Tumor-targeted knockdown of KRAS mutants with novel Centyrin:siRNA conjugates

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Introduction

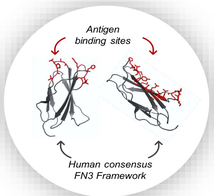
Aro Biotherapeutics is a preclinical stage biotechnology company focused on discovery and development of Centyrins, a new class of small, structurally simple, highly stable and soluble proteins engineered to specifically bind antigens with high affinity.[1]

We demonstrate Centyrins have a longer residence time in the early endosome relative to antibodies which are rapidly shunted to lysosomes after binding to the same receptor. Using Centyrins targeted to cell surface receptors on tumor cells, we also demonstrate efficient internalization and trafficking of Centyrins to the cytosol via protein complementation as demonstrated using GFP complementation assays.

Centyrins provide a means to specifically deliver oligonucleotides to cell types beyond hepatocytes which enables access to intracellular targets that have been considered "undruggable". Our Centyrin-KRAS siRNA conjugates are designed to inhibit a variety of solid tumors driven by KRAS mutations and are released in early endosomes over an extended time period. These data highlight the broad utility of this platform.

Centyrins for Delivering siRNA

Small interchangeable protein scaffolds, optimized for multi-antigen targeting and delivery, of complex drug payloads, including RNA drugs



Small and Simple	Excellent tissue and tumor penetration; cleared through kidney
	No glycosylation sites
	Site specific drug conjugation
Unique Biophysical Properties	Soluble, heat and pH stable
	E coli production with high expression levels
Flexible	Rapid screening of Centyrin libraries using *in vitro* CIS-display and intracellular delivery
	Centyrins selectively bind proteins with high affinity and specificity
	Easily formatted into bi- and multi- specific molecules

Centyrins Reside in Endosomes Longer than Antibodies

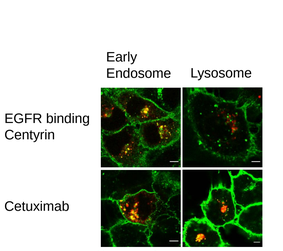


Figure 1 : CAL 27-SCCHN cells were transiently transfected with LAMP1 or Rab5a rfp and were treated with an EGFR targeting Centyrin or Cetuximab labeled with Alexa-Fluor 488.

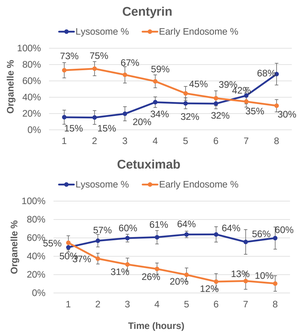
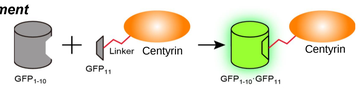


Figure 2: Single cell imaging was used to track the trafficking of each construct. An average of 10 cells were used for each point.

Centyrins Traffic into the Cytoplasm

"Split GFP" experiment



- GFP (1-10) expressed in cytoplasm and only fluoresces upon binding of GFP (11) peptide

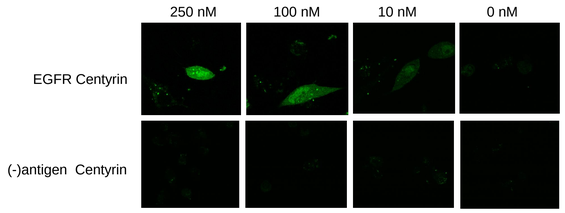


Figure 3: An EGFR binding Centyrin and a control (-) antigen negative Centyrin were expressed with GFP11 amino acid sequence at the C-terminus. HCC872 cell were treated for 24 h with each Centyrin and then imaged.

Targeting Mutant and Wild Type KRAS

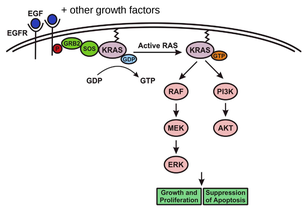


Figure 4. KRAS is a critical regulator of the MAPK/ERK pathway responsible for tumor oncogenesis in many solid tumors.

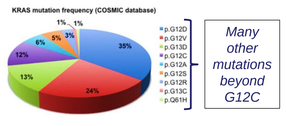
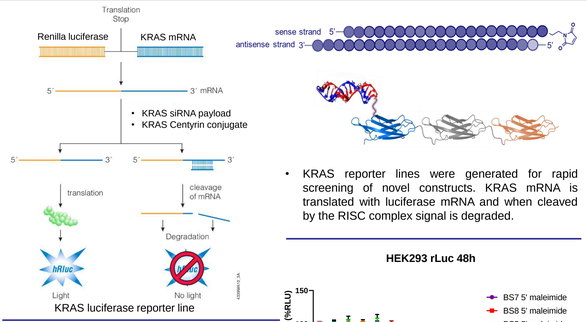


Figure 5. While therapies are emerging for G12C, ~12 % of mutant forms, other KRAS mutant cancers are still undruggable. [2]

siRNA Sequence/Chemistry SAR



- Over 3500 siRNA sequences were computationally evaluated for off target effects and potency against pan-KRAS mRNA.
- 87 siRNAs were tested as fully modified and protected sequences via transfection into HEK293 luciferase based reporter cell line.
- The most potent and selective sequences were further modified with maleimide linker chemistry to enable conjugation to the targeting Centyrin, Figure 6.

- KRAS reporter lines were generated for rapid screening of novel constructs. KRAS mRNA is translated with luciferase mRNA and when cleaved by the RISC complex signal is degraded.

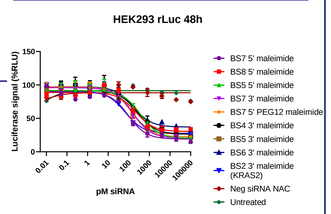


Figure 6: Sequences identified from a high throughput transfection study (not shown) were incorporated into linker and novel chemistry designs. Linker site and linkage chemistry were evaluated by transfection using the HEK293 luciferase cell line. ABXP-03-12 (KRAS2) was taken on as a lead siRNA linker

ABX300: Dual Antigen Targeting

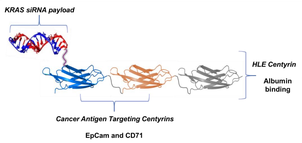


Figure 7: ABX300:Targets both EpCam and CD71 cancer antigens.

- ABX300 is a bispecific Centyrin targeting EpCam and human transferrin (CD71) conjugated to a potent pan-KRAS siRNA.
- The siRNA was selected for potency and specificity for KRAS mRNA and is fully modified to evade innate immune detection.
- An additional Centyrin was selected for half-life extension via binding to albumin, a serum protein with a 19-21 day half-life in humans.

Advantages of dual targeting

1. Avidity increases internalization rate and accumulation of therapeutic payload.
2. Dual antigen specificity further differentiates targeting to tumor specific tissue.
3. Ease of Centyrin production allows for rapid screening of Centyrin combinations for the most effective combination of targets.

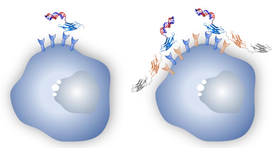


Figure 8: Single vs dual antigen targeting.

Time Dependent Knockdown Consistent with Slow Endosomal Release

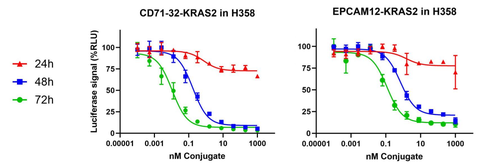


Figure 9: The H358 luciferase reporter cells were treated with EpCam Centyrin – KRAS2 siRNA or CD71 Centyrin-KRAS siRNA drug conjugates. The plates were read at 24h, 48 h, and 72 h. A time dependent effect was observed consistent with receptor mediated uptake and accumulation of the conjugate in the cell.

Knockdown of Many KRAS Mutants Evaluated: G12C, G12D, G12S, G12V, G13D, Q61H

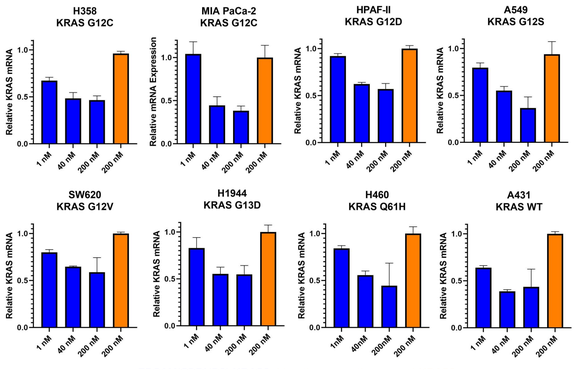


Figure 10: H358-NSCLC (G12C), MIA PaCa-2-pancreatic (G12C), HPAF II-pancreatic (G12D), A549-NSCLC (G12S), SW620-CRC (G12V), H1944-NSCLC (G13D), H460-NSCLC (Q61H) and A431-skin (KRAS WT) cancer lines were treated with Centyrin-KRAS2 siRNA conjugates for 72 h. Cells were harvested and cDNA was prepared using Cells-to-Ct kits. Quantitative RT-PCR was performed using Taqman primer/probe sets for KRAS and Ubiquitin C (endogenous control). Dose-dependent knockdown of endogenous KRAS mRNA was observed in all tumor cell types that were evaluated, including a wide variety of KRAS mutations.

ABX300 Knocks Down KRAS Protein

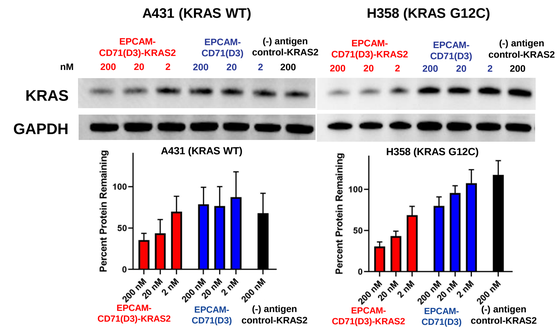


Figure 11: A431 and H358 cells were treated with bispecific Centyrins conjugated to KRAS2 at 2, 20 and 200 nM concentrations. Western blots were used to detect KRAS protein at 72 hours. A good correlation between siRNA mRNA silencing and protein was observed.

Evaluation of lead KRAS siRNA sequence for specificity with RNA-seq

Transcriptional profiling on 3 different KRAS siRNAs performed on H358 cells

- KRAS2 (**BS2**) lead siRNA was most specific for KRAS (2 other siRNAs were not specific and led to off target knockdown or mRNAs, **282**)
- KRAS mRNA was most suppressed mRNA with KRAS2
- Other genes known to be regulated by KRAS also reduced with KRAS2

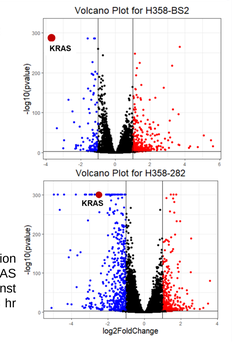


Figure 12: Volcano plot representation of the differential expression analysis for gene knockdown after treatment with varying KRAS siRNA sequences. H358 cells were lipofected with siRNA against KRAS, **BS2** and **282**, at 10 nM, n=5. Cells were collected at 24 hr for analysis.

Conclusions

- Centyrins possess differentiated trafficking vs. antibodies facilitating siRNA delivery.
- ABX300 demonstrated receptor specific delivery of KRAS siRNA.
- Centyrin:siRNA conjugates demonstrated potent knockdown of all mutant forms of KRAS evaluated.
- ABX300 inhibits KRAS-driven tumor cell proliferation.
- RNAseq data demonstrate excellent specificity for the knockdown of KRAS mRNA with our lead siRNA **BS2**.
- Potential broad utility of Centyrins to deliver siRNA or other payloads into many internalizing receptor positive cells.

References

1. Diem, M. et al. "Selection of high-affinity Centyrin 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. Hobbs, A.; Der, C.; Rossman, K. "RAS isoforms and mutations in cancer at a glance" *J. Cell. Sci.*, **2016**, 1287.

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