Extra-hepatic delivery of Centyrin-targeted oligonucleotides

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Abstract

Using Aro's proprietary Centyrin platform, we have demonstrated potent functional delivery of siRNA oligonucleotides into skeletal and heart muscle while sparing knockdown in other tissues. Centyrins are ~10kDa protein ligands that combine the affinity and specificity properties of antibodies with significantly improved biophysical properties. Centyrins' small size, lack of immunogenicity, scalable, cost-efficient manufacturing in E. Coli, and site-specific conjugation make them ideally suited for targeted delivery of oligonucleotides to extra-hepatic tissues. Thus, Centyrins represent a potential solution to the well-recognized challenge of delivery of oligonucleotide medicines to tissues outside of the liver.

Using large libraries of Centyrin variants, we have identified a panel of Centyrin leads that bind to human transferrin receptor 1 (CD71, TfR1) and are not competitive with transferrin. Centyrin leads were evaluated for immunogenicity and were found to be devoid of T cell epitopes. Cysteine residues were introduced at specific amino acid sites in the Centyrin framework which enabled homogeneous, site-specific conjugation to siRNAs and ASOs. In animal models, Centyrins were shown to efficiently target conjugated siRNAs or ASOs to muscle, leading to robust knockdown of target genes in skeletal muscle and heart, with excellent tolerability profiles.

Given that Centyrins are of a similar size as the oligonucleotide cargo, Centyrin oligonucleotide drug conjugate doses are significantly lower than antibody or antibody fragment-based conjugates, reducing the potential for acute infusion reactions and immunogenicity. As a result of these unique properties, Centyrins hold promise as a new class of oligonucleotide conjugate therapies, with superior developability properties and potential for best-in-class efficacy and safety.

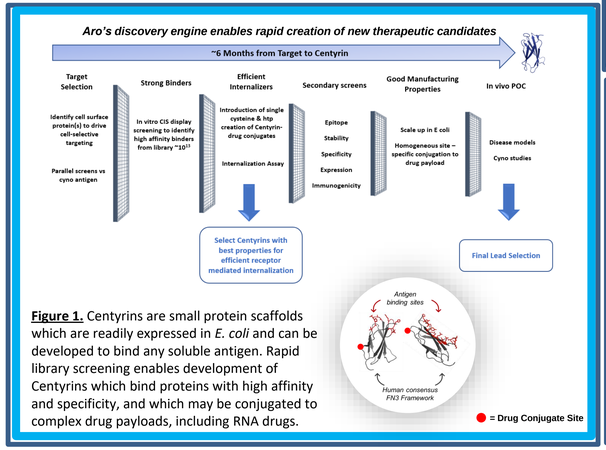


Figure 1. Centyrins are small protein scaffolds which are readily expressed in *E. coli* and can be developed to bind any soluble antigen. Rapid library screening enables development of Centyrins which bind proteins with high affinity and specificity, and which may be conjugated to complex drug payloads, including RNA drugs.

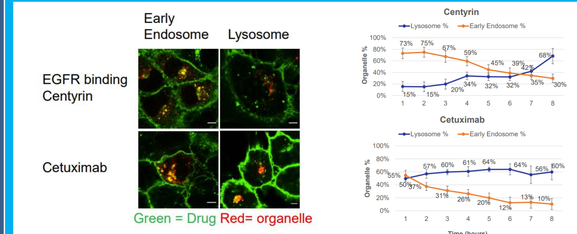


Figure 2. Centyrins reside in endosomes longer than antibodies. CAL 27-SCCHN cells were transiently transfected with LAMP1 or Rab5a RFP and were treated with an EGFR targeting Centyrin or Cetuximab labeled with AlexaFluor 488. Single cell imaging was used to track the trafficking of each construct. An average of 10 cells were used for each point.

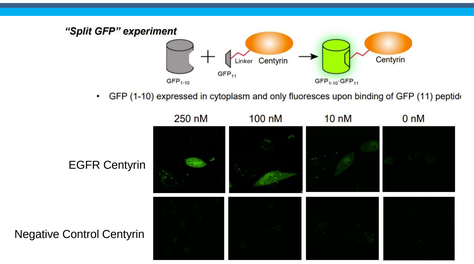
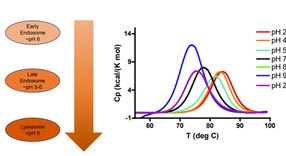


Figure 3. Centyrins traffic into the cytoplasm. An EGFR binding Centyrin and a non-binding Centyrin (negative control) were expressed with GFP11 amino acid sequence at the C-terminus. HCC827 cells were treated for 24 h with each Centyrin and then imaged.



B. Centyrins have low potential for immunogenicity

Percentage Stimulation above Background ≥0.5%, SEM=2			
Protein ID	**Percentage Antigenicity**	**Strength of Response (Mean %Stimulation)**	**Response Index (RI)**
Ctrl 1 PPD	100.00	61.39	61.394
Ctrl 2 KLH	100.00	32.05	32.053
Tencon40	25.00	1.36	0.341

Figure 4. Centyrins are exceptionally stable proteins which lack immunogenicity in human T cell assays. (A) Centyrin melting temperatures indicate stability at physiological pH of endocytic compartments. (B) Dendritic cell:Tcell co-culture assay was used to assess Centyrin immunogenicity potential (ProImmune, Inc). 20 donor PBMC samples were HLA typed and the allele distribution frequency of HLA class II resembled the global population. T cell activation was assessed after 7 days.

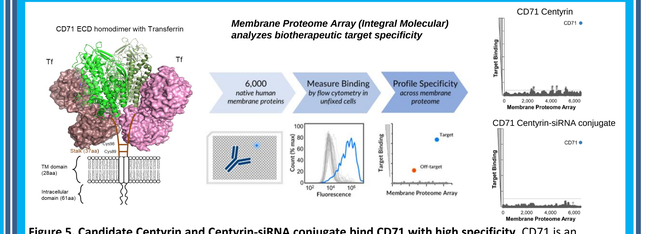


Figure 5. Candidate Centyrin and Centyrin-siRNA conjugate bind CD71 with high specificity. CD71 is an essential and ubiquitously expressed receptor responsible for iron transport into cells. We have generated a large diversity of CD71 Centyrins to enable efficient and customized targeting of various CD71+ cell types, delivering siRNA payloads to target tissues. Candidate CD71-targeting Centyrins were screened against a Membrane Proteome Array of 6,000 human proteins (Integral Molecular) and shown to specifically bind CD71 with no off-target interactions.

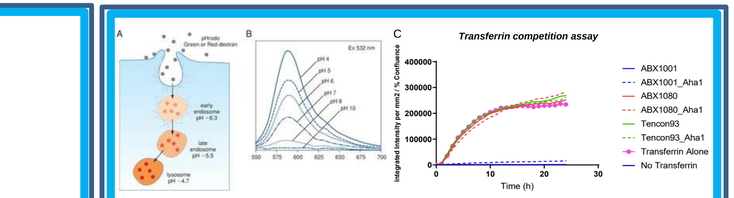


Figure 6. pHrodo-Transferrin uptake assay identifies Centyrins which do not compete with Tf. A) Commercially-available Transferrin conjugated to pHrodo Red dye fluoresces upon uptake into acidic cell compartments, and B) increases fluorescence intensity with decreasing pH. C) pHrodo-Tf was co-incubated with Centyrin and Centyrin-siRNA conjugates and fluorescence was measured using a live cell imager (Incucyte). Transferrin uptake is reduced in presence of competitive molecules, and noncompetitive Centyrins and conjugates are selected via high-sensitivity assay.

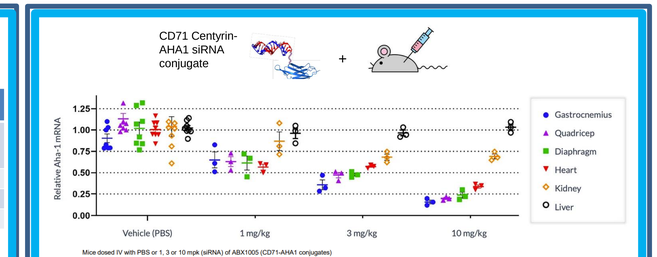
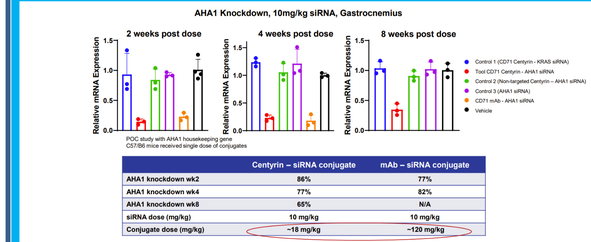


Figure 7. Proof-of-concept Centyrin-siRNA conjugate generates robust and selective gene knockdown in skeletal and cardiac tissue *in vivo*. Mice dosed with CD71 Centyrin-AHA1 siRNA conjugate demonstrate strong dose-response relationship in target gene knockdown by RT-qPCR. Up to 80% gene knockdown is observed 2 weeks after a single dose in skeletal and cardiac muscle, with limited or no effect in off-target tissues kidney and liver.



	Centyrin – siRNA conjugate	mAb – siRNA conjugate
AHA1 knockdown wk2	86%	77%
AHA1 knockdown wk4	77%	82%
AHA1 knockdown wk8	65%	N/A
siRNA dose (mg/kg)	10 mg/kg	10 mg/kg
Conjugate dose (mg/kg)	~18 mg/kg	~120 mg/kg

Figure 8. Proof-of-concept CD71 Centyrin conjugate drives sustained gene knockdown at a fraction of mAb conjugate dose. Centyrin-siRNA conjugate generates sustained gene knockdown after a single dose, with similar activity as a CD71 mAb-siRNA conjugate. The smaller size of the Centyrin-oligonucleotide drug conjugate requires significantly lower doses than antibody conjugates for equivalent activity.

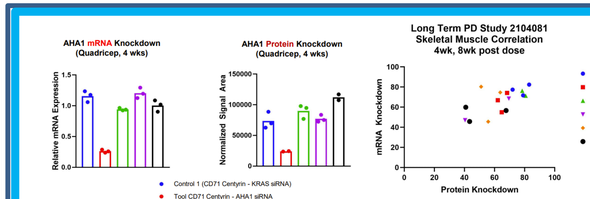


Figure 9. *In vivo* mRNA and protein knockdown by Centyrin-siRNA conjugate are durable and well correlated. Centyrin-siRNA generates substantial knockdown of both mRNA and protein after a single dose. Both mRNA and protein were quantified by RT-qPCR and quantitative Western blot, respectively, in tissues collected at 4 and 8 weeks after a single dose. A strong correlation is observed between mRNA and protein knockdown across various skeletal muscles.

Summary and Conclusions

- CD71 Centyrin-AHA1 siRNA conjugate demonstrates receptor-specific delivery of siRNA to skeletal and cardiac tissues
- siRNA-mediated knockdown of target mRNA and protein is robust and long-lasting after a single dose
- Rapid generation of Centyrin libraries allowed for the selection of potent leads which do not compete with transferrin and have low immunogenicity potential
- Centyrin-siRNA conjugates may be administered at a fraction of the dose required for an equivalent mAb conjugate

References

1. Diem, M. et al. "Selection of high-affinity FN3 domains from a simple library diversified at a combination of strand and loop positions." *Protein Engineering, Design and Selection*, 2014, **27**, 419-429.
2. Tortorella, S.; Karagiannis, T.C. "Transferrin-Mediated Endocytosis: A Useful Target for Cancer Therapy." *J. Membrane Biol.*, 2014, **247**:291–307