

Title: Development and Characterization of a Novel Small Molecule KDM6A Inhibitor for Targeting Castration-Resistant Prostate Cancer

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BACKGROUND Androgen deprivation therapies (ADT) although effective, fail to impede progression of prostate cancer (PC) to the lethal castration-resistant prostate cancer (CRPC) stage. Prostate cancers exhibit an ability to rapidly alter their epigenetic programs in response to ADT as well as other combination therapies. Among the pathways dysregulated in drug-resistant lethal prostate cancers are often those associated with increased transcriptional output such as AR, HOXB13 and Lysine-specific demethylases (KDMs). Among the KDMs, KDM6A, a H3 lysine (K) 27 (H3K27me3) demethylase is found to be upregulated in CRPCs. KDM6A erases transcriptionally repressive histone H3 lysine (K) 27 (H3K27me3) marks to promote gene activation. Preliminary analysis revealed elevated expression of KDM6A, a lysine-specific demethylase 6A, in CRPC patients that correlates with increased AR expression and at sites of tumor invasion. While a potent Jumonji H3K27 demethylase inhibitor, GSK-J4, was previously identified that targeted multiple KDM6 subfamily members including KDM5A, it is not specific. The lack of a specific KDM6A inhibitor led us to screen for novel KDM6A-specific inhibitors that compromised CRPC xenograft tumor growth and examine their mechanism of action.

METHODS Chromatin immunoprecipitation, quantitative RT-PCR, immunoblotting, immunohistochemistry, single-cell and bulk RNA sequencing, bioinformatic analysis, human prostate tissue, xenograft tumor growth inhibition, flow cytometry and statistical analysis.

RESULTS KDM6A antagonizes deposition of the transcriptionally repressive H3K27 methylation marks at an *AR* enhancer to promote its expression, uncovering a new mechanism of *AR* gene regulation. To target KDM6A as selective inhibitors are not available, we performed a high throughput screening of 450,000 compounds and have identified a small subset of compounds with histone demethylase inhibitory activity. We undertook structure activity relationship studies and generated multiple derivatives of selective inhibitors to improve potency which were then again tested for their activity against KDM6A. A compound KNM2 was identified as a top 'hit' that exhibited high potency (IC₅₀ 1.3 μM). We discovered KNM2 shows high specificity for KDM6A. It inhibits KDM6A demethylase activity but not any other KDMs including the closely related ortholog, KDM6B. To identify its mechanism of action in prostate cancer, we performed RNA sequencing and chromatin-immunoprecipitation analysis in PC cells treated with KNM2. We not only uncovered AR as a major target of KNM2 in CRPCs but observed that this compound had potent anti-proliferative activity. Further, KNM2 significantly inhibits CRPC xenograft tumor growth.

CONCLUSIONS Our studies credential KDM6A inhibitor, KNM2 as a new therapeutic modality to target lethal prostate cancers. These pre-clinical studies have advanced KNM2 to investigational new drug studies for the treatment of castration-resistance prostate cancers.

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CONFLICT OF INTEREST K.M. and N.M. are co-founders of Technogenesys, a startup company that controls the intellectual property and patents on the ACK1 inhibitor (R)-9b. Washington University has filed for intellectual property for KNM2.