Modulating Androgen Receptor-Driven Transcription in Prostate Cancer with Selective CDK9 Inhibitors

Highlights

- KI-ARv-03 reduces AR protein levels and AR-driven transcription
- KI-ARv-03 is deduced to be a potent, ultraselective inhibitor of CDK9
- Optimization led to the orally bioavailable and selective CDK9 inhibitor KB-0742
- KB-0742 displays potent anti-tumor activity in cancer models in vitro and in vivo

Graphical Abstract

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In Brief

In the pursuit of hormone receptor modulators in prostate cancer, a potent, ultraselective CDK9 inhibitor is discovered. This study describes the most selective inhibitors of CDK9 known to date and provides compelling preclinical in vitro and in vivo support for CDK9 as a therapeutic target.
Castration-resistant prostate cancers (CRPCs) lose sensitivity to androgen-deprivation therapies but frequently remain dependent on oncogenic transcription driven by the androgen receptor (AR) and its splice variants. To discover modulators of AR-variant activity, we used a lysate-based small-molecule microarray assay and identified KI-ARv-03 as an AR-variant complex binder that reduces AR-driven transcription and proliferation in prostate cancer cells. We deduced KI-ARv-03 to be a potent, selective inhibitor of CDK9, an important cofactor for AR, MYC, and other oncogenic transcription factors. Further optimization resulted in KB-0742, an orally bioavailable, selective CDK9 inhibitor with potent anti-tumor activity in CRPC models. In 22Rv1 cells, KB-0742 rapidly downregulates nascent transcription, preferentially depleting short half-life transcripts and AR-driven oncogenic programs. In vivo, oral administration of KB-0742 significantly reduced tumor growth in CRPC, supporting CDK9 inhibition as a promising therapeutic strategy to target AR dependence in CRPC.
of acquired ADT resistance. These AR-Vs produce constitutively active forms of AR against which there are no known inhibitory small molecules (Hörnberg et al., 2011). The most frequent AR-V, AR-V7, is highly expressed in patients that experience resistance to ADTs (Antonarakis et al., 2014; Sharp et al., 2019) and has been shown to heterodimerize with AR to repress growth suppressor genes in CRPC (Cato et al., 2019). Importantly, these tumors are dependent on both AR-V7 and full-length AR (AR-FL), and still express oncogenic AR-driven gene expression programs. Therefore, agents that inhibit the function of AR and its splice variants independent of the LBD or indirectly via modulating shared interactome members may be efficacious for CRPC patients.

In the absence of their LBDs, AR-Vs are largely unstructured and considered classically undruggable (McEwan, 2012). Thus, innovative approaches are required to identify small molecules that can bind to and modulate the activity of AR-V complexes in cells. Small-molecule microarrays (SMMs) are a robust and scalable screening platform for protein–small-molecule binders in which small molecules are displayed on a glass slide and protein binders are fluorescently detected (Bradner et al., 2006; Vegas et al., 2008). Previously, this approach has led to the identification of small-molecule binders across a broad range of target classes, including transcription factors such as ETV1 and MAX (Pop et al., 2014a; Struntz et al., 2019), epigenetic regulators like histone deacetylases (Boskovic et al., 2016), secreted proteins like Sonic hedgehog (Stanton et al., 2009), and DNA/RNA targets such as G-quadruplexes (Felsenstein et al., 2016).

Results

**Discovery of KI-ARv-03 as a Modulator of AR Transcriptional Output**

We used SMMs to screen whole-cell lysates containing an N-terminal hemagglutinin (HA)-tagged AR-ΔLBD truncate (AR-V) overexpressed in HEK293T cells against a set of 50,000 immobilized compounds (Figure 1A). This library included fragments, lead-like molecules, and precompetitive drug-like structures as well as an overrepresentation of rapamycin, an FKBP12 binder that is unrelated to AR and AR signaling, to represent a null distribution (Figures S1A and S1B). The screen yielded 1,316 putative AR-V and interactome binders with an average robust Z score of ≥1.96 across four replicates (Figure 1B).

Using a medium-throughput qRT-PCR assay, we evaluated the ability of these hits to reduce prostate-specific antigen (PSA; encoded by KLK3) transcript levels in the AR-dependent LNCaP prostate cancer cell line (Figures 1S and S1C). This

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**Figure 1. Discovery of KI-ARv-03 as Modulator of AR Transcriptional Output**

(A) SMM assay principle. Small-molecule compounds are immobilized on a glass surface and a subset is capable of binding the desired target either directly or indirectly via an essential interaction partner.

(B) Scatterplot of high-throughput SMM screening data of 50,000 compounds against an N-terminal HA-tagged AR-ΔLBD truncate. assay positives, blue; validated compounds, orange.

(C) Critical path to hit determination leads to three validated probe candidates after multiple rounds of secondary screening.

(D) Chemical structure of KI-ARv-03. Intrinsic nucleophiles for SMM surface immobilization are highlighted in gray.

See also Figures S1 and S2.
Figure 2. KI-ARv-03 Engages and Selectively Inhibits CDK9 in Biochemical and Cellular Assays

(A) Phylogenetic tree representation of molecular kinase targets of KI-ARv-03 profiled against 413 kinases (Eurofins KinaseProfiler). Circles indicate >50% inhibition upon treatment with 10 μM KI-ARv-03.

(B) Dose-response curves for biochemical inhibition of a focused CDK panel (Eurofins KinaseProfiler, ATP at Km) after KI-ARv-03 treatment (n = 2 technical replicates, error bars represent mean ± SD).

(C) Michaelis-Menten and Lineweaver-Burk plots of the kinetic study of CDK9 treatment with KI-ARv-03 in the presence of various ATP concentrations (HotSpot Kinase Assay, Reaction Biology Corp.).

(D) Melt curve of CDK9 protein levels in the soluble fraction in intact 22Rv1 cells exposed to different temperatures (25°C–61°C) prior to lysis visualized by immunoblotting (top). CETSA CDK9 isothermal fingerprint of the soluble fraction of intact 22Rv1 cells following 1 h KI-ARv-03 dosing treatment (0–40 μM) at 49°C (bottom).

(legend continued on next page)
marker was chosen as a proxy for AR activity because its transcription is primarily regulated by AR through androgen-responsive elements present in the promoter region of the KLK3 gene body. PSA is also utilized in prostate cancer patients as a blood-based biomarker for monitoring disease progression (Gelmini et al., 2003b). LNCaP cells were starved of androgens in charcoal-stripped serum medium (low androgen levels) and AR activation and PSA expression were subsequently induced with synthetic androgen R1881 (Bonne and Raynaud, 1975; Gelmini et al., 2003a). Eighty-five small molecules at 10 μM decreased PSA levels by 20% or more after 24 h treatment compared with the R1881-induced condition (Figure S1C).

We evaluated these molecules in a mouse mammary tumor virus (MMTV)-driven stable luciferase reporter gene assay in LNCaP cells with a doxycycline-inducible element for exogenous expression of AR-V. Androgen-starved cells (suppressed AR-FI activity) were treated with R1881 and concomitantly induced for AR-V expression using doxycycline and cotreated with DMSO or our putative binders for 24 h. We prioritized 32 compounds that both attenuated R1881-induced PSA expression in LNCaP by >20% and inhibited the AR-V-driven luciferase signal in the reporter cell line by at least 40% (Figure S1C). These were then subjected to 10-point dose evaluation using qPCR for PSA and luciferase reporter, yielding nine compounds that exhibited dose-dependent reduction of PSA transcripts and AR-responsive promoter occupancy (Figure S1D). We then assessed favorable chemotype characteristics such as drug likeliness, cLogP, and synthetic accessibility for compounds that scored with area under the curve (AUC) values of <0.80 in both assays and prioritized seven compounds for further study. Three probe candidates were selected based on their single-digit micromolar half-maximal inhibitory concentration (IC50) values in both the MMTV-driven reporter gene assay and the viability assay for AR-positive cell lines (Figures S1D and S2A).

KI-ARv-03 was selected based on reasonable physicochemical properties and its single-digit micromolar inhibitory activity in both the reporter assay (IC50 = 7.6 μM) and the viability assays for AR-dependent over AR-independent prostate cancer cell lines (VCaP and LNCaP versus DU145 and PC3, Figures 1D, S1D, and S2A). In VCaP-16, an enzalutamide-resistant prostate cancer cell line expressing increased levels of AR-V7, treatment with 5 μM KI-ARv-03 dramatically decreased KLK3 transcript levels (Figure S2B). The decrease in KLK3 expression coincided with decreases in AR-V7 transcript and protein, while AR-FI transcript and protein levels remained unchanged (Figures S2C and S2D). Similar decreases in AR-V7 protein levels were observed in the castration-resistant cell line 22Rv1 in response to KI-ARv-03 treatment (Figure S2D). Unlike VCaP-16, the decreases in AR-V7 protein levels in 22Rv1 did not coincide with decreases in KLK3 transcript, which could be due to the higher level of AR-V7 protein expression seen in 22Rv1 compared with VCaP-16. In summary, we identify KI-ARv-03 as a putative interactor with the AR-V7 complex in lysates that has attractive drug-like properties and the ability to potentially downregulate AR and AR-V7-dependent transcription in CRPC models.

**Kinase Profiling and Cellular Target Engagement Identify CDK9 as the Molecular Target of KI-ARv-03**

We next sought to determine the molecular target of our lead candidate KI-ARv-03 using in vitro and cell-based techniques. Structurally, KI-ARv-03 features a pyrazolopyrimidine core with multiple potential H-bond-accepting groups that is substituted with molecular entities incorporating H-bond-donating amino groups. The overarching composition incorporates molecular features that are typically found in ATP-competitive kinase inhibitors (Traxler et al., 1997; Traxler and Furet, 1999). Therefore, we performed in vitro kinase selectivity profiling against a panel of 413 kinases (Table S1) using 10 μM KI-ARv-03 and ATP concentrations at or within 15 μM of the apparent K_in of each kinase to determine if kinase inhibition might give rise to the observed AR-related cellular effects.

Surprisingly, we observed selective reduction of enzymatic activity of the transcriptional CDKs CDK9 and CDK7 to 7% and 47% compared with DMSO treatment (Figure 2A). Given the high structural conservation within the kinase and particularly the CMGC family of kinases, we evaluated KI-ARv-03 against 13 CDKs at 10-point dose to assess selectivity within the CDK family of kinases (Figure 2B). The compound selectively inhibited CDK9 (IC50 = 0.15 μM at 45 μM ATP) over all other tested CDKs with a minimum of 130-fold selectivity. Kinetic studies and Michaelis-Menten analysis for CDK9 inhibition by KI-ARv-03 in the presence of varying ATP concentrations illustrate that the apparent K_m is increased when inhibitor concentration is increased (Figures 2C and S3A). Lineweaver-Burk plotting illustrates increased slopes for the linear fit with increasing concentration of KI-ARv-03 and all linear regressions converging on the y axis (Figure 2C). Overall these data suggest CDK9 as the molecular target of KI-ARv-03 and confirm an ATP competitive mode of CDK9/cyclin T1 inhibition.

Next, we investigated the ability of KI-ARv-03 to directly engage CDK9 in live cells by employing a cellular thermal shift assay (CETSA) in AR-V7-positive 22Rv1 prostate cancer cells (Martinez Molina et al., 2013). A small molecule that binds a cellular target protein generally leads to a protein-ligand complex with increased heat stability over the unbound protein, which increases the soluble amount of target protein following heat shock. Protein populations of CDK9, AR-FL, and splice variants in 22Rv1 cells become undetectable in the soluble fraction by immunoblotting after heat shock at temperatures between 46°C and 51°C (Figures 2D and S3B). Isothermal dose-response fingerprints were generated in subsequent CETSA studies at 49°C using increasing concentrations of KI-ARv-03 (0–40 μM, 1 h treatment). At 10 μM compound concentration we observed significant stabilization of CDK9 as illustrated by increased protein levels compared with DMSO treatment. We did not observe any significant stabilization of AR-FL or AR-V7 at any applied
dose (Figure S3B), supporting our hypothesis that KI-ARv-03 does not directly engage AR species but rather affects AR levels through impaired CDK9 enzymatic activity.

To evaluate whether KI-ARv-03 engages CDK9 only, CDK9 and AR independently, or any complex of these, we utilized a bead-based approach for target engagement from 22Rv1 cell lysates (and others, Figures 2E, 2F, and S3C–S3F). We used NHS-activated agarose beads to attach KI-ARv-03 (Figures 2E and S3F) to a solid support through its terminal primary amine in the same way that our SMM surface chemistry captured and displayed the compound in the lysate-based screening (Figure S3C). We used high-salt RIPA buffer for 22Rv1 cell lysis to break up any protein-protein and protein-DNA/RNA interactions and engage single targets only. We observed robust and specific enrichment of the most abundant isoform of CDK9 (43 kDa) from 22Rv1 cell lysate by KI-ARv-03 compared with ethanalamine-blocked control beads (Figure 2F). CDK9 engagement was reversible as demonstrated by soluble competition experiments using 40 μM free KI-ARv-03. There was no engagement of AR-FL or its truncate versions. Using mild MIPP lysis buffer, which maintains protein-protein and protein-DNA/RNA interactions in lysates (Pop et al., 2014b), we observed engagement of both isoforms of CDK9 compared with blocked beads (Figure S3D). This interaction was reversible upon competition with free KI-ARv-03 as well as structurally unrelated ATP-competitive CDK9 reference inhibitors NVP-2 (Barsanti et al., 2011), BAY-1143572 (atuveciclib) (Lucking et al., 2017), and MC180295 (Zhang et al., 2018) (Figure S3E, Table S2).

In addition, we observed engagement of both AR-FL and truncate versions such as AR-V7. These interactions were irreversible, as neither free KI-ARv-03 nor any of the structurally unrelated CDK9 inhibitors was able to attenuate binding of engaged AR protein. Furthermore, coimmunoprecipitation experiments on AR-FL and AR-V7 from 22Rv1 cells under mild lysis conditions did not demonstrate a stable protein-protein interaction with CDK9 in our hands in the presence or absence of KI-ARv-03 (Figure S3G). Therefore, we speculate that AR and CDK9 do interact transiently and directly in the regulation of Ser81 in the N terminus of AR (Koryakina et al., 2014). The enrichment for AR-FL and AR-V7 observed with KI-ARv-03 beads is most likely CDK9 independent, either through transient interactions between immobilized KI-ARv-03 and the N terminus of AR or between immobilized KI-ARv-03 and AR-containing complexes.

CDK9 Regulates AR Stability and Is Required for Downstream AR Target Gene Expression

Among known AR interactome members of potential therapeutic advantage are regulatory kinases. CDK9, for instance, is a serine/threonine kinase from the CDK family, of which multiple members are involved in the regulation of either cell-cycle progression or gene transcription (Lim and Kaldys, 2013; Malumbres, 2014). CDK9 extends the AR half-life and activity through its N-terminal phosphorylation (Ser81). Unphosphorylated AR is exported from the nucleus, where it is ubiquitinated and degraded by the proteasome (Koryakina et al., 2014). Inhibition of CDK9 therefore may offer an indirect route for AR modulation in CRPC. KI-ARv-03 treatment (5 μM) leads to reduction of both AR-FL and AR-V7 phosphorylation at Ser81 after 4 h, concurrent with loss of protein levels for both AR species starting at 6 h of treatment (Figure S4C). AR protein and phosphorylation levels are rescued upon cotreatment with the proteasome inhibitor MG132 (Figure S4D).

CDK9 and cyclin T1 form the positive transcription elongation factor b (P-TEFb), which facilitates phosphorylation of Ser2 of the C-terminal domain of RNA polymerase II (RNA Pol II), ultimately initiating transcriptional elongation (Hirose and Ohkuma, 2007). P-TEFb inhibition enables selective downregulation of oncogenes by decreasing RNA Pol II Ser2 phosphorylation, resulting in universal RNA Pol II stalling that disproportionately downregulates sites of heavy transcriptional recruitment and transcriptional cooperativity at superenhancers within transcriptional condensates or insulated environments (Bradner et al., 2017; Cho et al., 2018; Guo et al., 2019; Jonkers and Lis, 2015; Lovén et al., 2013; Weintraub et al., 2017). Consequently, transcriptional CDK inhibitors, including those that preferentially inhibit CDK9, have shown strong potential as therapeutic agents owing to their ability to selectively downregulate oncogenic transcription programs and target tumors addicted to transcription factors such as AR or MYC (Huang et al., 2014). We demonstrate that treatment of 22Rv1 cells with KI-ARv-03 (5 μM) leads to significant reduction of RNA Pol II phosphorylation after 6 and 24 h (Figures S4C and S4D). Translational inhibition with cycloheximide (20 μg/mL) depletes AR levels, similar to CDK9 inhibition. Proteasome inhibition with MG132 (10 μM) rescues AR protein levels, but cotreatment with KI-ARv-03 does not decrease AR pS81 levels. Together, these data suggest downregulation of AR transcription as the key mechanism for KI-ARv-03-mediated effects on AR protein levels.

Transcriptional CDK inhibitors have been explored clinically but have had limited success. It is unclear whether these limitations are due to small therapeutic index, lack of selectivity, or unfavorable pharmacology. Notably, most clinical transcriptional CDK inhibitors have hit multiple CDKs. CDK9 has been shown to be a promising and druggable target in oncology for attenuating oncogenic transcription for a variety of indications (Blake et al., 2019; Brisard et al., 2018; Franco et al., 2018; Hashiguchi et al., 2019; Mitra et al., 2016; Morales and Giordano, 2016; Wang et al., 2019). However, as CDK9 also plays a global role in transcription, a sufficient therapeutic index for clinical benefit has not yet been demonstrated. KI-ARv-03 treatment (5 μM) leads to robust and sustained impact on AR transcript levels in 22Rv1 cells compared with treatments with the structurally unrelated CDK9 inhibitor NVP-2 or a separate RNA Pol II inhibition mechanism using THZ1, a covalent inhibitor of CDKs 7, 12, and 13 that elicits transcriptional defects by inhibition of superenhancer-associated gene expression (Kwiatkowski et al., 2014; Olson et al., 2019; Zhang et al., 2016) (Figure S5A).

Prior clinical investigation of transcriptional CDK inhibitors has been confounded by off-target interactions with other kinases, especially other CDKs that play important roles in transcription and the cell cycle. Due to the high structural conservation of the ATP-binding cleft in kinases it is challenging to accomplish selective inhibition for single CDKs across the family of CDKs as well as across the kinome. We demonstrated that KI-ARv-03 is a selective CDK9 inhibitor that ultimately modulates AR and AR-V7-dependent transcription in vitro.
Structure-Based Considerations and Medicinal Chemistry Optimization Yield the Potent and Selective CDK9 Inhibitor KB-0742

Given the remarkable up-front selectivity of KI-ARv-03, we aimed to optimize its potency and simultaneously retain its selectivity for CDK9. To better understand structure-activity relationships and molecular recognition, we undertook crystallization efforts using recombinant CDK9/cyclin T1 and KI-ARv-03. Due to the low resolution of the resulting hexagonal crystals, we were not able to generate a high-quality complex structure. Therefore, we utilized molecular modeling to assess the binding mode of KI-ARv-03 in complex with CDK9/cyclin T1 (Figures 3D and E).

Figure 3. KB-0742 Is a Potent and Selective CDK9 Inhibitor
(A) Chemical structures of parent compound KI-ARv-03 and optimized molecule KB-0742.
(B) Dose-response curves for biochemical CDK9/cyclin T1 inhibition (at 10 μM ATP concentration; HotSpot Kinase Assay, Reaction Biology Corp.) after treatment with KI-ARv-03 and KB-0742 (n = 2 technical replicates, error bars represent mean ± SD).
(C) Heatmap representation of biochemical inhibition of CDK family members by KI-ARv-03 and KB-0742 (HotSpot Kinase Assay).
(D and E) Molecular docking of (D) KI-ARv-03 (green sticks) and (E) KB-0742 (green sticks) into the ATP-binding cleft of CDK9 (PDB: 3MY1). Hinge, teal; DFG motif, blue; helix αC, lime; hydrogen bonds, yellow spheres.
(F) Molecular interaction map of KI-ARv-03 (left) and KB-0742 (right) with key amino acids for ligand binding within the binding cleft of CDK9.
and 3F). Our modeling suggests that the compound binds to the catalytic cleft of CDK9 in a type I fashion, establishing H-bond interactions through the terminal primary amine with the hinge backbone amides of His108 and Glu107, as well as an ionic interaction with the side chain of Asp109. The R,R configuration of the cyclopentane-1,3-diamine prevents the establishment of additional H bonds through the pyrazolopyrimidine core of KI-ARv-03. Consecutive compound optimization efforts resulted in the identification of the more potent CDK9 inhibitor KB-0742 (CDK9/cyclin T1 inhibition IC_{50} = 6 nM at 10 μM ATP, Figures 3A and 3B).

KB-0742 incorporates beneficial features to increase potency that include: (1) a branched 3-pentane extension of the propane alky chain that increases Van der Waals interactions with the glycin-rich loop and (2) inverted stereochernistry of the cyclopentane-1,3-diamine, which preserves the terminal primary amine interactions with Asp109 and Glu107 while also enabling two H-bond interactions between the backbone amide of Cys106 (NH and CO) and the bridging secondary amine and pyrazolopyrimidine core of KB-0742 (Figures 3E and 3F). The compound was subjected to kinase selectivity profiling and found to be selective for CDK9/cyclin T1 with >50-fold selectivity over all CDKs profiled and >100-fold selectivity against cell-cycle CDKs (CDK1-6) in the panel (Figure 3C).

**KI-ARv-03 and KB-0742 Show Cytostatic Effects in Prostate Cancer and Leukemia Cell Lines**

Next, we performed growth rate inhibition (GR) studies with our CDK9 inhibitors in 22Rv1 prostate cancer cells over time and at 72 h of treatment compared with the known CDK9 inhibitors NVP-2, BAY-1143572, and AZD4573 (Cidado et al., 2020) (Figures 4A, S4A, and S4B). These studies reflect the 10-fold in vitro improvement of CDK9 inhibitory potency of KI-ARv-03 versus KB-0742, which translated into an 18-fold improvement of antiproliferative activity in 22Rv1 cells (GR_{50} = 3.26 μM versus 0.18 μM). Monitoring induction of apoptosis by caspase-3/7 activation, we observed a similar trend with KI-ARv-03 only moderately increasing apoptotic cell counts at high doses (10 and 3 μM), while no apoptotic cells were produced at submicromolar doses (Figure 4B). KB-0742 follows the same trend, with stronger induction of apoptosis at higher doses and additionally little induction at doses as low as 0.3 μM. Analogous behavior was observed for BAY-1143572, while NVP-2 and AZD4573 drove 22Rv1 cells into massive events of apoptosis even at double-digit nanomolar concentrations, with NVP-2 generally inducing apoptotic cell counts at high doses (10 and 4 μM) and KB-0742 diminishing phosphorylation at Ser5 and only at the highest applied dose (2.5 μM). Total RNA Pol II and CDK9 protein levels were not affected by treatment with any inhibitor utilized in this study. To assess our CDK9 inhibitors with respect to their effects on transcription factors regulated by CDK9 activity, we performed a time-course study (Figure 4F) with fixed concentrations of KI-ARv-03 (5 μM) and KB-0742 (1 μM). Global AR-FL and AR-V protein levels are significantly reduced starting at 6 h treatment time, which is accompanied by the reduction of phospho-AR levels (Ser81). Complete depletion of AR levels is obtained after 16 h of treatment for both compounds. This result is in line with previous findings demonstrating that lack of AR phosphorylation at Ser81 entails nuclear export of AR, its subsequent ubiquitination, and proteasomal degradation (Koryakina et al., 2014). From our observations, however, this AR effect appears to be proteasome independent and is also observed with CDK9 inhibitors of unrelated structural scaffolds (e.g., NVP-2, Figures S4C and S4D), suggesting that KI-ARv-03 and KB-0742 achieve this effect primarily through CDK9-mediated transcriptional regulation of the AR locus versus CDK9-mediated regulation of the AR gene product.

**KI-ARv-03 and KB-0742 Block Nascent Transcription and Downregulate Oncogenic Prostate Cancer Gene Expression Programs**

CDK9 inhibition or degradation has been shown to globally downregulate nascent transcription (Olson et al., 2018; Winter et al., 2017). To place inhibition of AR-driven transcriptional programs in the overall context of global transcriptional response, we performed nascent transcriptional profiling using SLAM-seq (thio(SH)-linked alkylation for the metabolic sequencing of RNA) (Muhar et al., 2018). SLAM-seq measures the incorporation of a metabolic uracil analog that is chemically converted and detected as a mutation upon sequencing. As a metabolic labeling assay, it enables a more direct measurement of nascent transcription than traditional RNA sequencing, which samples cellular mRNA composition. We performed SLAM-seq at concentrations (KI-ARv-03 4 μM and KB-0742 1.2 μM) sufficient to reduce RNA Pol II Ser2 phosphorylation and induce growth arrest and sampled cells at early time points (2, 4, and 8 h) to capture more immediate and direct changes in gene expression that occur prior to gross changes in cellular phenotype.

Across the 8 h time course, we observe a steady increase in nascent mRNA metabolic labeling under DMSO conditions that is blunted by either KI-ARv-03 or KB-0742 treatment (Figures 5A and S5B). By 8 h, 95% of high-confidence actively transcribed genes show downregulated nascent transcription in treatments versus DMSO, consistent with a global role for CDK9. Based on nascent transcription levels, samples tend to cluster by time point and then by treatment, with little difference between KI-ARv-03 and KB-0742 (Figure S5C). The strongest divergence between DMSO and CDK9 inhibition is observed at 8 h. Across genes, changes in nascent transcription correlate
Figure 4. KI-ARv-03 and KB-0742 Induce Apoptosis in Prostate Cancer and Leukemia Cell Lines

(A and C) Dose-response curves and growth rate inhibition of (A) 22Rv1 prostate cancer cells and (C) MV-4-11 AML cells after treatment with KI-ARv-03, KB-0742, and reference CDK9 inhibitors AZD4573, BAY-1143572, and NVP-2 (0–40 μM, n = 3 technical replicates).

(B and D) Induction of apoptosis in (B) 22Rv1 and (D) MV-4-11 cells monitored as a function of caspase 3/7 activation after treatment with KI-ARv-03, KB-0742, and reference CDK9 inhibitors (0–10 μM, n = 3 technical replicates).

(E) Immunoblot of dose-response study of CDK9 and (phospho)-RNA Pol II levels in 22Rv1 after 6 h treatment with KI-ARv-03, KB-0742 and reference CDK9 inhibitors.

(F) Immunoblot of time course of CDK9 downstream target and interactome protein levels in 22Rv1 in response to treatment with KI-ARv-03 (5 μM) and KB-0742 (1 μM).

See also Figures S3 and S4.
well between KI-ARv-03 and KB-0742 (Figure S5D). These data suggest that selective CDK9 inhibition blunts nascent RNA Pol II transcription and that at respective dose/time points, KI-ARv-03 and KB-0742 elicit similar transcriptional responses.

Differential analysis identifies a subset of genes in which CDK9 inhibition has a more pronounced impact (Figures 5B and 5C). Comparing highly with lowly transcribed genes, we do not observe differential effects on mRNA synthesis rates (Figures 5B and 5C). However, changes in mature mRNA levels for the top transcribed differential genes versus other differential genes demonstrate distinct selective effects on mature mRNA levels (p = 0.0007 and p = 0.0001) for KB-0742 and KI-ARv-03, respectively (Figure S5E), suggesting that these genes are more influenced by CDK9 inhibition. Although we observe downregulation

See also Figure S5.
SOX4 are highly transcribed transcription factors, including markers of AR and PSA locus genes (KLK family genes), we also note that these genes are not highly transcribed under control (DMSO) conditions. Instead we find significant downregulation of several highly transcribed transcription factors, including KLF6, FOXA1, SOX4, MAFG, and ZNF3. SOX4 and FOXA1 serve as lineage-defining transcription factors in prostate cancer (Adams et al., 2019; Parolia et al., 2019; Scharer et al., 2009). MAFG is associated with chemotherapy resistance in other cancers (Vera-Puente et al., 2016). Interestingly, KLF6 is a tumor suppressor that is recurrently mutated in prostate cancer (at least 25% of all tumors) (Liu et al., 2012). These data are consistent with a preferential effector role of CDK9 inhibition on highly transcribed genes, which often include master transcription factors associated with tumor or lineage identity.

Inspection of key prostate cancer loci, including PSA (KLK3) and FOXA1, shows regions of hyperacetylated and accessible chromatin (H3K27ac chromatin immunoprecipitation sequencing [chip-seq] and assay for transposase-accessible chromatin sequencing [ATAC-seq]) bound by both FOXA1 and AR-V7 (Figures 5D and 5E). Interestingly, across the 8 h time course, lineage transcription factors such as FOXA1 show more immediate-early downregulation that is followed by later downregulation of AR and PSA genes (KLK3 and KLK4) (Figure 5C). From these data we conclude that CDK9 inhibition suppresses nascent RNA Pol II transcription and collapses AR-driven transcription programs and their upstream master regulators (e.g., FOXA1).

**KB-0742 Exhibits Efficacy in Prostate Cancer and AML-Derived Xenograft Models**

To assess the therapeutic potential of KB-0742 we performed in vivo efficacy studies using subcutaneous 22Rv1 human prostate cancer cell line-derived xenograft (CDX) models in male CB17-SCID mice (Figures 6A and 6B, Tables S3 and S4). Subsequent to tumor establishment, mice (n = 10) were treated with vehicle (by mouth [p.o.], once a day [QD]), standard docetaxel chemotherapy (intraperitoneally, 15 mg/kg, once a week), or escalating doses of KB-0742 (p.o., 3, 10, and 30 mg/kg, QD) over 21 days. KB-0742 does not lead to any tumor or body weight reduction at 3 and 10 mg/kg daily dosing compared with vehicle treatment. At 30 mg/kg, we observe stagnating tumor burden reflected as 82% (p = 0.000003) tumor growth inhibition (TGI), which is superior to standard docetaxel treatment (TGI = 70%, p = 0.000002). We observe body weight loss in the first week of treatment with KB-0742, which then stabilizes at 15% overall body weight loss over the course of treatment. Body weight loss was minimized to 7.5% by applying high-dose KB-0742 on a scheduled treatment regimen (30 mg/kg, 3 days on/4 days off) but tumor growth reduction was less pronounced here (TGI = 58%, p < 0.00005). These studies demonstrate that KB-0742 is well tolerated even at high dose, while significantly reducing tumor burden in 22Rv1-driven CDX models.

In addition, we evaluated KB-0742 in a subcutaneous MV-4-11-driven human AML CDX model with adjusted treatment regimen (Figures 6C and 6D, Tables S3 and S6). KB-0742 treatment was well tolerated across all groups (n = 10) with applied treatment strategies spanning vehicle (p.o., QD), KB-0742 (p.o., 3, 10, and 30 mg/kg, QD) as well as 3 days on/4 days off QD treatments using escalating doses of KB-0742 (p.o., 15, 30, and 60 mg/kg). We did not observe any significant body weight changes over the course of the 20 day study (Figure 6D). Daily treatment with 25 mg/kg KB-0742 leads to substantial reduction in tumor volume, which is reflected in 74% TGI (p < 0.00002). Solely high-dose cyclic dosing of KB-0742 (60 mg/kg, 3 days on/4 days off QD) led to more substantial tumor volume reduction (TGI = 81%, p < 0.00001). Cyclic treatment at half (30 mg/kg) and quarter (15 mg/kg) KB-0742 doses resulted in 40% and 24% reduction of tumor burden, respectively. In conclusion, our xenograft studies demonstrate that KB-0742 treatment is well tolerated in vivo at doses of at least 25–30 mg/kg daily, at which significant reduction of tumor burden across 22Rv1 and MV-4-11 CDX models was accomplished.
DISCUSSION

Here we sought to identify small-molecule modulators of AR-V activity as a means to overcome resistance to ADT in CRPC. We took advantage of the unbiased and context-based SMM binding platform to identify KI-ARv-03, an inhibitor of the AR-V cofactor CDK9. KI-ARv-03 inhibits CDK9 in cells and leads to downregulation of AR-driven transcription and AR protein phosphorylation and abundance. Interestingly, as a low-molecular-weight screening hit, KI-ARv-03 displayed remarkable up-front selectivity versus other kinases and in particular other CDKs. In our experience, hits from in vitro kinase screens often require significant medicinal chemistry optimization and structural elaboration to achieve selectivity. Optimization of KI-ARv-03 into KB-0742 increased shape complementarity with the CDK9 ATP-binding pocket without significant increase in molecular weight or at the expense of selectivity, resulting in an ultraselective and single-digit nanomolar potent CDK9 inhibitor. We suspect that the up-front selectivity observed for KI-ARv-03 may be attributed to the SMM platform, which screened for binding in a more native lysate environment that maintained context-specific structure of the AR-V/CDK9 complex. These findings suggest that the SMM may be particularly advantageous for targets residing in large complexes that are difficult to reconstitute in vitro.

CDK9 is a core component of the P-TEFb transcription elongation complex and is required for all active transcription (Olson et al., 2018; Rahl et al., 2010; Winter et al., 2017). We and others have previously shown that targeting core members of the transcriptional complex can have surprisingly selective effects on gene expression, especially on highly transcribed short-half-life genes (Erb et al., 2017; Jaeger et al., 2020; Kwiatkowski et al., 2014; Lovén et al., 2013; Sharifnia et al., 2019; Winter et al., 2017). In 22Rv1 cells, treatment with either KI-ARv-03 or KB-0742 results in global downregulation of nascent transcription and AR-driven prostate cancer gene expression programs and displays in vivo efficacy in CRPC- and AML-derived xenograft models. Therefore, our selective and orally bioavailable CDK9 inhibitor KB-0742 is a promising candidate for further clinical development in CRPC and other transcriptionally addicted cancers.

SIGNIFICANCE

Castration-resistant prostate cancers (CRPCs) are insensitive to classical androgen-deprivation therapies but commonly dependent on androgen receptor (AR)-driven oncogenic transcriptional programs. One eminent resistance mechanism is the expression of constitutively active AR splice variants (AR-Vs) lacking the ligand-binding domain typically addressed by anti-androgen inhibitors. AR-Vs are mostly unstructured in solution and do not feature druggable binding pockets; therefore, targeting druggable AR interactome members constitutes a suitable and promising path to abrogate AR activity. We have successfully applied our unbiased small-molecule microarray platform in a context-specific approach to identify direct modulators of an N-terminal truncate of AR and its interactome members in lysate-based screenings. We identified KI-ARv-03 and developed KB-0742, which both modulate AR transcriptional programs by reducing AR and AR-V protein levels through selective inhibition of its cofactor CDK9, an essential member of the transcriptional elongation machinery. Moreover, CDK9 facilitates the N-terminal phosphorylation of AR and thereby regulates its half-life as well as downstream AR target gene expression, which nominations CDK9 as a suitable and druggable therapeutic target in CRPC. Many of the clinically explored transcriptional CDK inhibitors target multiple CDKs and have had limited success. KB-0742 is a remarkably selective CDK9 inhibitor that leads to global downregulation of nascent transcription and AR-driven prostate cancer gene expression programs and displays in vivo efficacy in CRPC- and AML-derived xenograft models. Therefore, our selective and orally bioavailable CDK9 inhibitor KB-0742 is a promising candidate for further clinical development in CRPC.

STAR METHODS

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MV-4-11 Human AML Xenograft in Female BALB/c Nude Mice

Chemical Synthesis

* QUANTIFICATION AND STATISTICAL ANALYSIS *

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.chembiol.2020.10.001.

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**AUTHOR CONTRIBUTIONS**

Conceptualization, A.R., S.K.D., C.Y.L., M.S.P. and A.N.K.; Methodology, S.K.D. and M.S.P.; Validation, A.R., S.K.D., F.K., J.U., and R.A.S.; Formal Analysis, A.R., S.K.D., D.B.F., B.C.H., and H.G.; Investigation, A.R., S.K.D., D.B.F., C.L., B.S.L., S.J., F.K., J.V.K., N.B.S., J.U., R.A.S., D.C., S.M., P.J.M., T.D.H., B.C.H., H.G., J.B., H.X., L.W., G.C.B., B.A.R., and J.W.R.; Resources, D.C., S.C., S.K., J.W.R., and S.P.B.; Data Curation, S.K.D. and C.Y.L.; Writing – Original Draft, A.R., S.K.D., C.Y.L., and A.N.K.; Writing – Review & Editing, A.R., S.K.D., D.B.F., B.C.H., C.Y.L., M.S.P., and A.N.K.; Visualization – S.K.D., A.R., B.C.H., H.G., and C.Y.L.; Supervision, A.R., S.K.D., K.L.K., C.M.O., J.V., C.M.W., W.B.T., D.C.S., N.B., S.K., I.H., J.R.B., M.M.G., S.P.B., C.Y.K., M.S.P., and A.N.K.; Project Administration, A.R., S.K.D., D.B.F., C.M.W., C.Y.K., M.S.P., and A.N.K.; Funding Acquisition, A.R., S.K.D., N.B.S., C.Y.L., and A.N.K.

**DECLARATION OF INTERESTS**

A.N.K. is a founder and member of the scientific advisory board of Kronos Bio, Inc. D.B.F., N.B.S., S.K.D., A.R., M.S.P., and A.N.K. are inventors on patent applications related to KI-ARv-03.

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### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse monoclonal anti-HA.11 (clone 16B12) | Covance | Cat#MMS-101R-1000, RRID:AB_291262 |
| Goat polyclonal Cy3-conjugated anti-mouse IgG | Millipore | Cat#AP124C, RRID:AB_11213281 |
| Mouse monoclonal anti-c-Myc (9E10) | Santa Cruz Biotechnology | Cat#sc-40, RRID:AB_627268 |
| Rat monoclonal anti-RNA Pol II subunit B1 (phospho-CTD Ser-2) (clone 3E10) | Millipore | Cat#04-1571, RRID:AB_11212363 |
| Rat monoclonal anti-RNA Pol II subunit B1 (phospho-CTD Ser-5) (clone 3E8) | Millipore | Cat#04-1572, RRID:AB_10615822 |
| Rat monoclonal anti-RNA Pol II subunit B1 (phospho-CTD Ser-7) (clone 4E12) | Millipore | Cat# 04-1570-I, RRID:AB_2801298 |
| Mouse monoclonal anti-Pol II (F-12) | Santa Cruz Biotechnology | Cat#sc-55492, RRID:AB_630203 |
| Rabbit polyclonal to RNA Pol II CTD repeat YSPTSPS (phospho S2) | Abcam | Cat#ab5095, RRID:AB_304749 |
| Mouse monoclonal anti-AR (441) | Santa Cruz Biotechnology | Cat#sc-7305, RRID:AB_626671 |
| Rabbit polyclonal anti-AR (N-20) | Santa Cruz Biotechnology | Cat#sc-816, RRID:AB_1563391 |
| Rabbit monoclonal anti-AR-V7 (clone EPR15656) | Abcam | Cat#ab198394, RRID:AB_2716436 |
| Rabbit monoclonal anti-AR-V7 (clone RM7) | RevMAb Biosciences | Cat#31-1109-00 |
| Rabbit polyclonal anti-AR (pSer81) | Sigma Aldrich | Cat#67-1375, RRID:AB_11211980 |
| Rabbit monoclonal anti-CDK9 (C12F7) | Cell Signaling Technology | Cat#2319, RRID:AB_2291505 |
| Rabbit monoclonal anti-GAPDH (clone 14C10) | Cell Signaling Technology | Cat#2118, RRID:AB_561053 |
| Mouse monoclonal anti-Mcl-1 (clone 22) | Santa Cruz Biotechnology | Cat# sc-27756, RRID:AB_627915 |
| Rabbit polyclonal to Histone H3 (acetyl K27) | Abcam | Cat#ab4729, RRID:AB_2118291 |
| Rabbit polyclonal anti-Histone H3 | Cell Signaling Technology | Cat#9719, RRID:AB_331563 |
| Goat polyclonalIRDye 800CW conjugated anti-rat IgG | LI-COR Biosciences | Cat#926-32219, RRID:AB_1850025 |
| Donkey polyclonal IRDye 800CW conjugated anti-rabbit IgG | LI-COR Biosciences | Cat#926-32213, RRID:AB_621848 |
| Horse HRP-conjugated anti-mouse IgG | Cell Signaling Technology | Cat#7076, RRID:AB_330924 |
| Goat polyclonal HRP-conjugated anti-rabbit IgG | Cell Signaling Technology | Cat#7074, RRID:AB_2099233 |
| Mouse monoclonal anti-beta Actin (clone mAbcam 8226) | Abcam | Cat#ab20272, RRID:AB_445482 |
| Rabbit polyclonal anti-FOXa1 | GeneTex | Cat#GTX100308, RRID:AB_1240823 |
| Rabbit monoclonal anti-Acetyl-Histone H3 (Lys27) (clone DSE4) | Cell Signaling Technology | Cat#8173, RRID:AB_10949503 |

### Chemicals, Peptides, and Recombinant Proteins

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| KI-ARv-03 | This paper | N/A |
| KB-0742 | This paper | N/A |
| NVP-2 | MedChemExpress | Cat#HY-12214A |
| BAY-1143572 (Atuveciclib) | Selleck Chemicals | Cat#S8727 |
| AZD4573 | Selleck Chemicals | Cat#S8719 |
| MC180295 | Resynthesized in the Koehler lab. | N/A |
| R1881 | Sigma Aldrich | Cat#R0908 |
| Enzalutamide | SelleckChem | Cat#S1250 |
| Protease and Phosphatase Inhibitor Cocktail | Thermo Scientific | Cat#78440 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| SYPRO-Orange        | Molecular Probes | Cat#S6651 |
| TRizol™ Reagent     | Thermo Scientific | Cat#15596018 |
| Halt™ Protease Inhibitor Cocktail | Invitrogen | Cat#1008D |
| Illumina Tagment DNA TDE1 Enzyme and Buffer | Illumina | Cat#20034198 |
| Proteinase K        | New England Biolabs | Cat#P81075 |
| DNA Clean & Concentrator-5 | Zymo Research | Cat#D4013 |
| DNase I (RNase-free) | New England Biolabs | Cat#M0303 |
| NEBNext® High-Fidelity 2X PCR Master Mix | New England Biolabs | Cat#M0541 |
| AMPure XP | Beckman Coulter | Cat#A63880 |

Critical Commercial Assays

| Dual-Glo® Luciferase Assay System | Promega | Cat#E2940 |
| CellTiter-Glo® Luminescent Cell Viability Assay | Promega | Cat#G7572 |
| IncuCyte® Caspase-3/7 Green Apoptosis Assay Reagent | Essen BioScience | Cat#4400 |
| QuantSeq 3’ mRNA-Seq Library Kit for Illumina | Lexogen | Cat#015 |
| NextSeq 500/550 High Output Kit v2.5 (75 Cycles) | Illumina | Cat#20024906 |
| NextSeq 500/550 High Output Kit v2.5 (150 Cycles) | Illumina | Cat#20024907 |

Deposited Data

| Structure of CDK9/cyclinT1 in complex with DRB | (Baumli et al., 2010) | PDB: 3MY1 |
| High throughput sequencing dataset | This paper, NCBI GEO: GSE156885 | https://www.ncbi.nlm.nih.gov/geo/ |

Experimental Models: Cell Lines

| Human: VCaP-16 (male) | Laboratory of S.P. Balk, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA | N/A |
| Human: VCaP (male) | ATCC | Cat#CRL-2876, RRID:CVCL_2235 |
| Human: HEK293T (female) | ATCC | Cat#CRL-3216; RRID: CVCL_0063 |
| Human: DU145 (male) | ATCC | Cat#HTB-81; RRID:CVCL_0105 |
| Human: LNCaP (male) | ATCC | Cat#CRL-1740; RRID:CVCL_1379 |
| Human: PC3 (male) | ATCC | Cat#CRL-1435; RRID:CVCL_0035 |
| Human: 22Rv1 (male) | ATCC | Cat#CRL-2505; RRID:CVCL_1045 |
| Human: MV-4-11 (male) | ATCC | Cat#HTB-189, RRID:CVCL_0064 |

Experimental Models: Organisms/Strains

| Mouse: CB17-SCID, sex: male | Beijing Vital River Laboratory Animal Technology Co., LTD | N/A |
| Mouse: BALB/c nude, sex: female | Shanghai Lingchhang Biotechnology Co., LTD | N/A |

Oligonucleotides: Primers

| GAPDH (hhsaCEP0041396) | Bio-Rad | N/A |
| KLK3 (qHsaCEP0024737) | Bio-Rad | N/A |
| KLK3 (Hs02576345_m1) | ThermoFisher | Cat#4331182 |
| AR (Hs00907243_m1) | ThermoFisher | Cat#4331182 |

Software and Algorithms

| GenePix Pro 6.0 | Molecular Devices | http://mdc.custhelp.com/app/answers/detail/a_id/18691/~/genepix%C2%AE-pro-6-microarray-acquisition-%26-analysis-software-download-page |
| ChemDraw Professional 19.0 | PerkinElmer | https://www.perkinelmer.com/product/chemdraw-professional-chemdrawpro |
RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Angela N. Koehler (koehler@mit.edu).

Materials Availability
KI-ARv-03 is available through http://koehlerlab.org/contact-us and upon completion of a material transfer agreement by the receiving institution.

Data and Code Availability
Full small molecule screening data sets, along with compound structures for the libraries, are available upon reasonable request. Raw and processed data for all high throughput sequencing experiments performed in this manuscript are available in the NCBI GEO database under the GSE156885 accession number. For analysis of high throughput sequencing data, custom analysis scripts can be found at (https://github.com/linlabcode/cdk9/).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal Studies
The protocol and any amendment(s) or procedures involving the care and use of animals in this study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of WuXi AppTec prior to conduct. During the study, the care and use of animals was conducted in accordance with the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The mice were kept in individual ventilation cages at constant temperature and humidity with 5 animals in each cage (Temperature: 20-26°C, Humidity 40-70%). Cages (300 mm × 200 mm × 180 mm) were made of polycarbonate. The bedding material was corn cob, which was changed twice per week. Diet: Animals had free access to irradiation sterilized dry granule food during the entire study period. Water: Animals had free access to sterile drinking water. The identification labels for each cage contained the following information: number of animals, sex, strain, date received, treatment, study number, group number and the starting date of treatment. Animal identification: Animals were marked by ear tags. After inoculation, the animals were checked daily for morbidity and mortality. At the time of routine monitoring, the animals were checked for any effects of tumor growth and treatments on normal behavior such as mobility, food and water consumption, body weight gain/loss (body weight was measured twice weekly, eye/hair matting and any other abnormal effect. Death and observed clinical signs were recorded on the basis of the numbers of animals within each subset. The efficacy studies used 6-8 week-old male CB17-SCID mice (Animal supplier: Beijing Vital River Laboratory Animal Technology Co., LTD., Animal certificate number: 20170011003869) or female BALB/c nude mice (Animal supplier: Shanghai Lingchang biotechnology co. LTD, Animal certificate number: 2018003006523). The cell lines used for transplantation experiments (22Rv1, #CRL-2505; MV-4-11, ATCC, #CRL-9591) were obtained from ATCC.
Cell Lines
Cell lines were obtained from ATCC with exception of VCaP-16 (male, human), which was generated by Joshua Russo in the laboratory of Steven P. Balk. Cell lines were not authenticated in our hands. Cell lines were tested intermittently throughout studies using MycoAlert Mycoplasma Detection Kit (Lonza, Cat#LT07–418), generally a few days after thawing and immediately before significant studies (e.g. RNA-seq experiment). Cells were cultured in a humidified incubator at 37.0 °C and 5.0% CO2. Cell lines used in this study: VCaP (male, human), HEK293T (female, human), DU145 (male, human), LNCaP (male, human), PC3 (male, human), 22Rv1 (male, human), and MV-4-11 (male, human).

METHOD DETAILS

Expression Vector for HA-Tagged Androgen Receptor N-terminal Truncate
The expression vector for HA-tagged AR-DLBD truncate was made by cloning the N-terminus of AR (amino acid residues 1-645) from the AR ORF in a pLEX backbone (acquired via the Broad Institute, Genetic Perturbation Platform) into a doxycycline inducible system with an HA tag.

SMM Screening of HA-Tagged AR N-terminal Truncate
Small molecule microarrays were manufactured as previously described (Clemons et al., 2010). Each SMM slide contained approximately 10,000 printed features including 5,000 unique compounds printed in duplicate. In total, 50,000 compounds were screened. Each slide was screened in duplicate for a total of four replicates per compound. HEK293-T cells were plated in 10 cm tissue culture dishes (Corning) in growth media (DMEM supplemented with 1% penicillin-streptomycin solution and 10% fetal bovine serum) at a density of 2 x 10^5 cells per dish. The following day, cells were transfected with the expression vector for HA-tagged AR-DLBD truncate (5 μg DNA per plate) using FuGENE® 6 (Promega) and Opti-MEM media according to the manufacture protocols. Doxycycline (3 μg/ml) was added 4 h after transfection. After 48 h, cells were lysed in 4x volume of RIPA buffer (modified to 250 mM NaCl supplemented with 2x Complete™ protease inhibitor cocktail tablets (Sigma Aldrich) with brief sonication on ice using a microtip sonicator (20 pulses, 30% power, 0.5 s cycle). Lysates were centrifuged for 20 min at 4 °C and the concentration of total protein was adjusted to 0.3 mg/mL in MIPP buffer (20 mM NaH2PO4, 1 mM Na2VO4, 5 mM NaF, 25 mM β-glycerophosphate, 2 mM EGTA, 2 mM EDTA, 1 mM DTT, 0.5% Triton X-100, pH 7.2, Complete™ protease inhibitor cocktail tablets) to maintain protein-protein complexes. Each slide was incubated with 3 mL of diluted lysate for 1 h, followed by three consecutive washes with TBS-T. Slides were incubated with an anti-HA.11 mouse monoclonal antibody (BioLegend, #901501) at 1:100 in TBS-T buffer (1X Tris buffered saline, 0.1% Tween-20), followed by three consecutive washes with TBS-T. Slides were then incubated with a Cy3-labeled anti-mouse secondary antibody (Invitrogen) for detection at 1:1000 in TBS-T, followed by three consecutive washes with TBS-T. Slides were briefly rinsed once each in TBS and distilled water and spin-dried. The slides were immediately scanned using a GenePix 4000B fluorescence scanner (Molecular Devices) at PMT 600. The slide images were analyzed using GenePix Pro software (Axon Instruments) to produce raw, numerical data for statistical analysis.

SMM Statistical Analysis
Raw data was analyzed based on the signal-to-noise ratio and reproducibility. For each feature, the signal to noise ratio (SNR) was defined as the median fluorescence intensity of the feature divided by the median fluorescence intensity of the surrounding slide area, defined as a radius 3 times the radius of the spot, excluding pixels within a certain overlap threshold of neighboring features. Then, a robust z-score (Zi) was calculated for each feature (i) using the following equation:

\[ Z_i = \frac{SNR_i - Mdn(SNR)}{MAD(SNR)} \times 1.486 \]

Where SNR_i is the SNR value for a given feature, Mdn(SNR) is the median of the SNR values for all features in the subarray, and the MAD(SNR) is the maximum absolute deviation of the SNR values for all features in the subarray. For each screen, a robust z-score threshold for calling assay positives for further study is chosen independently based on the performance of control feature groups (which serve as “null distribution”) in the unique assay.

RT-qPCR for PSA in LNCaP Cells
LNCaP cells were grown in androgen-stripped media (RPMI media supplemented 1% penicillin-streptomycin solution and 10% charcoal stripped fetal bovine serum) for 24 h prior to seeding in 384-well plates at a density of 10,000 cells/well in the same media. After 24 h post-seeding, cells in assay wells were co-treated with 1 nM synthetic androgen (R1881) and compound stocks dissolved in DMSO using a Tecan Freedom EVO 150 S liquid handling system. Following a 24 h compound incubation, cells in control wells were treated either with DMSO alone (‘’uninhibited’’) or DMSO and 1 nM R1881 (‘’induced’’), again using the same liquid handling system. Following a 24 h compound incubation, media was aspirated from all wells and all wells were rinsed briefly in PBS. Cells were lysed using the BioRad SingleShot Cell Lysis Kit (12 μL total lysis solution per well). Cellular mRNA was reverse transcribed to cDNA using the iScript™ Advanced cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) and RT-qPCR was performed using the SsoAdvanced™ Universal Super Probes Mix (Bio-Rad) with PrimePCR™ Probe Assay (Bio-Rad) probes for GAPDH (FAM conjugated, BioRad Unique Assay ID: qHsaCEP0041396) and KLK3 (i.e. PSA, Cy5 conjugated, BioRad Unique Assay ID: qHsaCEP0024737) on the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad) according to manufacturer specified protocols.
RT-qPCR Data Analysis

Data collected via the CFX Manager™ software (Bio-Rad) was exported to Microsoft® Excel® for analysis. For uninduced and induced control wells, the Ct (cycle threshold) value for each fluorescence channel was taken to be the average Ct value across all respective control wells ($n \geq 10$). The ΔCt value for all wells was calculated as the difference between the Ct value in the Cy5 channel (PSA) and the FAM channel (GAPDH) and the ΔΔCt value was calculated as the difference between the ΔCt value of the assay well and the ΔCt value of the induced wells to normalize expression relative to the induced wells. The expression fold change for each well was defined as $2^{\Delta \Delta Ct}$. Average expression fold change for qPCR technical replicates ($n = 3$) were used to compare compound performance.

Luciferase Reporter Gene Assay

A mouse mammary tumor virus (MMTV)-driven firefly luciferase construct (MMTV-FLuc2P) was used to generate a stable reporter cell line containing doxycycline-inducible AR-V and CMV-driven Renilla luciferase in LNCaP. These cells were grown in androgen-starved media (RPMI media supplemented with 1% penicillin-streptomycin solution and 10% charcoal stripped fetal bovine serum) for 24 h prior to seeding in 384-well plates at a density of 10,000 cells/well in the same media to reduce the activity of endogenous AR-FL. After seeding for 24 h, cells in assay wells were treated with DMSO, 2 μg/mL doxycycline, or cotreated with 2 μg/mL doxycycline and compound stocks dissolved in DMSO using a Tecan Freedom EVO 150 S liquid handling system. After 24 h post-treatment, luciferase signal was measured using a dual-luciferase reporter assay system (Promega) and a compatible plate reader (Tecan). Raw firefly luciferase signal was normalized for cell count by dividing this value by the raw Renilla luciferase signal on a well-by-well basis. In the absence of compound treatment, the normalized luciferase signal showed ~8-fold induction in the presence of doxycycline compared with vehicle treated controls. Averages for replicate wells were calculated and normalized to define doxycycline-treated wells as 1.0 on a plate-by-plate basis to allow comparison of compound performance.

Cell Viability Assays

Prostate cancer cells (LNCaP, VCaP, DU145, PC3) were plated in 384-well plates in appropriate cell culture media (ATCC) at a density empirically determined to produce wells of ~90% confluency five days after seeding for each cellular model. After 24 h post-seeding, cells in assay wells were treated either with DMSO or compound stocks dissolved in DMSO using a Tecan Freedom EVO 150 S liquid handling system. After 72 h of compound exposure, the ATP content in each well was measured as a proxy for cell viability using the CellTiter-Glo® assay system (Promega) and a compatible plate reader (Tecan). Raw luminescence values for each well were averaged across replicate wells and average values were normalized to define DMSO treated wells as 1.0 on a cell line-by-cell line, plate-by-plate basis to compare compound performance.

Lead Evaluation in Cellular CRPC Models

The CRPC cell line VCaP-16 was maintained in DMEM with 10% FBS and 16 μM enzalutamide (SelleckChem, #S1250). 22Rv1 was maintained in RPMI1640 with 10% FBS. For RT-qPCR assays to quantitatively measure PSA, AR-FL, and AR-V7 expression levels, CRPC cells (VCAp-16 or 22Rv1) were plated into 6-well plates in maintenance media at a density of 50%. After 48 h, cells in assay wells were washed twice with PBS then treated either with DMSO or 16 μM enzalutamide plus compound stocks dissolved in DMSO at 5 μM compound concentration. After 18 h of compound exposure, media was aspirated from all samples and each was rinsed briefly in PBS. RNA was isolated and purified using the Qiagen RNeasy Kit (Qiagen) following manufacturer’s instructions. Quantitative real-time RT-PCR amplification was performed with TaqMan One-Step RT-PCR reagents (ThermoFisher Scientific) and results were normalized to coamplified β-actin. Primers and probes were purchased from ThermoFisher Scientific and are listed as follows: KLK3 (Hs02576345_m1, FAM-MGB), AR (Hs00907243_m1, FAM-MGB), AR-V7 (Assay ID: A6ROCI). qRT-PCR was performed on an Applied Biosystems StepONEPlus Real-Time PCR System Thermocycler, according to the manufacturer’s specified protocols. The expression fold change for each well was defined as $2^{\Delta \Delta Ct}$ in the manner described above, using actin as the reference gene to define a value of 1.0. Average expression fold change across biological replicates ($n = 3$) tested in qPCR technical duplicate were used to compare compound performance.

For western blot assays to qualitatively measure AR-FL and AR-V7 protein levels, CRPC cells (VCAp-16 or 22Rv1) were plated in 6-well plates in maintenance cell culture media at a density of 50%. After 48 h, cells in assay wells were washed twice with PBS then treated either with DMSO or 16 μM enzalutamide plus compound stocks dissolved in DMSO at 5 μM compound concentration. After 18 h of compound exposure, media was aspirated from all samples and each was rinsed briefly in PBS. Cells were lysed in RIPA buffer with protease and phosphatase inhibitor cocktail (ThermoScientific, #P78440) on a vortex shaker for 30 min at 4°C. Lysates were spun at 16,000 g for 15 min at 4°C to clarify and pellet aggregated protein. The supernatant containing the soluble protein fraction was transferred to a new tube. Protein lysates were added to loading buffer (2x Laemmli Sample Buffer, Bio-Rad, #1610737) and the samples were boiled at 90°C for 7 min. Samples were electrophoresed at 200 V using a 4-20% denaturing PAGE gel (Bio-Rad). Relative AR-FL, AR-V7 (both detected with AR-N20, Santa Cruz Biotechnology, #sc-816), and β-actin (Abcam, #ab20272) protein levels were determined via immunoblotting.

In Vitro Kinase Activity Assays

KinaseProfiler™ assay to assess broad kinase activity was conducted by Eurofins using 10 μM K1-ARv-03 and ATP concentrations within 15 μM of the apparent $K_m$ for each kinase. IC50Profiler™ assays were conducted at Eurofins for all kinases showing greater...
than 40% reduction of activity in the presence of KI-ARv-03. In addition, all available CDKs were tested using ICsoProfiler to generate data included in Figure 2B.

Kinetic studies for ATP competition of KI-ARv-03 towards CDK9 were conducted using the HotSpot Kinase assay from Reaction Biology Corporation. Here, five different ATP concentrations (1 µM, 10 µM, 50 µM, 100 µM, 200 µM) and four concentrations of KI-ARv-03 (0.123 µM, 0.370 µM, 1.11 µM, 3.33 µM) were used and the reduction of CDK9 activity monitored over 120 min. These raw data were suggestive of a competitive mode of inhibition and were formally investigated for mode of inhibition using Prism 8.0 (GraphPad Software, LLC) to fit these data to a competitive inhibition model described by the following system of equations:

\[ K_{m}^{\text{obs}} = K_m \left(1 + \frac{|I|}{K_i}\right) \]

\[ v = \frac{v_{\text{max}} \cdot |S|}{K_{m}^{\text{obs}} + |S|} \]

Where \(|I|\) is the concentration of inhibitor, variable across KI-ARv-03 doses tested; \(|S|\) is the concentration of substrate, variable across ATP doses tested; \(v\) is the enzyme velocity; \(K_m^{\text{obs}}\) is the apparent Michaelis-Menten constant; \(v_{\text{max}}\) is the maximum enzyme velocity without inhibitor; \(K_i\) is the Michaelis-Menten constant. Global fit parameters for \(K_i\), \(K_m\), and \(v_{\text{max}}\) are reported in Figure 2C (R² = 0.99).

Additional testing of 16 CDK’s were performed by Reaction Biology Corporation (RBC) using their HotSpot Kinase Assay to profile KI-ARv-03 side-by-side with KB-0742 (Figures 3B and 3C). Compounds were tested in 10-dose in duplicate with a 3-fold serial dilution starting at 10 µM. Control compound, Staurosporine, was tested in 10-dose with 4-fold serial dilution starting at 20 µM. Alternate control compound, THZ531, was tested in 10-dose with 3-fold serial dilution starting at 10 µM. Reactions were carried out at \(K_m\) ATP according to the RBC \(K_m\) binning structure (CDK1/cyclin A (10 µM ATP), CDK2/cyclin K (30 µM ATP), CDK3/cyclin K (5 µM ATP), CDK4/cyclin Y (15 µM ATP), CDK6/cyclin Y (10 µM ATP), CDK7/cyclin Y (20 µM ATP), CDK8/cyclin Y (20 µM ATP), CDK9/cyclin C (20 µM ATP), CDK10/cyclin A (10 µM ATP), CDK12/cyclin A (10 µM ATP), CDK13/cyclin D1 (100 µM ATP), CDK14/cyclin D1 (100 µM ATP), CDK15/cyclin D1 (50 µM ATP), CDK16/cyclin D1 (50 µM ATP), CDK17/cyclin H (50 µM ATP), CDK8/cyclin C (10 µM ATP), CDK9/cyclin T1 (10 µM ATP)). Normalized data were plotted and analyzed to produce ICso values in GraphPad PRISM. For all compound:kinase pairs where the top compound concentration tested (10 µM) did not inhibit the kinase by >65% or where the ICso value was outside the range of concentrations tested, the ICso value was set to 10 µM for plotting in the heatmap comprising Figure 3C.

**Compound-Anchored Target Engagement**

*Functionalization of Beads with KI-ARv-03*

NHS-functionalized Affi-Gel 10 beads (Bio-Rad Laboratories, Inc., 500 µL, 50% suspension in isopropyl alcohol (IPA)) were pelleted in a 1.5 mL reaction tube by centrifugation (400 x g, 2 min, RT) and washed three times with cold IPA by repeated centrifugation (400 x g, 2 min, RT). Subsequently, the beads were washed three times with anhydrous DMSO (repeated centrifugation, 400 x g, 2 min, RT). After the final wash, the supernatant was removed, and the bead pellet resuspended in 250 µL of anhydrous DMSO to gain a 50% suspension. 200 µL of this suspension each were distributed into fresh 1.5 mL reaction tubes for a) KI-ARv-03 functionalization as well as b) aminooethanol blocking for control purposes (unspecific protein binding to beads). A solution of KI-ARv-03 in DMSO (18 µL, 10 mM, theoretical bead loading: 12%) or 5 µL ethanolamine (blocked beads) was added. To each vial Et3N (2.1 µL) was added. The resulting suspensions were shielded from light and allowed and rotate overnight (~16 h) at RT on an overhead rotator. Then 5 µL of ethanolamine were added to each reaction tube in order to block any unreacted NHS-ester. After another 4 h shielded from light on the overhead rotator, the beads were pelleted (400 x g, 2 min, RT). The supernatant was removed, and the beads were washed three times with anhydrous DMSO by repeated centrifugation (400 x g, 2 min, RT). The beads were then washed with PBS three times by repeated centrifugation (400 x g, 2 min, RT) and the pellet was then taken up in as much PBS to account for a final total volume of 200 µL. Distribute beads (40 µL or 60 µL per sample) into new 1.5 mL reaction tubes and add 22Rv1 cell lysate subsequently.

**Compound-Anchored Pull-Downs**

22Rv1 prostate cancer cells were plated at 200k/mL in 15 cm dishes (30 mL total volume) using phenol red free RPMI media supplemented with 10% FBS (VWR), 1x Pen/Strep (Invitrogen). Cells were harvested at 90% confluency (approx. 2-3 days). Briefly, cells were washed with PBS, and finally collected into 500 µL PBS (2 mL reaction tube) using a cell scraper and then pelleted (300 x g, 3 min, 4°C). The pellet was resuspended in high salt RIPA buffer (50 mM Tris HCl, 400 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 1 mM EDTA, 0.1% (w/v) SDS and 0.01% (w/v) Na2EDTA, pH 7.4) or MPP buffer (20 mM Na2HPO4, 1 mM Na2HPO4, 5 mM NaF, 50 mM β-glycerophosphate, 2 mM EGTA, 2 mM EDTA, 1 mM DTT, 0.5% (v/v) Triton X-100, pH 7.2) supplemented with complete protease inhibitor cocktail (add 4-times volume of pellet). After 30 min of incubation on ice, the cells were sonicated briefly and then centrifuged (15 min, 4000 x g, 4°C). The supernatant was transferred to fresh reaction tubes and the protein concentration quantified using Pierce BCA Protein Assay. For each target engagement assay condition 500 µg lysate were diluted in lysis buffer to make a final volume of 455 µL (40 µL beads used) or 435 µL (60 µL beads used) respectively. For target engagement experiments 5 µL DMSO were added to achieve a final concentration of 1%. For soluble competition experiments 2 µL of 10 mM KI-ARv-03 in DMSO as well as 3 µL DMSO were added to achieve a final concentration of 40 µM KI-ARv-03. The lysates were added to either
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40 μL or 60 μL KI-ARv-03-anchored beads (50% in PBS) to reach a final volume of 500 μL for each target engagement experiment. Samples were incubated shielded from light at 4°C with end-over-end rotation overnight. Samples were then gently centrifuged, and the supernatant removed. The beads were washed three times with cold PBS by repeated centrifugation (200 x g, 3 min) and the supernatant discarded. The beads were resuspended in 100 μL 2x SDS loading buffer and boiled at 95°C for 10 min. After centrifugation (200 x g, 3 min) the supernatant was transferred to fresh tubes and 30 μL of each sample were used for subsequent SDS-PAGE (4-20% denaturing PAGE gel, Bio-Rad) and Western blot analysis.

Cellular Thermal Shift Assay (CETSA)
Protocol adapted from Jafari et al. (Jafari et al., 2014).

Melting Curve
22Rv1 (1 x 10^6) cells were washed and harvested with a cell scraper in cold PBS. After transferring to falcon tubes these were spun down (300 x g, 5 min) and the supernatant discarded. The cell pellet was resuspended in 100 μL PBS containing protease inhibitor cocktail and transferred to PCR tubes. Cells were shock heated in a thermal cycler for 3 min (37°C, 38.6°C, 41.5°C, 46.1°C, 51.5°C, 55.9°C, 59.0°C, 61.0°C, and no heat). The cell suspensions were then subjected to three freeze-thaw cycles with liquid nitrogen to lyse cells. For uniform thawing this step was performed in a thermal cycler at 25°C. Lysates were spun at 21,000 g for 45 min at 4°C to clarify and pellet aggregated protein. The supernatant containing the soluble protein fraction was transferred to a new tube. Protein lysates (7.5 μL) was added to loading buffer (7.5 μL, 2x Laemmli Sample Buffer, Bio-Rad, #1610737) and the proteins were heated to 90°C for 5 min. Samples were electrophoresed at 200 V using a 4-20% denaturing PAGE gel (Bio-Rad). Relative AR (sc-7305, Santa Cruz Biotechnology) and CDK9 (2316S, Cell Signaling Technology) protein levels were determined via immunoblotting.

Dose Response Curve
22Rv1 (1 x 10^6 cells were treated with DMSO or KI-ARv-03 (0.625 μM, 1.25 μM, 2.50 μM, 5.00 μM, 10 μM, 20 μM, 40 μM) for 1 h at 37°C. Cells were then washed and harvested with a cell scraper in cold PBS. After transferring to falcon tubes these were spun down for 5 min at 300 x g, and the supernatant discarded. The cell pellet was resuspended in 100 μL PBS containing protease inhibitor cocktail (Millipore Sigma) and transferred to PCR tubes. Cells were shock heated in a thermal cycler at 49.0°C (no heat) for 3 min. The cell suspensions were then subjected to three freeze-thaw cycles with liquid nitrogen to lyse cells. For a uniform thawing this step was performed in a thermal cycler at 25°C. Lysates were spun at 21,000 g for 45 min at 4°C to clarify and pellet aggregated protein. The supernatant containing the soluble protein fraction was transferred to a new tube. Protein lysates (7.5 μL) was added to loading buffer (7.5 μL, 2x Laemmli Sample Buffer, Bio-Rad, #1610737) and the proteins were boiled at 90°C for 5 min. Samples were electrophoresed at 200 V using a 4-20% denaturing PAGE gel (Bio-Rad). Relative AR (sc-7305, Santa Cruz Biotechnology) and CDK9 (2316S, Cell Signaling Technology) protein levels were determined via immunoblotting.

Thermal Shift Assays (TSA)
Thermal melting experiments were carried out with an Mx3005p PCR machine (Agilent). CDK9/cyclin T was expressed and purified as described (Baumli et al., 2008) and assayed in a 96-wellplate at a final concentration of 2 μM in a 20 μL volume. Inhibitors were added at a final concentration of 10 μM. SYPRO-Orange (Molecular Probes) was added at a dilution of 1 in 1000. Excitation and emission filters were set to 465 nm and 590 nm, respectively. The temperature was raised with a step of 3°C per minute, and fluorescence readings were taken at each interval as described before (Fedorov et al., 2012).

Immunoblotting
Samples for immunoblotting studies were prepared for electrophoresis by heating (90°C, 5-10 min) in a final concentration of 1x Laemmli loading buffer (supplemented with 5% 2-mercaptoethanol, freshly added). Samples were then electrophorized by SDS-PAGE (4-15% or 4-20% Criterion™ TGX™ Precast gel, BioRad) at a constant voltage within the range of 160 – 200 V. Proteins were transferred to a nitrocellulose membrane (BioRad) using the Trans-Blot® Turbo™ transfer system (BioRad). Membranes were blocked with 5% BSA in TBS-T for a minimum of 1 h (RT) before incubating with the following primary antibodies (unless otherwise specified): Pol II Antibody (F-12) #sc-55492 (Santa Cruz Biotechnology), RNA polymerase II CTD repeat YSPTPS (pSer2) antibody #ab5095 (Abcam), AR Antibody (441) #sc-7305 (Santa Cruz Biotechnology), anti- phospho-Androgen Receptor (pSer81) #07-1375 (Sigma Aldrich), Histone H3 antibody #9715 (Cell Signaling Technology), Pol II pS2 #04-1571 (Sigma Aldrich), Pol II pS5 #04-1572 (Sigma Aldrich), Pol II pS7 #04-1570-L (Sigma Aldrich), CDK9 #2316 (Cell Signaling Technology), GAPDH #2118 (Cell Signaling Technology), MYC #sc-40 (Santa Cruz Biotechnology), MCL-1 #sc-12756 (Santa Cruz Biotechnology). Secondary antibodies were either anti-mouse or anti-rabbit HRP linked antibodies (BioRad) for imaging on a ChemiDoc™ imaging system (BioRad) or antimouse, anti-rabbit IR800 antibodies or anti-rat 800CW and 680LT (LI-COR Biotechnology) for all for imaging on an Odyssey CLx (LI-COR Biotechnology). For crossblotting, Restore™ PLUS (Thermo Fischer Scientific) or NewBlot™ (LI-COR Biotechnology) Western Blot Stripping Buffer was used after re-probing with the appropriate primary antibody.

Growth Rate Inhibition Studies
22Rv1 cells were plated in 96-well plates, adding 100 μL of a 75,000 cells/mL cell suspension in RPMI media (7,500 cells per well). After 24 h, cell culture media for 22Rv1 cells was removed and replaced with media containing 5 μM of IncuCyte® Caspase-3/7 Green Apoptosis Assay Reagent (Essen Bioscience) according to manufacturer protocol and either DMSO or CDK9 inhibitor dissolved in DMSO at the appropriate final concentration. MV-4-11 cells were plated in 96-well plates after treating plates with fibro-
nectin (1 μg/cm² in PBS, 40 μL/well, 1 h, RT), removing excess PBS, and adding 80 μL of a 62,500 cells/mL cell suspension in IMDM media (5,000 cells per well). Plate was spun for 1 min at 200 x g after plating of cells. After 24 h, 40 μL of a mixture containing IncuCyte® Caspase-3/7 Green Apoptosis Assay Reagent (Éssen Bioscience, 5 μM final concentration) and CDK9 inhibitor in DMSO at the appropriate concentrations or DMSO alone in IMDM were added. Plates were then imaged over a period of 72 h (22Rv1) or 48 h (MV-4-11), collecting four images per well every 3 h with a 10x objective using the IncuCyte® S3 in a standard tissue culture incubator. The resultant images were analyzed for confluency and apoptotic signal using the associated IncuCyte® S3 software, adjusting the mask and filter settings for image analysis using a small training set of images (~12) from DMSO control wells and high doses of compound at early and late time points. To determine growth rate inhibition (GR) metrics, confluency data for compound-treated wells at the assay endpoint and DMSO wells at t = 0 and the assay endpoint were used as inputs to GRcalculator, an online tool for calculating and mining dose-response data hosted by Harvard Medical School (HMS) LINCS (Library of Integrated Network-based Cellular Signatures) Center. Finally, the number of caspase-3/7 positive cells for each condition over time were used to calculate AUC values for cleaved caspase accumulation using GraphPad PRISM 8.0.

Thiol(SH)-Linked Alkylation for the Metabolic Sequencing of RNA (SLAM seq)
SLAM seq experiments were performed as described before (Herzog et al., 2017). In brief, 22Rv1 carcinoma cells were seeded at 60% confluence 18 h before being pretreated with 800 μM s4U (Sigma, St. Louis, MO) to start the pulse. The cells were incubated in the media with s4U and the 0 h samples were collected after 30 min by washing the cells with PBS and lysing directly with TRIzol (Invitrogen, Carlsbad, CA). The media was replaced with media containing 800 μM s4U along with either DMSO, KB-0742 (1.2 μM) or KI-ARv-03 (4 μM) and they were collected similarly after 2, 4 or 8 h post incubation. The RNA from the cells were harvested by total RNA extraction in the presence of 0.1 mM DTT to maintain the samples in reducing conditions and the extracted RNA was resuspended with 1 mM DTT. The ERCC RNA Spike-in control mix was added to each sample and thiol modifications were performed by treating 1.5 μg of total RNA with freshly prepared iodoacetamide (IAA) at a final concentration of 10 mM. The reactions were quenched, and the thiol modified RNA was ethanol precipitated. The libraries were prepared using the QuantSeq 3′ mRNA-Seq Library Prep Kit (Lexogen, Vienna, Austria) and were sequenced with NextSeq 500/550 High Output Kit v2.5 (75 Cycles) (Illumina, San Diego, CA). Reads were aligned to a bed of 3′ UTR regions (hg19_RefSeq_Curated_3UTR.bed) drawn from the UCSC hg19 genome reference using the SLAM Seq processing pipeline SlamDunk (https://t-neumann.github.io/slamdunk/) (Neumann et al., 2019). Custom analysis scripts can be found at (https://github.com/linlabcode/cdk9/).

Analysis was performed on a set of 4,852 highly transcribed active genes. These were defined as having at least one sample with total reads (counts per million) in the top 25 percentile for which nascent (converted reads) transcripts could be detected. To quantify levels of nascent transcription, we utilized the fraction converted reads as output by SlamDunk to represent the nascent mRNA fraction. For instance, a value of 0.1 would indicate that 10% of reads for a gene are metabolically labeled and therefore nascently transcribed.

Samples were hierarchically clustered by nascent mRNA fraction levels at top active transcribed genes (Figure S5C). Profiles of mean log₂ fold change at each time point relative to matched DMSO control were hierarchically clustered by both row (genes) and columns (samples) (Figure S5A). In both cases a distance metric of 1 – Pearson correlation was used.

To identify differentially transcribed genes, nascent mRNA fraction was compared between treatment and DMSO control samples. A p-value threshold of <0.01 was used to identify differential genes. Changes in nascent mRNA fraction were compared between KI-ARv-03 and KB-0742 and assessed using a Pearson correlation (Figure S5D). Genes in the top 50 by nascent mRNA fraction in DMSO control samples were annotated as top transcribed genes (Figure 5B). For changes in nascent mRNA fraction at individual genes, bar plots are used with the error bars representing standard error of the mean (Figure 5C).

Chromatin Immunoprecipitation (ChIP)
Chromatin immunoprecipitations were performed using the ChIPmentation protocol as described previously (Schmidt et al., 2015) with minor modifications. Briefly, 15 × 10⁶ (for FOXA1 ChIP) or 8 × 10⁵ (for H3K27ac ChIP) 22Rv1 carcinoma cells were treated for 24 h with DMSO and were crosslinked by incubating in formaldehyde to a final concentration of 0.8% for 5 min at room temperature. The fixation was terminated by adding Glycine to a final concentration of 125 mM and incubation on a rocker at RT for 5 min. The cells were washed in ice cold PBS buffer thrice and the cells were harvested using a cell lifter. The crosslinked cells were then lysed in L1 buffer (50 mM HEPES, 140 mM NaCl, 1 mM EDTA, 10% Glycerol, 0.5% IGEPAL and 0.25% Triton X-100) with protease inhibitor (PI) at 4°C in a rotor. The cells were pelleted and resuspended in L2 buffer (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA in 10 mM Tris) with PI and incubated at RT in a rotor. The nuclei were pelleted by centrifugation at 4°C and were washed twice with the sonication buffer (SE) (10 mM Tris, 1 mM EDTA, 0.1% SDS). The pellet was resuspended in SE with PI and was sheared using a covaris LE220 sonicator (Covaris, Woburn, MA) for 12-14 minutes (FOXa1 assay) or 6.5 min (H3K27ac assay). Sonicated lysates were supplemented with salts and detergents to a final concentration of 1% Triton X-100, 150 mM NaCl and 0.1% Na-deoxycholate. The chromatin was then cleared by centrifugation and incubated with Dynabeads™ Protein A (Thermo Fisher Scientific, Waltham, MA) for 1 h to preclear the lysate. After collecting the input, the supernatant was incubated with end-over-end rotation overnight at 4°C with Dynabeads™ Protein A magnetic beads prebound with antibody (2 μL anti-H3K27ac (Cell Signaling Technology. #8173) for H3K27ac ChIP and 6 μL anti-FOXa1 (GeneTex. #GTX100308) for FOXA1 ChIP). The antibody was bound to the beads by incubating the beads with the antibody in 200 μL of bead binding buffer (10 mM Tris, 1 mM EDTA, 0.2% IGEPAL) in a rotor at 4°C for 2 h. Beads were washed once with low salt wash buffer (250 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 0.1% SDS, 0.1% Na-deoxycholate, 0.1% Triton X-100), once with...
high salt wash buffer containing 500 mM NaCl, once with LiCl wash buffer (20 mM Tris pH 8.0, 1 mM EDTA, 250 mM LiCl, 0.5% NP-40, 0.5% Na-deoxycholate), once with wash buffer containing 10 mM Tris, 1 mM EDTA, 0.1% Triton X-100 and twice with ice cold 10 mM Tris-HCl. The beads and the input were then incubated at 37°C with the Tagment DNA enzyme (Illumina) following tagmentation. The beads were washed twice with the low salt wash buffer and DNA was eluted with 10 mM Tris, 1 mM EDTA, 250 mM NaCl and 0.3% SDS. Cross-links were reversed by initial incubation at 55°C and continued with protein digestion with the addition of Proteinase K (New England Biolabs, Boston, MA) for 10 h at 64°C. DNA was purified using a DNA Clean & Concentrator-5 Kit (Zymo Research, Irvine, CA).

ChIP data was processed using Genialis platform and the data analysis pipeline resembles the AQUAS pipeline (https://github.com/kundajelab/chipseq_pipeline). ChIP-seq, ChIPmentation, and ChIP-exo undergo the same procedure: Reads are aligned to hg19 genome using BWA-ALN. Then reads are filtered and only mapped reads, primary alignments, and reads with mapping quality greater than 30 are kept. In case of paired-end reads only properly mapped pairs are kept. Subsequently, duplicate reads are removed using picard-tools MarkDuplicates and samtools. Fragment length is estimated using spp. Finally, peaks are called using MACS2 with p-value set to $1 \times 10^{-6}$ and without building a model, using the previously estimated fragment length.

**Omni Assay for Transposase-Accessible Chromatin (Omni-ATAC)**

Omni ATAC-seq was performed as previously described (Corces et al., 2017). 50,000 live cells in culture medium were treated with DNase at a final concentration of 200 U/mL at 37°C for 30 minutes. The cells were washed thrice with PBS to remove DNase followed by lysis with RSB (10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl$_2$) containing 0.1% NP40, 0.1% Tween-20, and 0.01% Digitonin and mixed by pipetting up and down thrice. The cells were lysed on ice for 3 min and the reaction was stopped by adding 1 mL of ice cold RSB containing 0.1% Tween-20 and mixed by inverting the tubes thrice. The nuclei were pelleted by centrifugation at 4°C and the pellet was resuspended in 50 μL of transposition mix (25 μL 2x TD buffer, 2.5 μL transposase, 16.5 μL PBS, 0.5 μL 1% digitonin, 0.5 μL 10% Tween-20, 5 μL H$_2$O) and was incubated at 37°C for 30 min with mixing. The reaction was then cleaned up with a Zymo DNA Clean and Concentrator-5 Kit.

ATAC data was processed using Genialis platform and the data analysis pipeline resembles the ATAC-seq pipeline (https://github.com/kundajelab/atac_dnase_pipelines). Reads are aligned to hg19 genome using Bowtie2. Then reads are filtered and only mapped reads, primary alignments, properly mapped pairs, and reads with mapping quality greater than 30 are kept. Subsequently, duplicate reads are removed using picard-tools MarkDuplicates and samtools. Then Tn5 transposon shifting is performed. Finally, peaks are called using MACS2 with p-value set to 0.01 and without building a model, using the fragment length of 150 and shiftsize of 75.

**Library Preparations**

Library preparations for the ChiP and ATAC samples were performed as previously described (Buenrostro et al., 2015) with minor changes. In brief, to generate multiplexed libraries, a preamplification cycle was performed with the transposed DNA. The DNA was amplified for five PCR cycles with Illumina indexed primers (Nextera index kit, Illumina), 25 μL of NEBNext High-Fidelity 2x PCR Master Mix (New England Biolabs). 5 μL of this amplified DNA was amplified further using qPCR to determine the appropriate number of PCR cycles needed to achieve maximum amplification with minimum duplicates. The number of cycles needed was calculated by determining the cycle number that corresponds to 25% of the maximum fluorescence intensity. The final amplification was performed with the leftover (45 μL) from the preamplification reaction using the number of cycles calculated in the qPCR. The amplified libraries were cleaned, and size selected using Ampure XP (Beckman Coulter, Indianapolis, IN) and were validated for quality and size distribution using a TapeStation 2200 (Agilent, Santa Clara, CA). The libraries were sequenced using NextSeq 500/550 High Output Kit v2 (75 cycles) (Illumina) for ChiP-seq and NextSeq 500/550 High Output Kit v2 (150 cycles) (Illumina) for ATAC-seq in a NextSeq 550 (Illumina).

**22Rv1 Human Prostate Cancer Xenograft Model in Male CB17-SCID Mice**

Xenograft studies were performed at WuXi AppTec Co., Ltd., (Shanghai, China).

**Cell Culture**

22Rv1 tumor cells (ATCC, CRL-2505) were maintained in vitro as a monolayer culture in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% Antibiotic-Antimycotic at 37°C in an atmosphere of 5% CO$_2$. The tumor cells were routinely subcultured twice weekly by 0.25% trypsin-EDTA treatment. The cells growing in an exponential growth phase were harvested and counted for tumor inoculation.

**Tumor Implantation and Treatment**

Each mouse (Mus musculus, male: CB17-SCID, age: 6-8 weeks, body weight: 18-22 g) was inoculated subcutaneously at the right flank with 22Rv1 tumor cells (2 x 10$^6$) in 0.2 mL of PBS supplemented with BD Matrigel (1:1) for tumor development. Animals were randomized and treatment started when the average tumor volume reached approximately 170 mm$^3$. Mice were treated at 10 μL/g body weight either with vehicle (10% EtOH, 20% PEG400, and 70% (20% HPβCD), p.o., QD, 21 days), 15 mg/kg Docetaxel (i.p., QW, 3 weeks), 3 mg/kg KB-0742 (p.o., QD, 21 days), 10 mg/kg KB-0742 (p.o., QD, 21 days), 30 mg/kg KB-0742 (p.o., QD, 21 days), or 3 mg/kg KB-0742 (p.o., QD, 21 days (3-day on/4-day off)).
MV-4-11 Human AML Xenograft in Female BALB/c Nude Mice

**Cell Culture**

MV-4-11 tumor cells (ATCC, CRL-9591) were maintained in vitro as a suspension culture in RPMI1640 medium supplemented with 10% heat inactivated fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in an atmosphere of 5% CO₂. The cells growing in an exponential growth phase were harvested and counted for tumor inoculation.

**Tumor Implantation and Treatment**

Each mouse (Mus musculus, female, strain: BALB/c nude, age: 6-8 weeks, body weight: 19-21 g) was inoculated subcutaneously at the right flank with MV4-11 tumor cells (10 x 10⁶) in 0.2 mL of PBS supplemented with BD Matrigel (1:1) for tumor development. The animals were randomized and treatment started when the average tumor volume reached 154 mm³. Mice were treated at 10 μL/g body weight either with vehicle (10% EtOH, 20% PEG400, and 70% (20% HPβCD), p.o., QD, 21 days), 25 mg/kg KB-0742 (p.o., QD, 21 days), 15 mg/kg KB-0742 (p.o., QD, 21 days (3-day on/4-day off)), 30 mg/kg KB-0742 (p.o., QD, 21 days (3-day on/4-day off)), or 60 mg/kg KB-0742 (p.o., QD, 21 days (3-day on/4-day off)).

**Tumor Measurement and Endpoints**

Tumor size was measured twice weekly in two dimensions using a caliper, and the volume was expressed in mm³ using the formula:

\[ V = 0.5 \times a \times b^2 \]

Where \(a\) and \(b\) are the long and short diameters of the tumor, respectively. Tumor growth inhibition (TGI) was calculated for each group using the formula:

\[ \text{TGI} (%) = \left[1 - \frac{(T_i - T_0)}{(V_i - V_0)}\right] \times 100 \]

Where \(T_i\) is the average tumor volume of a treatment group on a given day, \(T_0\) is the average tumor volume of the treatment group on the first day of treatment, \(V_i\) was the average tumor volume of the vehicle control group on the same day with \(T_i\), and \(V_0\) was the average tumor volume of the vehicle group on the first day of treatment. Tumor volume was measured at study termination. The T/C value (in percent) was calculated where \(T\) and \(C\) are the mean tumor volume of the treated and control groups, respectively. Animal body weight was monitored regularly as an indirect measure of toxicity. On day nine of treatment, one mouse of the Docetaxel (15 mg/kg) and one mouse of the KB-0742 (30 mg/kg) treatment regimen were sacrificed due to the body weight loss >20% in the 22Rv1 Human Prostate Cancer Xenograft Model.

**Statistical Analysis**

Summary statistics, including mean and the standard error of the mean (SEM), are provided for the tumor volume of each group at each time point (Tables S3 and S5). Statistical analysis of difference in the tumor volume among the groups was conducted on the data obtained at the best therapeutic time point after the final dose (day 21 of treatment 22Rv1, day 16 of treatment MV-4-11, Tables S4 and S6). A one-way ANOVA was performed to compare the tumor volume and the tumor weights among groups, comparisons between groups were carried out with Games-Howell (22Rv1) or Dunnett t (2-sided) (MV-4-11). All data were analyzed using SPSS 17.0. \(p < 0.05\) was considered to be statistically significant.

**Chemical Synthesis**

**KI-ARv-03 Synthesis**

![Scheme 1: Synthetic Procedure to Yield Probe Molecule KI-ARv-03](image-url)
A solution of 3-Aminopyrazole (14.5 g, 175 mmol) and ethyl 3-oxoethanoate (29.4 mL, 184 mmol) in glacial acetic acid (100 mL) was refluxed for 3 h. After cooling to room temperature, the solvent was removed under reduced pressure and residuals were suspended in EtOAc. The resulting mixture was filtered and the remaining solid was washed with EtOAc (3 x 100 mL) to yield 1 as an off-white solid (25.1 g, 142 mmol, 81%).

**1H NMR** (400 MHz, DMSO-d6): δ 12.22 (s, 1H), 7.82 (d, J = 2.0 Hz, 1H), 6.10 (d, J = 2.0 Hz, 1H), 5.58 (s, 1H), 2.55 – 2.49 (m, 2H), 1.66 (h, J = 7.4 Hz, 2H), 0.91 (t, J = 7.4 Hz, 3H).

**13C NMR** (101 MHz, DMSO-d6): δ 156.53, 153.79, 142.75, 141.71, 94.28, 88.46, 34.21, 21.27, 13.31. **LC-MS (ES+)**: m/z 178.1.

7-chloro-5-propylpyrazolo[1,5-a]pyrimidine (2).

To a suspension of 5-propylpyrazolo[1,5-a]pyrimidin-7-ol (1, 801 mg, 4.52 mmol) in dry MeCN were added phosphorous oxychloride (1.68 mL, 18.1 mmol, dropwise), pyridine (438 µL, 5.42 mmol), and dimethylaminopyridine (28 mg, 0.23 mmol). The resulting suspension was refluxed for 3 h. After cooling to room temperature, the solvent was removed in vacuo and the remaining residue was treated with ice water and immediately extracted with EtOAc (3 x 100 mL). The combined organic layers were dried with Na2SO4 and the crude was purified by silica gel flash column chromatography (0-30% EtOAc/hexane) to yield 2 as yellow/green liquid (611 mg, 3.12 mmol, 69%). The product was used immediately for subsequent reactions.

**1H NMR** (500 MHz, DMSO-d6): δ 8.27 (d, J = 2.3 Hz, 1H), 7.36 (s, 1H), 6.75 (d, J = 2.3 Hz, 1H), 2.77 (t, J = 7.5 Hz, 2H), 1.74 (h, J = 7.4 Hz, 2H), 0.92 (t, J = 7.4 Hz, 3H). **13C NMR** (DMSO-d6): δ 162.27, 149.05, 145.14, 137.33, 108.79, 97.04, 39.08, 21.23, 13.58. **LC-MS (ES+)**: m/z 197.1 and 198.0 [M+H]+

tert-butyl ((1R,3R)-3-((5-propylpyrazolo[1,5-a]pyrimidin-7-yl)amino)cyclopentyl) carbamate (3).

To a solution of 7-chloro-5-propylpyrazolo[1,5-a]pyrimidine (2, 400 mg, 2.04 mmol) in MeCN were added tert-butyl ((1R,3R)-3-aminocyclopentyl)carbamate (429 mg, 2.14 mmol) and K2CO3 (563 mg, 4.08 mmol). The resulting suspension was stirred at 60 °C for 16 h. After cooling to room temperature, the reaction mixture was diluted with water and extracted with DCM (3 x 100 mL). The combined organic layers were dried over Na2SO4 and the crude was purified by flash column chromatography (0-70% EtOAc/hexane) to yield 3 as a light-brown resin (481 mg, 1.34 mmol, 66%).

**1H NMR** (500 MHz, DMSO-d6): δ 7.99 (d, J = 2.2 Hz, 1H), 7.61 (d, J = 7.9 Hz, 1H), 6.97 (d, J = 7.5 Hz, 1H), 6.28 (d, J = 2.3 Hz, 1H), 6.05 (s, 1H), 4.17 (h, J = 7.3 Hz, 1H), 3.97 (h, J = 6.6 Hz, 1H), 2.61 (dd, J = 8.4, 6.7 Hz, 2H), 2.13 (dt, J = 12.6, 7.9, 4.6 Hz, 1H), 2.08 – 1.94 (m, 1H), 1.96 – 1.84 (m, 2H), 1.76 – 1.64 (m, 3H), 1.52 – 1.41 (m, 1H), 1.38 (s, 9H), 0.92 (t, J = 7.3 Hz, 3H). **LC-MS (ES+)**: m/z 360.5 [M+H]+
tert-butyl ((1R,3R)-3-(5-propylpyrazolo[1,5-a]pyrimidin-7-yl)amino)cyclopentyl carbamate (3, 481 mg, 1.34 mmol) was treated with a solution of 4 M HCl in MeOH for 1.5 h at room temperature. The solution was basified with saturated Na2HCO3 solution and extracted with DCM. The combined organic layers were dried over Na2SO4 and the solvent was removed in vacuo to yield KI-ARv-03 as brown syrup (343 mg, 1.33 mmol, 99%).

**1H NMR** (DMSO-d6): δ 7.99 (d, J = 2.3 Hz, 1H), 7.44 (s, 1H), 6.28 (d, J = 2.2 Hz, 1H), 6.03 (s, 1H), 4.21 (t, J = 7.3 Hz, 1H), 3.43 (p, J = 5.8 Hz, 1H), 2.61 (dd, J = 8.4, 6.7 Hz, 2H), 2.20 (dt, J = 12.9, 7.9, 4.9 Hz, 1H), 2.00 – 1.83 (m, 3H), 1.83 – 1.58 (m, 5H), 1.30 (dd, J = 13.2, 8.0, 5.4 Hz, 1H), 0.92 (t, J = 7.4 Hz, 3H).

**13C NMR** (101 MHz, DMSO-d6): δ 162.26, 148.77, 146.00, 142.99, 93.59, 84.91, 51.07, 41.97, 39.84, 34.19, 30.66, 21.93, 13.76.

**LC-MS (ES+): m/z 260.4 [M+H]+**

**KB-0742 Synthesis**

![Scheme 2: Synthetic Procedure to Yield Advanced Molecule KB-0742](image)
overnight. In a second flask MgCl₂ (6.14 g, 64.6 mmol) and potassium 3-ethoxy-3-oxo-propanoate (17 g, 100.1 mmol) were mixed with THF (150 mL) and stirred under argon overnight at 50°C. The resultant white suspension was cooled to rt and solution A was added dropwise over 10 min and the reaction mixture was stirred for 16 h at rt. After several minutes a sticky, amorphous solid appeared whereupon after several hours the reaction mixture becomes homogenous in appearance. The reaction mixture was concentrated to about a third, taken up in half sat. potassium bisulphate solution and extracted twice with ethyl acetate. The combined organic layers were washed with a sat. sodium bicarbonate solution, combined, dried over anhydrous sodium sulfate, filtered and the solvent was evaporated. Purification by column chromatography gave ethyl 4-ethyl-3-oxo-hexanoate (4.3 g, 23.1 mmol, 36%) as a clear liquid. The RM was monitored by TLC (10% EtOAc/hexane, Product Rf = 0.6, SM Rf = 0.1). 1H NMR (400 MHz, DMSO-d₆) δ 4.08 (q, J = 7.1 Hz, 2H), 3.59 (s, 2H), 2.49 – 2.38 (m, 1H), 1.68 – 1.34 (m, 4H), 1.18 (t, J = 7.0 Hz, 3H), 0.84 – 0.75 (m, 6H).

To a suspension of ethyl 4-ethyl-3-oxo-hexanoate (4, 4.4 g, 23.6 mmol) in acetic acid (11 mL) at 70°C was added 1H-pyrazol-5-amine (4.71 g, 56.7 mmol) in two portions (the second portion was added after 2 h of stirring the first portion) over a 4 h period. The reaction was cooled to rt and the solvent was evaporated under reduced pressure. The residue was treated with ethyl acetate and filtered to give 5-(pentan-3-yl)pyrazolo[1,5-a]pyrimidin-7(4H)-one (3.7 g, 17.7 mmol, 75%) as an off-white solid. The reaction mixture was monitored by TLC (5% MeOH/DCM, Product Rf = 0.3, SM Rf = 0.8). 1H NMR (400 MHz, DMSO-d₆) δ 12.05 (s, 1H), 7.84 (s, 1H), 6.12 (s, 1H), 5.59 (s, 1H), 2.42–2.31 (m, 1H), 1.71–1.52 (m, 4H), 0.81 (t, J = 7.4 Hz, 6H).

13C NMR (100 MHz, DMSO-d₆) δ 156.38, 156.31, 142.49, 141.57, 93.60, 88.49, 46.71, 26.30, 11.52. LC-MS (m/z 205.12, found 206.4 [M+H+]).

7-chloro-5-(pentan-3-yl)pyrazolo[1,5-a]pyrimidine (6).

A stirred solution of 5-(pentan-3-yl)pyrazolo[1,5-a]pyrimidin-7(4H)-one (5, 3.7 g, 18.0 mmol) in POCl₃ (33.7 mL, 360.5 mmol) was heated to reflux for 4 h. The reaction mixture was cooled to rt, excess reagent was evaporated in a rotary evaporator, and the residue was treated with ice-water. The chlorinated product was immediately extracted with DCM. The organic layer was separated, dried over anhydrous Na₂SO₄, filtered and purified by column chromatography to yield 7-chloro-5-(pentan-3-yl)pyrazolo[1,5-a]pyrimidine (3.1 g, 13.9 mmol, 77%) as a light yellow liquid. The reaction mixture was monitored by TLC (20% EtOAc/hexane, Product Rf = 0.6, SM Rf = 0.1). 1H NMR (400 MHz, CDCl₃) δ 8.16 (s, 1H), 6.81 (s, 1H), 6.69 (s, 1H), 2.69–2.52 (m, 1H), 1.84–1.64 (m, 4H), 0.87–0.81 (m, 6H).

13C NMR (100 MHz, CDCl₃) δ 165.61, 149.62, 145.25, 138.35, 107.49, 97.50, 51.59, 27.64, 11.99. LC-MS (m/z 223.09, found 224.3 [M+H+]).

 tert-butyl ((1S,3S)-3-((5-(pentan-3-yl)pyrazolo[1,5-a]pyrimidin-7-yl)amino)cyclopentyl) carbamate (7).

To a stirred solution of 7-chloro-5-(pentan-3-yl)pyrazolo[1,5-a]pyrimidine (6, 500 mg, 2.24 mmol) in MeCN (10 mL) tert-Butyl ((1S,3S)-3-aminocyclopentyl)carbamate (447.7 mg, 2.24 mmol) and K₂CO₃ (925 mg, 6.71 mmol) were added and the resulting reaction mixture was heated to reflux for 16 h. The reaction mixture was filtered, concentrated under reduced pressure and purified by column chromatography (30% EtOAc/hexane) to give tert-butyl ((1S,3S)-3-((5-(pentan-3-yl)pyrazolo[1,5-a]pyrimidin-7-yl)amino)cyclopentyl)carbamate (850 mg, 2.19 mmol, 98% yield) as an off-white solid. The reaction mixture was monitored by TLC (40% EtOAc/hexane, Product Rf = 0.5, SM Rf = 0.7). 1H NMR (400 MHz, CDCl₃) δ 7.92 (d, J = 2.3 Hz, 1H), 6.41 (d, J = 2.2 Hz, 1H), 6.17 (d, J = 6.8 Hz, 1H), 5.73 (s, 1H), 4.54 (s, 1H), 4.19–4.06 (m, 2H), 2.53–2.40 (m, 1H), 2.41–2.18 (m, 2H), 2.15–1.99 (m, 2H), 1.78–
1.68 (m, 4H), 1.63–1.49 (m, 1H), 1.44 (s, 9H), 0.84 (t, J = 7.4 Hz, 6H). $^{13}$C NMR (400 MHz, DMSO-$d_6$) δ 165.12, 154.91, 148.83, 145.94, 142.93, 93.66, 84.83, 77.43, 51.18, 50.98, 49.84, 38.40, 31.18, 30.06, 28.19, 27.29, 12.01.

13C NMR (400 MHz, DMSO-$d_6$)

(1S,3S)-N$^1$-(5-(pentan-3-yl)pyrazolo[1,5-a]pyrimidin-7-yl)cyclopentane-1,3-diamine hydrochloride (KB-0742 x 2 HCl).

To tert-butyl ((1S,3S)-3-((5-(pentan-3-yl)pyrazolo[1,5-a]pyrimidin-7-yl)amino)cyclopentyl) carbamate (7, 1.0 g, 2.58 mmol) in 1,4-dioxane (0.2 mL) 4 M HCl in dioxane (3.22 mL, 12.9 mmol) was added and stirred at room temperature for 4 h. The reaction mixture was evaporated in vacuo, triturated with pentane and lyophilized from MeCN:H$_2$O to yield (1S,3S)-N$^1$-(5-(pentan-3-yl)pyrazolo[1,5-a]pyrimidin-7-yl)cyclopentane-1,3-diamine hydrochloride (0.9 g, 2.5 mmol, 97% yield) as a pale-yellow sticky solid. The reaction mixture was monitored by TLC (100% EtOAc, Product R$_f$ = 0.1, SM R$_f$ = 0.8).

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 15.00 (s, 1H), 9.93–9.86 (m, 1H), 8.51 (s, 3H), 8.30 (s, 1H), 6.84 (s, 1H), 6.58 (s, 1H), 4.95 (q, J = 7.8 Hz, 1H), 3.77–3.66 (m, 1H), 2.84–2.71 (m, 1H), 2.29–2.05 (m, 4H), 1.94–1.63 (m, 6H), 0.81 (t, J = 7.4 Hz, 6H). $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 161.23, 148.95, 145.40, 139.54, 91.18, 85.59, 52.49, 49.15, 47.47, 38.80, 29.85, 28.96, 26.77, 11.70.

LC-MS (m/z 287.21, found 288.0 [M+H$^+$]).

(1S,3S)-N$^1$-(5-(pentan-3-yl)pyrazolo[1,5-a]pyrimidin-7-yl)cyclopentane-1,3-diamine (KB-0742).

To (1S,3S)-N$^1$-(5-(pentan-3-yl)pyrazolo[1,5-a]pyrimidin-7-yl)cyclopentane-1,3-diamine hydrochloride (0.2 g, 0.56 mmol) aqueous NH$_3$ (4.0 mL, 0.56 mmol) was added and the resulting reaction mixture stirred at rt for 4 h. The reaction mixture was evaporated in vacuo, triturated with pentane and lyophilized from MeCN:H$_2$O to give (1S,3S)-N$^1$-(5-(pentan-3-yl)pyrazolo[1,5-a]pyrimidin-7-yl)cyclopentane-1,3-diamine (140 mg, 0.49 mmol, 87.8% yield) as a pale-yellow sticky solid. The reaction mixture was monitored by TLC (100% EtOAc, Product R$_f$ = 0.1, SM R$_f$ = 0.1). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.95 (d, J = 2.2 Hz, 1H), 6.86 (d, J = 2.2 Hz, 1H), 5.95 (s, 1H), 4.31–4.19 (m, 1H), 3.57–3.44 (m, 1H), 2.52–2.44 (m, 1H), 2.36–2.22 (m, 1H), 2.09–1.79 (m, 3H), 1.80–1.59 (m, 5H), 1.58–1.24 (m, 3H), 0.83 (t, J = 7.4 Hz, 6H). $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 165.12, 148.82, 145.89, 142.90, 93.65, 84.85, 51.50, 50.99, 50.92, 41.74, 34.00, 30.53, 27.27, 11.98.

LC-MS (m/z 287.21, found 288.5 [M+H$^+$]).

QUANTIFICATION AND STATISTICAL ANALYSIS

For relevant experiments, number of replicates, error bars, statistical test, and P values are noted in the respective figure legends and in the corresponding experimental methods sections.