IHC and flow cytometry quantifies BRD4 levels in surrogate tissues after *ex-vivo* and *in-vivo* dosing with a BRD4 degrading PROTAC

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ABSTRACT

Background. The Bromodomain and Extra Terminal (BET) protein BRD4 is a key transcriptional regulator of cancer oncogenes (eg *MYC*) in numerous human malignancies and BET inhibitors have entered clinical trials. We have developed a proteolysis targeting chimera (PROTAC) molecule, ARCC-29, which induces degradation of BRD4 protein in several human malignant cell lines and in ex-vivo rodent xenograft tumors. In rodent xenograft models of prostate (22Rv1), DLBCL (SU-DHL-8) and ovarian (A2780) tumors, this molecule demonstrates a strong PROTAC-efficacy relationship. In clinical trials targeting tumor tissue can be invasive and difficult, sometimes impossible. Looking ahead to clinical trials we have targeted PBMC and skin as surrogate tissues to monitor PROTAC mode-of-action and pharmacodynamic biomarkers.

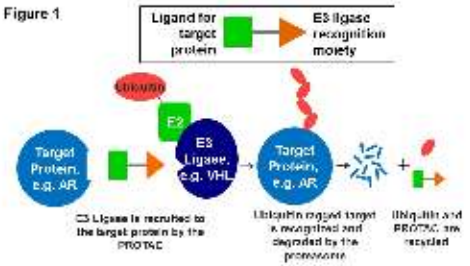
Methods and Results. We have developed a flow cytometry protocol to quantitatively assess BRD4 levels in human and rat perchlated blood mononuclear cells (PBMCs). Human (*ex-vivo*) and rat (*in-vivo*) PBMCs treated with ARCC-29 were fixed and permeabilized for BRD4 staining. ARCC-29 dramatically reduced BRD4 levels in fresh and frozen/thawed DLBCL human (SU-DHL-8) showed a marked increased levels of BRD4 compared to vehicle controls, as did monitor stereoisomers of ARCC-29. Immunohistochemistry (IHC) can evaluate BRD4 degradation in skin biopsies of rats dosed *in-vivo* following ARCC-29 treatment demonstrating a dose-dependent reduction BRD4 protein.

Conclusions. BRD4 degradation can be evaluated in surrogate tissue. Flow cytometry using PBMCs and skin IHC provide alternative methods for confirming PROTAC mechanism-of-action in the clinic using pre- and post-treatment patient samples.

PROTAC: PROteolysis TArgeting Chimera

- Technology developed by Prof. Craig Crews, Yale University
- Platform licensed to Arvinas in 2013.

Figure 1

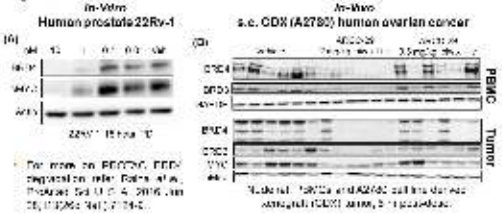


Selected publications on PROTAC technology:
- PNAS 2011 Jun 28;108:E657-65.
- Nat Chem Biol. 2015 Aug;11:611-7.
- Nat Rev Drug Discov. 2017 Feb;16:101-114.

BET PROTAC DEGRADES BRD4

- ARCC-29 PROTAC degrades BRD4 and BRD3 in rat PBMCs *in vivo*, a viable surrogate tissue for tumor to monitor drug mode-of-action in clinical trials (Fig 2).

Figure 2

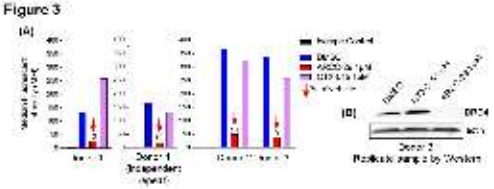


- For more on PROTAC, BRD degradation refer: Raina et al., Proc Natl Acad Sci U S A. 2016 Jun 28;113(26):7124-9.

A BRD4 FACS assay with fresh PBMCs

- Depleted BRD4 can be reproducibly measured by fluorescence activated cell sorting (FACS) in the lymphocyte subset of fresh human PBMC treated *ex-vivo* with ARCC-29 and the BET CTX-016 (Fig 3).

Figure 3



FACS Materials and Methods
- Human PBMCs dosed *ex-vivo* for 6 hours, fixed & permeabilized (TF Buffer Set, BD Biosciences). Blocked with human AB serum (Zenbio).
- Primary antibody: BRD4 (probe), (E2A7, Cell Signal) + 2° goat anti-rabbit-AF488 (Abcam, ab 50077).
- Isotype: primary rabbit IgG (ab 199091) + 2° anti-rabbit AF488 (ab 50077).
- Marker: anti-HD4 (ab 5076, Abcam) + 2° AF647-goat anti-rabbit.
- Gated lymphocyte BRD4 events are shown (FACSCalibur).

BRD4 FACS in frozen/thawed PBMCs

- Depleted BRD4 can be reliably detected by FACS in *ex vivo* ARCC-29 treated frozen/thawed lymphocyte subset of normal human PBMCs (ZenBio) (Fig 4, 5).

Figure 4

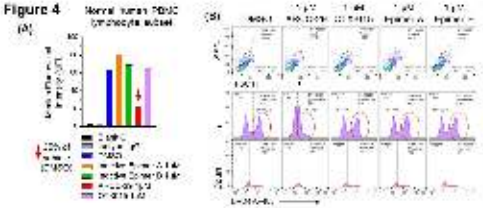
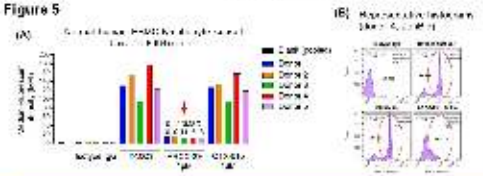


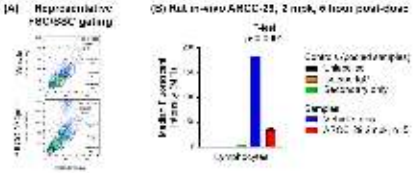
Figure 5



In-vivo BRD4 depletion in rat PBMCs

- Depletion of BRD4 can be reproducibly measured by FACS in the lymphocyte subset of nude rat PBMCs dosed *in-vivo* with ARCC-29 (Fig 6).

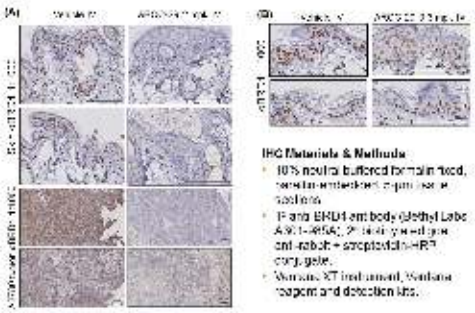
Figure 6



Skin BRD4 depletion evaluation by IHC

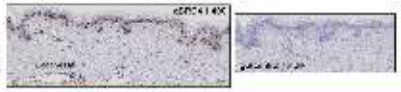
- Nude rat skin biopsies show a dose dependent depletion of BRD4 *in vivo* following 3 and 6.3 mg/kg (mpk) IV, 5 hours post-dose (Fig 7). A2780 (ovarian cancer) human xenograft tumors used as controls. Figure 8 shows example staining in normal untreated human skin.

Figure 7



IHC Materials & Methods
- 10% neutral buffered formalin fixed, paraffin embedded, 4 µm tissue sections.
- IF and BRD4 antibody (Bethyl Labs A301-985A), 2° rabbit-goat anti-rabbit + streptavidin-HRP conjugate.
- Vectors XT Instrument, Ventana reagent and detection kits.

Figure 8



- BRD4 IHC on normal human skin

Conclusion

We have developed two assays to monitor BET PROTAC mode-of-action in clinical trials using PBMCs and skin as surrogate tissues for tumor. PBMCs and skin biopsies are easily acquired, stored and transported for analysis.

Acknowledgements

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