Small molecule microarray screening identifies novel androgen receptor ligands

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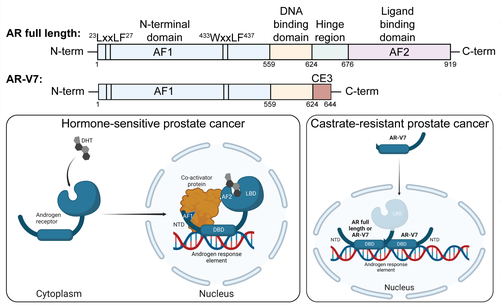
Abstract

The androgen receptor (AR) is a well-established oncogenic driver of prostate cancer, and AR-targeting hormone therapy has proven an efficacious treatment option for patients with metastatic prostate cancer. Due to the importance of AR in prostate cancer disease progression, we leveraged small molecule microarray (SMM) screening technology to identify compounds that bind to transcriptional complexes of a common AR splice-variant, AR-V7, present along with AR in castration-resistant prostate cancer cell lysates. A subset of SMM hits were identified to modulate the AR Transcription Regulatory Network (TRN) in hormone-sensitive, but not castration-resistant prostate cancer models using a functional cell-based transcription signature approach. Several AR SMM hits directly engaged the AR ligand binding domain in a biophysical assay. One of these AR SMM hits altered AR nuclear translocation and antagonized steroid receptor co-activator recruitment to AR but did not antagonize the glucocorticoid receptor. Leveraging structural data for known AR ligands, we propose a computational model for binding of this molecule to the known AR ligand binding domain. Together, the data demonstrates that the AR SMM lysate screen successfully identified structurally novel small molecules that modulate the AR TRN and bind the AR ligand binding domain.

Androgen receptor SMM lysate screen design and hit triage strategy

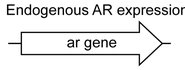
AR is a pivotal transcription factor in prostate cancer



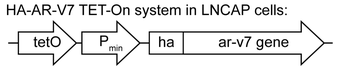
- Androgen receptors are the primary driver of cell growth and differentiation in most prostate cancers
- AR is activated by androgens binding to the ligand binding domain (LBD)
  - Testosterone or 5α-dihydrotestosterone (DHT) are common androgens
- Activated AR translocates into the nucleus, binds DNA at AR-binding sites and drives an AR-dependent transcriptional regulatory network
- Androgen therapy can induce the progression of hormone-sensitive prostate cancer to castration-resistant prostate cancer, limiting therapeutic options
- Many forms of castration-resistant prostate cancer produce AR variants that are constitutively active due to loss of regulatory C-terminal LBD
  - AR-V7 is the most common variant expressed
- Targeting constitutively active AR variants would help curb progression to castration-resistant prostate cancer

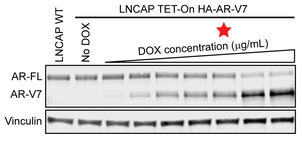
A Engineer an AR-V7 expressing prostate cancer cell line for SMM screening

Endogenous AR expression in LNCAP cells:

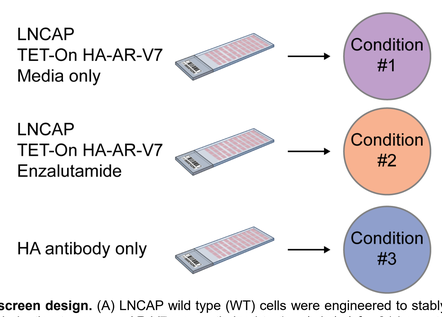


HA-AR-V7 TET-On system in LNCAP cells:



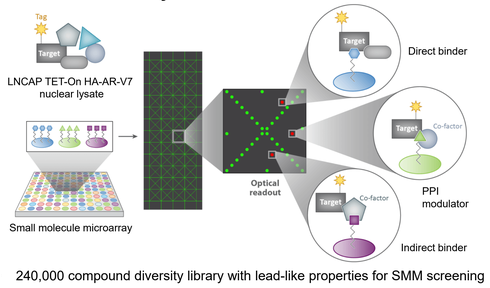


B AR SMM lysate screen design

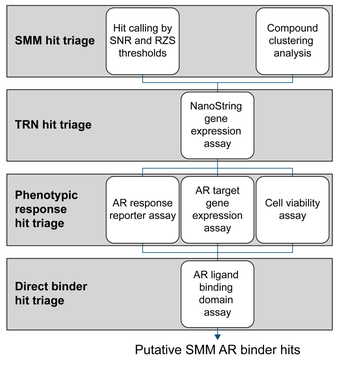


Prostate cancer cell line engineering and AR SMM lysate screen design. (A) LNCAP wild type (WT) cells were engineered to stably express doxycycline inducible HA-tagged AR-V7. Doxycycline induction to express AR-V7 was optimized to 1 μg/mL (★) for 24 hours to mimic AR-V7 expression levels observed in other AR-V7 expressing prostate cancer cell lines. (B) The AR SMM screen was designed to include three separate conditions, *i*) LNCAP HA-AR-V7 cells in regular media, *ii*) LNCAP AR-V7 cells treated with 3 μM enzalutamide, and *iii*) HA antibody-only as a counter screen. Nuclear lysates were derived from LNCAP AR-V7 cells using common cell fractionation techniques and SMM slides were incubated with ~0.3 mg/mL of nuclear lysate for 1 hour at 4°C. AR-V7 containing complexes were readout on the SMM slides using a fluorophore-conjugated antibody targeting the HA peptide sequence.

SMM lysate screen schematic



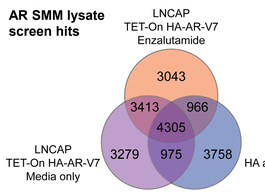
Hit triage strategy



- 240,000 compound diversity library with lead-like properties for SMM screening
- SMM offers an expanded modality range for drug target types:
  - Direct binders to target
  - Indirect binders to target (e.g. cofactor)
  - Protein-protein interaction (PPI) modulators (e.g. target::cofactor)

SMM screen hit triage identifies AR TRN modulators with putative AR binding mode of action

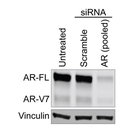
AR SMM screen hit calling



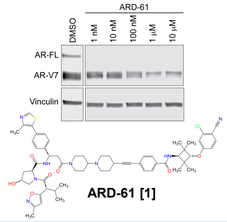
- 3413 SMM hits were identified between conditions #1 and #2
  - LNCAP TET-On HA-AR-V7 +/- enzalutamide
  - 1.43% hit rate
- SMM hits were first triaged at readout by assessing the SNR and RZS scores of each spot
  - SNR cut-off at x ≥ 2
  - RZS cut-off at x ≥ 3
- Additional SMM hit triaging used compound clustering analysis to select compounds with the most favorable molecular properties and structural diversity

AR TRN-focused hit triage

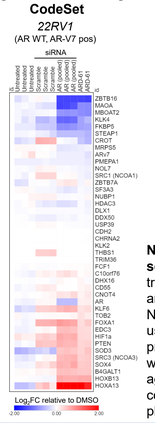
A Knockdown of AR-FL and AR-V7 using siRNA



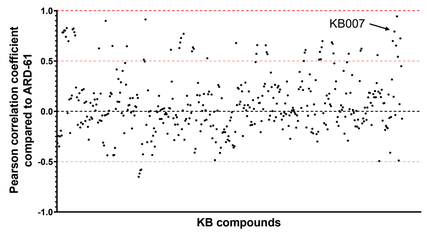
B ARD-61 degrader preferentially degrades AR-FL



C Validation of AR TRN signature NanoString CodeSet



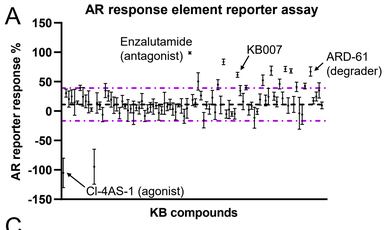
D Transcriptional signature-based screening identified AR TRN modulators (NanoString)



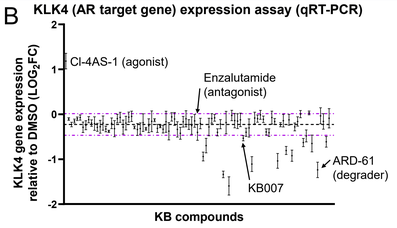
NanoString gene expression profiling adapted for high-throughput screening to rapidly identify bioactive compounds. (A) 22RV1 cells were transfected with siRNA for 48 hours or (B) treated with ARD-61 for 24 hours and lysates were immunoblotted with the specified antibodies. (C) AR TRN NanoString CodeSet validated in 22RV1 cells with genetic (AR knockdown using siRNA) and pharmacologic (ARD-61) perturbation. (D) SMM hits were profiled by NanoString AR TRN CodeSet in 22RV1 cells. Cells were treated with 20 μM compound for 24 hours. Compound signatures were compared against the ARD-61 signature and scored as a Pearson correlation coefficient. Compounds that scored between 1 ≥ x ≥ ~0.5 were selected for phenotypic response and direct binder profiling.

Confirmation of AR TRN modulation using orthogonal assays

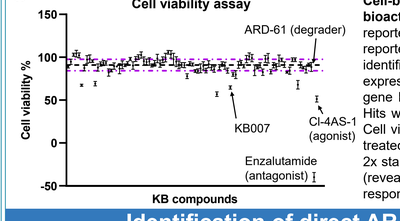
A AR response element reporter assay



B KLK4 (AR target gene) expression assay (qRT-PCR)



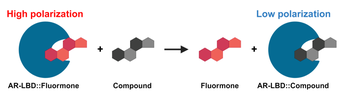
C Cell viability assay



Cell-based phenotypic response profiling confirmed the identity of several bioactive compounds that targeted AR activity. (A) Cignal AR luciferase reporter (Qiagen) was transfected into 22RV1 cells to generate the AR response reporter assay. Cells were treated with 20 μM compound for 24 hours. Hits were identified using 2x standard deviation from the median. (B) AR target gene expression assay monitored the change in gene expression of the AR target gene KLK4 in 22RV1. Cells were treated with 20 μM compound for 24 hours. Hits were qualified as outliers from 2x standard deviation from the median. (C) Cell viability was assessed using CellTiter-Glo (Promega) in 22RV1. Cells were treated with 20 μM compound for 72 hours. Hits were qualified as outliers from 2x standard deviation from the median. All assays were run in duplicate. KB007 (revealed below as a direct AR binder) was highlighted across all phenotypic response assays as a bioactive compound.

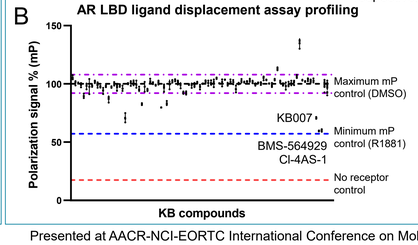
Identification of direct AR ligand binding domain binders

A AR-LBD ligand displacement assay schematic

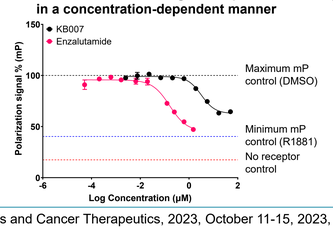


A subset of bioactive compounds targeting AR activity directly bind AR via the C-terminal LBD. (A) PolarScreen AR competitor assay (Thermo Fisher Scientific) schematic. (B) The AR-LBD ligand displacement assay was used to profile all bioactive compounds from the TRN triage. AR-LBD was treated with 20 μM compound and incubated for 4 hours. Hits were identified using 2x standard deviation from the median. The assay was run in duplicate. (C) KB007 was selected as a putative AR binder and subjected to dose response profiling with the AR-LBD ligand displacement assay. A 10-point dose series was performed where the IC_{50} values for KB007 and enzalutamide were 3.2 μM and 0.16 μM, respectively.

B AR LBD ligand displacement assay profiling



C KB007 is active in the AR-LBD ligand displacement assay in a concentration-dependent manner



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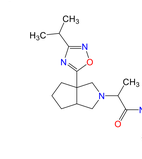
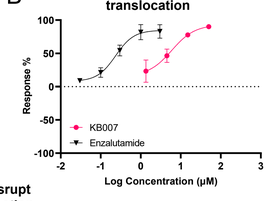
KB007 is an AR antagonist with activity across prostate cancer cell models

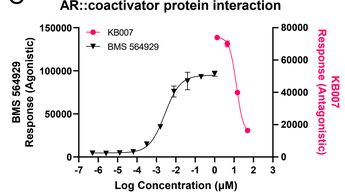
A KB007 chemical structure



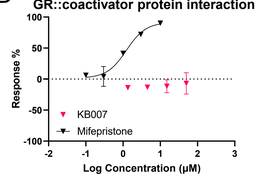
B KB007 modulates AR nuclear translocation



C KB007 treatment disrupts AR::coactivator protein interaction

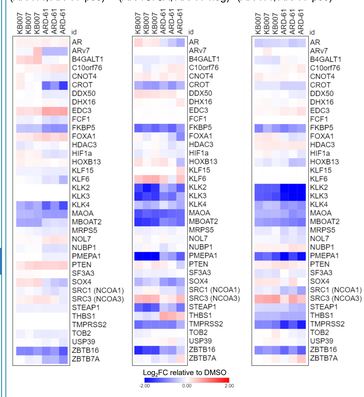


D KB007 treatment does not disrupt GR::coactivator protein interaction



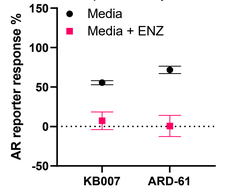
KB007 is a bona fide AR antagonist. (A) Chemical structure of KB007. (B) KB007 inhibited AR nuclear translocation stimulated by DHT. The IC_{50} values for KB007 and enzalutamide were 5.9 μM and 0.24 μM, respectively. (C) KB007 blocked the protein-protein interaction between AR and a steroid receptor co-activator (SRC) peptide containing LXXLL motifs. Cells were pretreated with KB007 at increasing concentrations followed by AR stimulation with BMS 564929. The IC_{50} value for KB007 was 13.3 μM. (D) KB007 does not antagonize glucocorticoid receptor (GR), hinting at target selectivity. GR is closely related to AR in the nuclear hormone receptor superfamily.

A AR TRN signature across various prostate cancer cell models (NanoString)

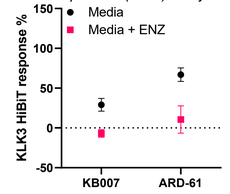


KB007 AR TRN response is antagonized by enzalutamide

B *AR response element reporter assay*

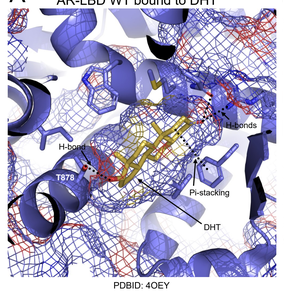


C *AR target gene KLK3 expression (HiBiT) assay*

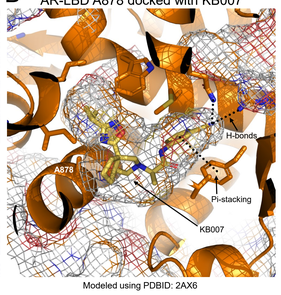


KB007 antagonizes AR activity across multiple prostate cancer cell models regardless of AR-V7 expression status. (A) NanoString expression profile of AR target genes in 22RV1, LNCAP and VCAP cells. Prostate cancer cells were treated with 20 μM compound for 24 hours. (B) AR response reporter revealed KB007 inhibition competes with enzalutamide (ENZ) inhibition on modulating AR activity. 22RV1 cells were pretreated with 5 μM enzalutamide before incubating cells with 20 μM KB007 for 24 hours. (C) KLK3 HiBiT response with KB007 was blocked by enzalutamide (ENZ). LNCAP cells were engineered to express N-terminal HiBiT tagged KLK3, an AR target gene. LNCAP cells were pretreated with 3 μM enzalutamide before incubating cells with 20 μM KB007 for 24 hours.

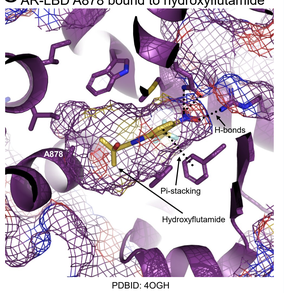
A AR-LBD WT bound to DHT



B AR-LBD A878 docked with KB007



C AR-LBD A878 bound to hydroxyflutamide



Docking analysis of KB007 with AR-LBD revealed a converging binding mode similar to AR agonists and antagonists alike. (A) Crystal structure of DHT bound to AR-LBD (B) Docking model of KB007 (single stereoisomer selected for best docking fit) bound to AR-LBD A878 mutant. (C) Crystal structure of hydroxyflutamide bound to AR-LBD A878 mutant.

Conclusions

- Leveraged SMM technology developed at Kronos Bio to identify AR binders from a prostate cancer cell nuclear lysate
  - Successfully implemented a targeted hit triage strategy using both cell and molecular pharmacological assay approaches
- Validated KB007 as a bona fide AR antagonist that was responsive across multiple prostate cancer cell models and regardless of AR-V7 expression status
- Docking analysis of KB007 with AR-LBD crystal structures revealed a converging binding mode similar to agonists and antagonists alike
  - KB007 is hypothesized to antagonize AR by competitive inhibition against AR agonists and maintain AR-LBD conformation in an open, inactive state

Acknowledgements

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- Eurofins Panlabs Discovery Services ran the AR translocation and GR co-activator assays with KB007; Eurofins DiscoverX Corporation ran the PathHunter® AR protein interaction assay with KB007
- Select figures were generated using BioRender.com, Adobe Illustrator, GraphPad, ChemDraw and PyMOL

AR = Androgen receptor; SMM = Small molecule microarray; TRN = Transcription regulatory network; AR-FL = AR full length; AR-V7 = AR variant 7; LBD = Ligand binding domain; DHT = 5α-dihydrotestosterone; WT = Wild type; PPI = Protein-protein interaction; SNR = Signal-to-noise ratio; RZS = Robust Z-score; ENZ = Enzalutamide

References

1. Xin Han *et al.*, Journal of Medicinal Chemistry, 2019, 62 (2), 941-964.