Bis-RNAi^{TM} Conjugates for Simultaneous Silencing of Two Different Gene Transcripts

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Abstract

siRNAs covalently conjugated to synthetic multivalent *N*-acetylgalactosamine (GalNAc) ligands represent a promising new class of RNAi therapeutics with demonstrated human proof-of-concept across multiple clinical programs.[1,2] Simultaneous silencing of two different gene transcripts could result in enhanced therapeutic benefit.[3-7] For example, in the case of an antiviral therapy the ability to simultaneously hit two different target sites may reduce viral resistance.

Here we present the design and evaluation of siRNA conjugates capable of silencing two different gene transcripts as a single chemical entity, termed bis-RNAi^{TM}. This approach ensures that cells receive both siRNAs simultaneously and with the same efficiency thereby maximizing the therapeutic benefit of dual target silencing. Multiple designs were evaluated and critical design parameters, such as placement of the ligand and the nature of the linker between the siRNAs, were identified. Through optimization of those features robust *in vivo* activity, comparable to a cocktail of individual siRNA conjugates, was achieved

Figure 1. Bis-RNAi Designs

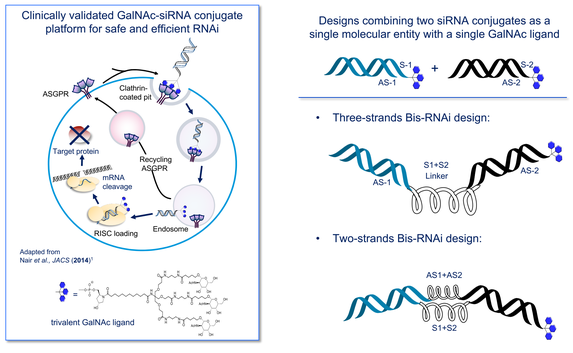


Figure 2. Evaluation of Two-Strand Designs: Effect of Linker Complementarity

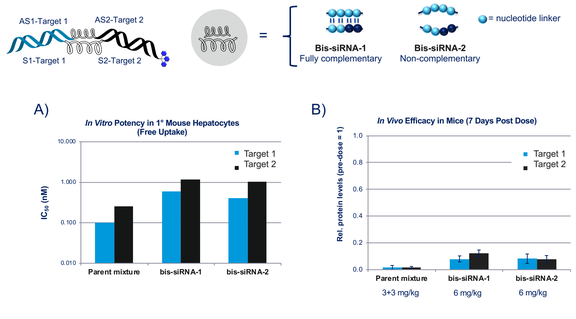


Figure 2. (A) *In vitro* potency (IC_{50}) of bis-siRNAs with complementary (bis-siRNA-1) and non-complementary (bis-siRNA-2) DNA linker targeting two distinct transcripts (Target 1 and Target 2) after free uptake into freshly isolated mouse hepatocytes; (B) Efficacy of bis-siRNAs in mice 7 days after s.c. administration compared to mixture of individual parent compounds (dose equivalent to 3 mg/kg of individual siRNAs).

- *In vivo* activity correlates well with *in vivo* potency; activity of bis-siRNAs similar to parent mixture
- No discernible difference between complementary and non-complementary linkers

Figure 3. Evaluation of Three-Strand Designs: Effect of Linker Structure and Ligand Placement

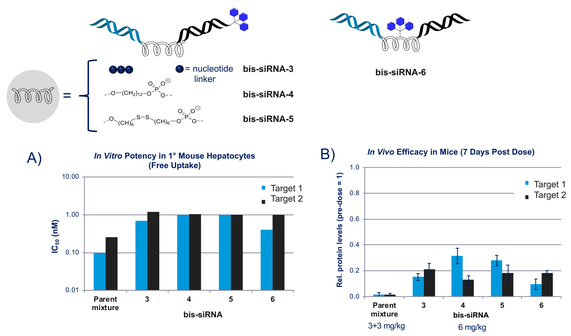


Figure 3. (A) Effect of linker structure and GalNAc ligand placement on *in vitro* potency (IC_{50}) of bis-siRNAs targeting two distinct transcripts (Target 1 and Target 2) after free uptake into freshly isolated mouse hepatocytes; (B) efficacy of bis-siRNAs in mice 7 days after s.c. administration compared to mixture of individual parent compounds (dose equivalent to 3 mg/kg of individual siRNAs).

- *In vitro* potency correlates well with observed *in vivo* efficacy
- Slightly better performance observed for bis-siRNA-3 and 6 compared to constructs 4 and 5 containing the alkyl-based linkers

Figure 4. Evaluation of Three-Strand Designs: Effect of Cleavability of Nucleotide Triplet Linker

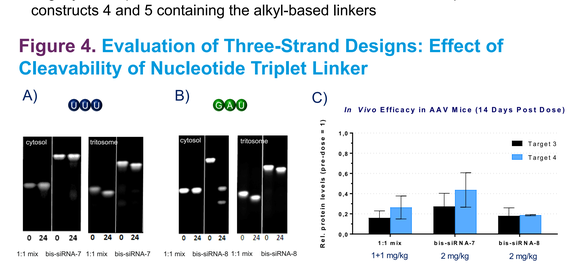


Figure 4. (A) and (B) Nucleolytic stability of bis-siRNAs in rat cytosol and rat liver tritosomes. Stability results displayed by non-denaturing 10% TBE Gel (100 V, 2 h at 4 °C) and revealed by staining with SYBR® Gold Nucleic Acid Gel Stain; (C) Efficacy of bis-siRNAs in AAV mice 14 days after s.c. administration of bis-siRNAs compared to mixture of individual parent compounds (dose equivalent to 1 mg/kg of individual siRNAs).

- Compartment-specific cleavability of the nucleotide linker (triplet) impacts potency *in vivo*
- Cytosolic nuclease-cleavable nucleotide linkers (2′-deoxy-2′-fluoro-triplet, green, bis-siRNA-8) show enhanced potency *in vivo* compared to non-cleavable linkers (2′-*O*-methyl-triplet, dark blue, bis-siRNA-7)
- The better performance observed for the cleavable linker bis-siRNA-8 compared to the non-cleavable construct 7 matches the potency of the siRNAs mixture *in vivo*

Figure 5. Evaluation of Three-Strand Designs in Non-Human Primates

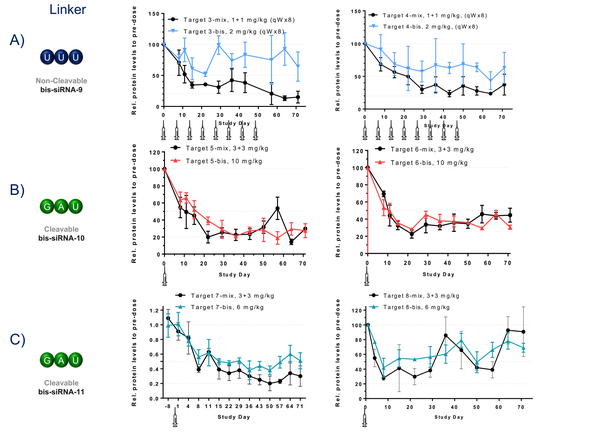


Figure 5. Efficacy of bis-siRNAs in NHP after s.c. administration of bis-siRNAs compared to mixture of individual parent compounds. (A) bis-siRNA-9 and mixture at a dose equivalent to 1 mg/kg of individual siRNAs. Eight weekly doses; (B) bis-siRNA-10 at 10 mg/kg and mixture at a dose equivalent to 3 mg/kg of individual siRNAs. Single dose; (C) bis-siRNA-11 and mixture at a dose equivalent to 3 mg/kg of individual siRNAs. Single dose.

Figure 6. Further Optimization of Cleavable Nucleotide Linkers

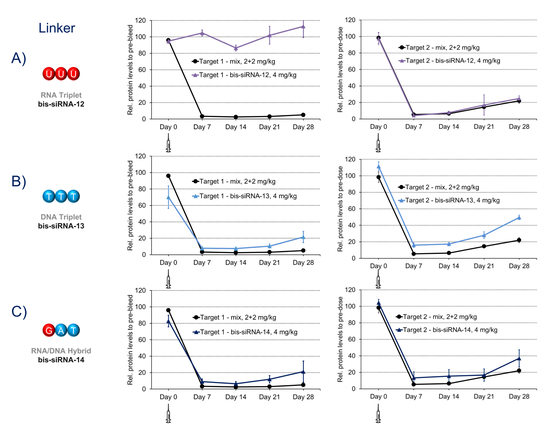
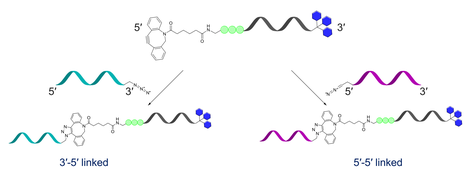


Figure 6. Efficacy of bis-siRNAs in mice after s.c. administration of bis-siRNAs compared to mixture of individual parent compounds. (A-C) bis-siRNAs-12-14 and mixture at a dose equivalent to 2 mg/kg of individual siRNAs.

Figure 7. "Click" Chemistry Linked Bis-RNAi Constructs



Objective

- Evaluate copper-free "click" cycloadditions for conjugation of two individually synthesized strands8
- Evaluate potential to improve overall synthesis yield to address scalability
  - *Example: for 98% stepwise coupling yield, overall crude yield for 24-mer strand: 62%, for 45-mer: 40%.*
- Nucleotide linker design (e.g. 2′-F triplet) can easily be incorporated
- Allows for 3′-5′ linked or 5′-5′ linked designs

Figure 8. Bis-siRNAs Do Not Induce Cytokine Response in CD1 Mice

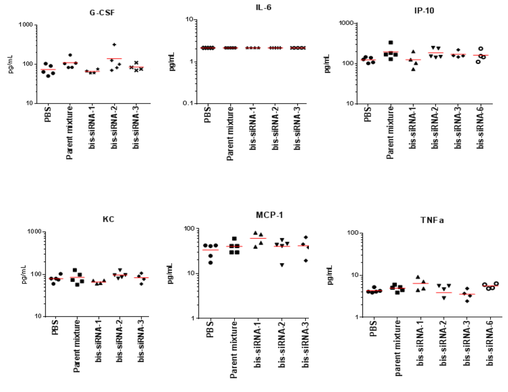


Figure 8. Panel of cytokines measured in CD-1 mice rel. to vehicle control after s.c. administration of 75 mg/kg dose of parent mixture or bis-siRNAs 1, 2, 3, or 6.

Summary

- We developed a simple and rapid screening system for evaluation of bis-RNAi conjugates capable of simultaneous silencing of two targets
  - *In vitro* free uptake and stability assays enable rapid pre-screening of compounds and may guide for further optimization
- Potent and durable silencing of both targets, comparable to the parent mixture, was achieved with bis-RNAi designs employing cleavable linkers
  - Successful synthesis of longer strands used for these designs requires adjustments in synthesis and purification conditions, incl. the use of alternate solid supports, which can accommodate larger oligonucleotides
- Bis-RNAi conjugates do not induce cytokine responses in human whole blood assay or CD1 mouse model
  - Profiles are comparable to parent mixture and untreated controls

References

1. Nair, J.K.; Willoughby J.L. et al. (2014). Multivalent *N*-acetylgalactosamine-conjugated siRNA localizes in hepatocytes and elicits robust RNAi-mediated gene silencing. *J. Am. Chem. Soc.* **136**, 16958-61.
2. Springer, A.D. and Dowdy, S.F. (2018). GalNAc-siRNA Conjugates: Leading the Way for Delivery of RNAi Therapeutics. Nucleic Acid Ther. **28**, 109-118.
3. Chang, C.I.; Kang, H.S. et al. (2009). Dual-target gene silencing by using long, synthetic siRNA duplexes without triggering antiviral responses. *Mol. Cells* **27**, 689-695.
4. Peng, W.; Chen, J. et al. (2013). Long double-stranded multiplex siRNAs for dual genes silencing. *Nucleic Acid Ther.* **23**, 281-288.
5. Avino, A.; Ocampo, J.C. et al. (2011). Branched RNA: A New Architecture for RNA Interference. *J. Nucleic Acids* **2011**, 1-7.
6. Chang, C.I.; Lee, T.Y. et al. (2012). Enhanced intracellular delivery and multi-target gene silencing triggered by tripodal RNA structures. *J. Gene Med.* **14**, 138-146.
7. Wu, Y.Y.; Chen, L. et al. (2013). Inhibition of hepatocellular carcinoma growth and angiogenesis by dual silencing of NET-1 and VEGF. *J. Mol. Histol.* **44**, 433-445.
8. Baskin, J.M; Presher, J.A. et al. (2007) Copper-free click chemistry for dynamic *in vivo* imaging. *PNAS* **104**, 16793-97.

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