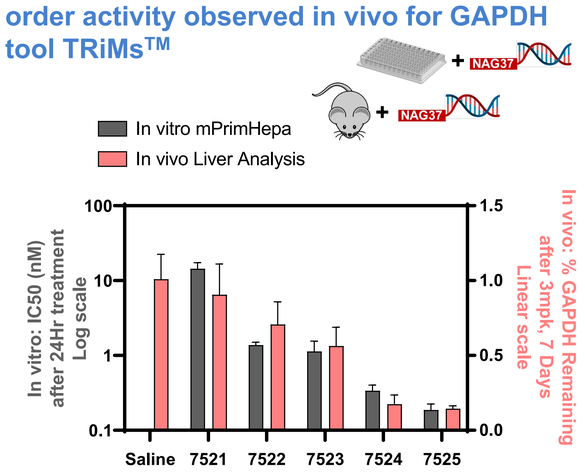
- "Tool" TRiMs™ targeting GAPDH expected to be highly active were interrogated to understand drivers of their abilities to support gene silencing
- TRiMs™ had identical modifying chemistries but showed a wide range of in vivo activity from highly active to poor GADPH knockdown in vivo
- Rank order generated in vivo was generally consistent in vitro using primary mouse hepatocytes, establishing this as a suitable model for analyzing this TRiM™ series
- TRiM™ cellular uptake was indistinguishable
- Occupancy of RISC by TRiM™ revealed a direct relationship to silencing activity: low association = low knockdown
- Addition of 5'-cyclopropyl-phosphonate (cPrp) to antisense strand of low activity TRiM™ improved KD activity and enhanced RISC association

Methods

- Mice were treated with the indicated GAPDH-targeted-N-Acetylgalactosamine (NAG) conjugate TRiM™ (3mpk) on day zero
- Livers were harvested on day 7 for RNA isolation and analysis of GAPDH expression by qPCR
- Primary hepatocytes were isolated using an IACUC-approved protocol and treated for the indicated duration at 1pM-1uM concentrations of TRiM™
- Passive TRiM™ uptake at the indicated concentration utilized 20,000 or 6,000,000 primary hepatocytes for knockdown assessment and streptavidin-mediated Ago2 isolation, respectively. Gene expression was analyzed using qPCR, Ago2 recovered by biotin-TRiM™ was visualized using SDS-PAGE and anti-Ago2 western blotting.

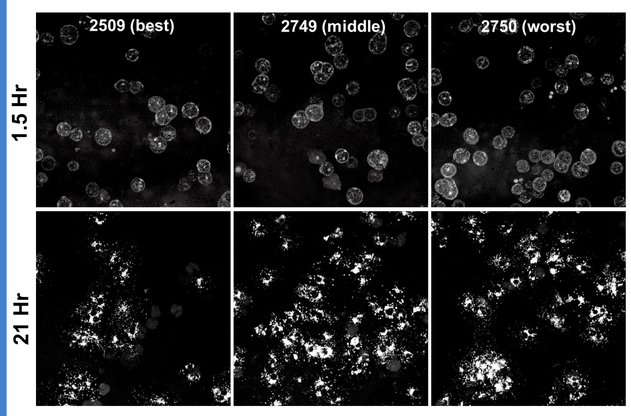
Results

Primary mouse hepatocytes recapitulate rank order activity observed in vivo for GAPDH tool TRiMs™



TRiMs™ utilizing the same NAG targeting ligand and identical chemical modifications but targeting distinct sequences within GAPDH mRNA were administered to mice (3mpk) or mouse primary hepatocytes (1pM-1uM titration). Liver tissue (7 days) or hepatocytes (24 hours) were processed for GAPDH expression analysis by qPCR; IC50 was calculated and reported. n=3

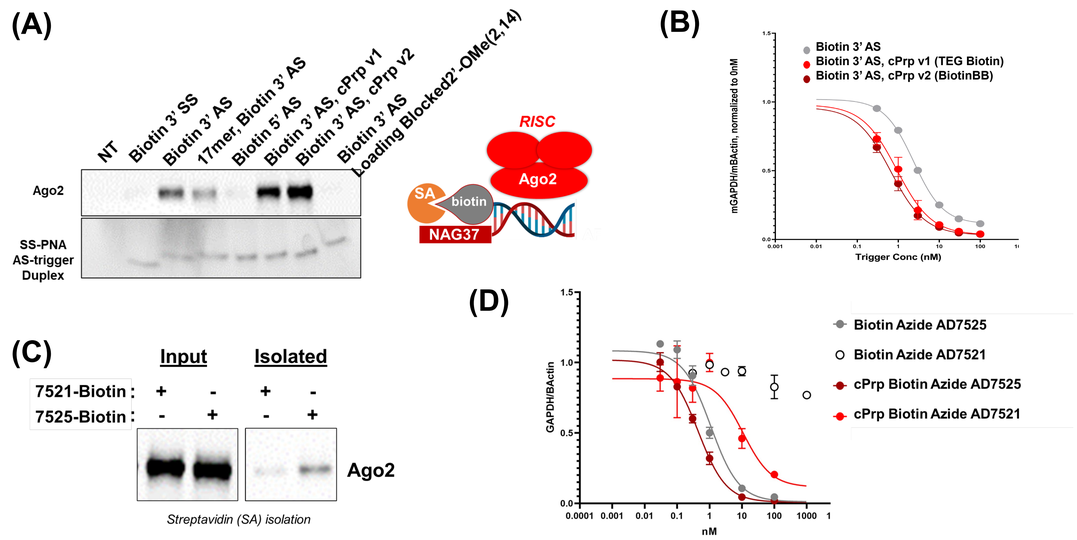
TRiM™ cellular uptake does not account for rank order



The TRiM™ series described above (containing the NAG targeting ligand and utilizing identical chemical modifications, targeting distinct sequences within GAPDH mRNA) were labeled with AlexaFluor647 (AF) and assessed for knockdown activity, revealing indistinguishable uptake (data not shown) and dose-response profiles (data not shown). Fluorescent TRiMs™ were incubated with mouse primary hepatocytes (10nM) for the indicated amount of time and imaged using a Yokogawa CQ1 (40x objective).

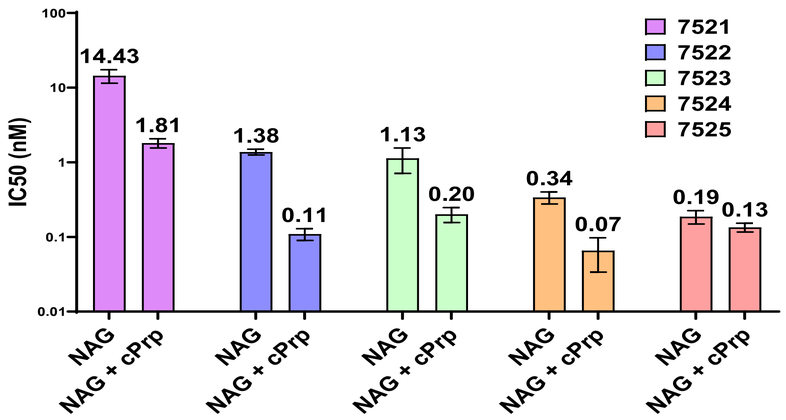
Results

Biotinylated TRiMs™ facilitate analysis of RISC loading, revealing correlation between knockdown activity and Ago2 association



Mouse primary hepatocytes were incubated with the indicated TRiM™ for 24 hours, streptavidin beads were used to isolate biotin-TRiM™ and associated Ago2 was detected using anti-Ago2 Western blotting (**A**). Biotinylation of the antisense strand (AS) on its 3' termini supported Ago2 recovery by this approach. In contrast, addition of biotin to the 3' end of the sense strand (SS), addition of biotin to the 5' of the AS, or use of TRiM™ blocked for RISC loading (and biotinylated 3' of the AS) all failed to recover Ago2 – supporting the utility of this approach for interrogating TRiM™-Ago2 association. 5'-cyclopropyl-phosphonate (cPrp) modification of the antisense strand enhanced both Ago2 isolation (**A**) and GAPDH knockdown (**B**). Streptavidin-mediated isolation of 7521 (lowest activity TRiM™) and 7525 (highest activity TRiM™) revealed a correlation between Ago2 recovery and activity – 7521 recovers less Ago2 than 7525 (**C**). Of note, cPrp modification of 7521 enhanced its potency to nearly that of 7525, while cPrp modification of 7525 had minimal impact on its activity (**D**). Together these data suggest a general relationship between RISC loading and knockdown activity, and furthermore the beneficial impact of cPrp suggests TRiM™ phosphorylation varies and represents a previously unappreciated rate-limiting variable contributing to RNAi silencing activity.

cPrp modification enhances TRiM™ IC50 to varying degrees, indicating 5' phosphorylation of non-cPrp AS is an important determinant of activity



Mouse primary hepatocytes were incubated with the indicated TRiM™ targeting GAPDH for 24 hours and processed for GAPDH expression analysis by qPCR. Addition of the 5' cPrp on the anti-sense enhanced TRiM™ IC50s across all TRiMs™. This difference suggests that the "native" form of 7525 is more phosphorylated than other TRiMs™ and reveals a possibility that the ability of a TRiM™ to be phosphorylated is sequence dependent. n=3.

Conclusions

These data support the interpretation that the ability of a TRiM™ to be phosphorylated is a variable that directly impacts TRiM™ binding to Ago2, a precursor event to gene silencing, and the efficacy of TRiM™-mediated gene silencing. These data further support an idea that the target sequence itself contributes to this variable. Deficiencies in phosphorylation of TRiM™ can be suppressed by implementation of Arrowhead's proprietary phosphomimetic cPrp modification.

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