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

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Introduction

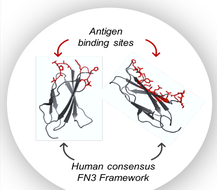
Aro Biotherapeutics is a preclinical stage biotechnology company focused on discovery and development of Centyrins, a new class of small, structurally simple, highly stable and soluble proteins engineered to specifically bind antigens with high affinity.[1] The ABX300 series of molecules provides tumor-targeted delivery of a KRAS siRNA.

We demonstrate Centyrins have a longer residency time in the early endosome relative to antibodies, which are rapidly trafficked 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 cytoplasm via protein complementation as demonstrated using GFP complementation assays.

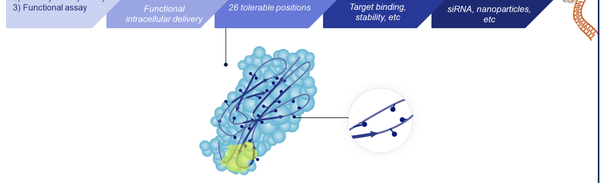
Centyrins provide a means to specifically deliver oligonucleotides to cell types beyond hepatocytes which enables access to intracellular targets that have been considered "undruggable". Our Centyrin-KRAS siRNA conjugates are designed to inhibit a variety of solid tumors driven by KRAS mutations. ABX300 targets all known oncogenic mutations of KRAS, addressing a major unmet need.

Centyrins for Delivering siRNA

Figure 1. Centyrins are 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

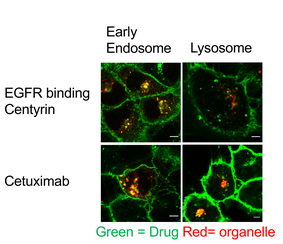


Figure 2: CAL27-SCCHN cells were transiently transfected with Rab5a (early endosome) or LAMP1 (lysosome) red fluorescent protein reporters and were treated with an EGFR-targeting Centyrin or Cetuximab labeled with Alexa-Fluor 488. Images taken at 1hr post-treatment. Centyrin is primarily located in endosomes while Cetuximab has moved to lysosomes.

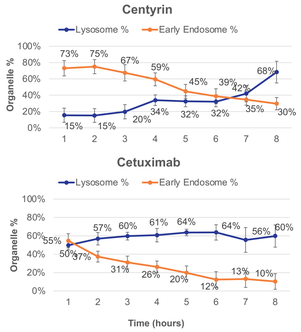
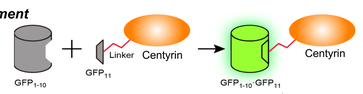


Figure 3: Single cell imaging was used to track the trafficking of each drug in CAL27 cells. An average of 10 cells were imaged at each point. Centyrins exhibit longer endosomal residence time than antibodies.

Centyrins Traffic into the Cytoplasm



GFP_{1-10} is expressed in cytoplasm and only fluoresces upon binding of the GFP_{11} peptide

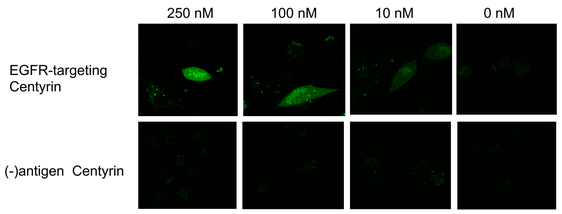


Figure 4: An EGFR-targeting Centyrin and a control antigen-negative Centyrin were expressed with GFP_{11} amino acid sequence at the C-terminus. HCC877 cells were treated for 24h with each Centyrin and then imaged. Cytoplasmic GFP signal indicates that Centyrins escape the endosome.

Targeting All Mutants of KRAS

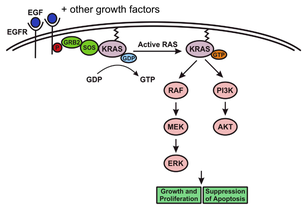


Figure 5. KRAS is a critical regulator of the MAPK/ERK pathway responsible for tumor oncogenesis in many solid tumors.

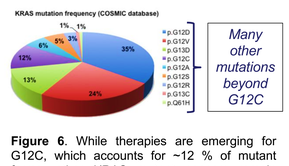
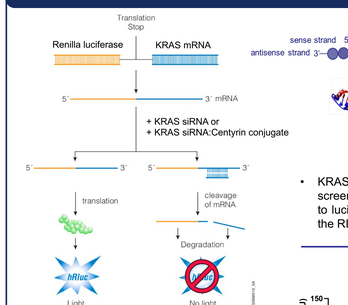
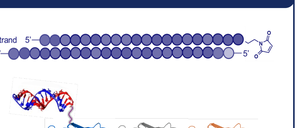


Figure 6. While therapies are emerging for G12C, which accounts for ~12 % of mutant forms, other KRAS mutant cancers remain undruggable. [2]

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.



- KRAS reporter lines were generated for rapid screening of novel constructs. KRAS mRNA is fused to luciferase mRNA. When this mRNA is cleaved by the RISC complex, luciferase signal is degraded.

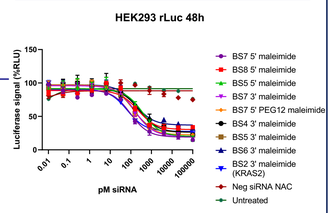


Figure 7: Sequences identified from a high throughput transfection study (not shown) were incorporated into linker and novel chemistry designs. Linker site and linkage chemistry were evaluated by transfection using the HEK293 luciferase cell line. BS2 3' maleimide (KRAS2) was advanced as a lead siRNA linker.

ABX300: Dual Antigen Targeting

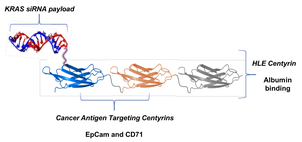


Figure 8: ABX300: Targets both EpCam and CD71 cancer antigens.

- ABX300 is a bispecific Centyrin targeting EpCam and CD71 (transferrin receptor) 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 cells versus normal tissue
3. Ease of Centyrin production allows for rapid screening of target combinations for the most effective combination of targets

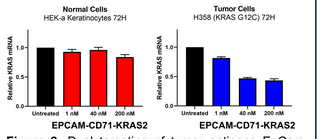


Figure 9: Dual targeting of tumor antigens EpCam and CD71 results in greater knockdown of endogenous KRAS mRNA in tumor cells than normal cells. KRAS mRNA was measured via qPCR following 72h of ABX300 treatment.

Time Dependent Knockdown Consistent with Slow Endosomal Release

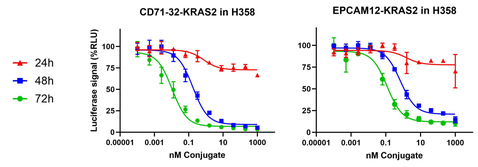


Figure 10: The H358 luciferase reporter cells were treated with EpCam Centyrin-KRAS2 siRNA or CD71 Centyrin-KRAS2 siRNA drug conjugates. The plates were read at 24h, 48h, and 72h. A time dependent effect was observed, consistent with receptor mediated uptake and slow release from endosomes into the cytoplasm.

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

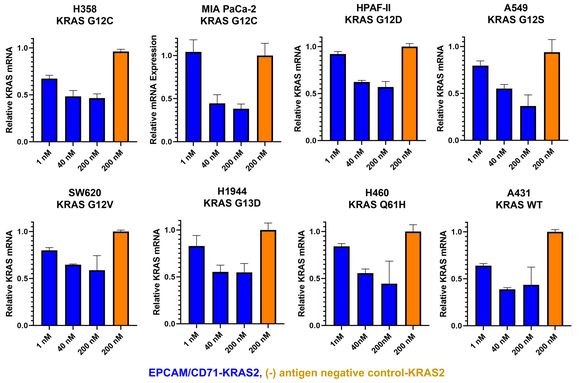


Figure 11: H358-NSCLC (G12C), MIA PaCa-2-pancreatic (G12C), HPAF II-pancreatic (G12D), A549-NSCLC (G12S), SW620-CRC (G12V), H1944-NSCLC (G13D), H460-NSCLC (Q61H) and A431-skin (KRAS WT) cancer lines were treated with Centyrin-KRAS2 siRNA conjugates for 72h. Cells were harvested and cDNA was prepared 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 12: H358 cells were treated with bispecific Centyrins conjugated to KRAS2 siRNA at 2, 20 and 200 nM concentrations. Western blots were used to detect KRAS protein at 72 hours. GAPDH was measured as a loading control. Dose dependent knockdown of KRAS protein was observed, correlating with mRNA knockdown.

ABX300 Inhibits Tumor Cell Proliferation via KRAS Knockdown

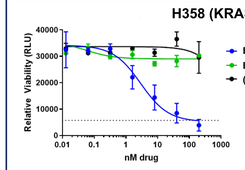


Figure 13: H358 cells in 3D spheroid culture were treated with Centyrin:siRNA conjugates for 21 days. Proliferation was assessed by CellTiter-Glo 3D. Dotted line notes starting population. Complete inhibition of proliferation was observed by 7 days (not shown) and sustained for 21 days.

Rescue Experiment to confirm anti-proliferative activity is driven by KRAS Knockdown

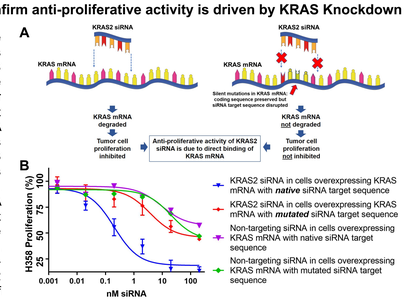


Figure 14: **A**) Overview of Rescue Experiment design. H358 cells were engineered to express KRAS mRNA with either the native KRAS2 siRNA target sequence or a mutated form in which silent mutations eliminated the siRNA binding site. **B**) KRAS2 siRNA was transfected into engineered H358 cells and 2D proliferation was assessed via CellTiter-Glo at 72h. Tumor cells with KRAS mRNA mutated at the siRNA target sequence were "rescued" from the effects of the KRAS2 siRNA, demonstrating that the anti-proliferative activity of KRAS2 siRNA is due to direct binding of KRAS mRNA.

Conclusions

- Centyrins possess differentiated trafficking vs. antibodies, facilitating siRNA delivery
- ABX300 demonstrates receptor-specific delivery of KRAS siRNA to tumor cells
- ABX300 demonstrates potent knockdown of all mutant forms of KRAS evaluated
- ABX300 knocks down KRAS protein and inhibits KRAS-driven tumor cell proliferation
- Rescue experiment confirms that anti-proliferative activity of KRAS2 siRNA is due to knockdown of KRAS rather than off-target genes
- 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, 129(7), 1287-92.

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