Targeting prostate cancer with compounds possessing dual activity as androgen receptor antagonists and HDAC6 inhibitors

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Abstract
While enzalutamide and abiraterone are approved for treatment of metastatic castration-resistant prostate cancer (mCRPC), approximately 20–40% of patients have no response to these agents. It has been stipulated that the lack of response and the development of secondary resistance to these drugs may be due to the presence of AR splice variants. HDAC6 has a role in regulating the androgen receptor (AR) by modulating heat shock protein 90 (Hsp90) acetylation, which controls the nuclear localization and activation of the AR in androgen-dependent and independent scenarios. With dual-acting AR–HDAC6 inhibitors it should be possible to target patients who don’t respond to enzalutamide. Herein, we describe the design, synthesis and biological evaluation of dual-acting compounds which target AR and are also specific towards HDAC6. Our efforts led to compound 10 which was found to have potent dual activity (HDAC6 IC50 = 0.0356 μM and AR binding IC50 = <0.03 μM). Compound 10 was further evaluated for antagonist and other cell-based activities, in vitro stability and pharmacokinetics.

It is widely recognized that chemotherapy drugs are most effective when given in combination. The rationale for combination therapy is to harness disparate mechanisms, thereby reducing the likelihood of resistance. Deriving from the same principles, it is possible to have a single molecule with dual activity. Cabozantinib is a small molecule tyrosine kinase inhibitor of cMet and VEGFR2; this dual activity has culminated in US FDA approvals for medullary thyroid cancer and advanced renal cell carcinoma and it is currently being tested in the clinic for various other cancers, including prostate cancer (PC).1 At the research level, a recent Letter describes the synthesis and evaluation of dual-acting estrogen receptor (ER) and histone deacetylase (HDAC) inhibitors (HDACi).2,3 These ER–HDAC inhibitors combined ERα antagonist activity with potent HDAC inhibitor activity, resulting in better anti-tumor efficacy in ERα positive breast cancer cells in vitro when compared to the approved drug Tamoxifen.

HDACs modulate histone acetylation, which controls gene expression. HDAC inhibitors have been studied and tested in cancer treatment with numerous agents approved and others undergoing clinical trials.4,5 HDAC6 has been implicated in the pathogenesis and treatment of cancer6 and its role in regulating the androgen receptor (AR) by modulating heat shock protein 90 (Hsp90) acetylation has also been studied.7,8 Hsp90 acetylation controls the stability, nuclear localization and activation of the AR in androgen-dependent and independent scenarios. Inhibition of HDAC6 therefore provides an opportunity to target castration resistant prostate cancer.6–9 Enzalutamide has proven to be clinically beneficial in metastatic castration-resistant prostate cancer (mCRPC).10–12 While enzalutamide and abiraterone are approved for treatment of mCRPC, approximately 20–40% of patients have no response to these agents. Moreover, it has been stipulated that the lack of response and the development of secondary resistance to these drugs may be due to the presence of AR splice variants.14 Meanwhile, the clinical evaluation of HDAC inhibitors as monotherapy for prostate cancer has not been promising. However using a
In the design of our dual AR–HDAC6 inhibitors, the intent was to maintain AR antagonist activity, while also inhibiting HDAC6. We first examined the binding mode of enzalutamide, which when compared to conventional agents like Bicalutamide, binds to the AR with higher affinity and demonstrates pure antagonist activity in preclinical models. After preparing the 3D structure of enzalutamide using LigPrep, we performed IFD to the ligand binding domain (LBD) of human AR (pdb code: 1T63) to understand its binding mode. The computational methods used are described in Supporting information (SI) section. Figure 1a illustrates the key interactions in 2D, highlighting the hydrophobic nature of the LBD and active site hydrogen bonds. The poses obtained from induced fit docking (IFD) shows that the trifluoromethyl group makes favorable van Der Waals (vDW) contacts with hydrophobic residues Val746, Met749, Phe764 and Leu873. Also the A ring of enzalutamide (Fig. 1b) forms a T-shaped pi-pi stacking interaction with Phe764. The cyano group forms H-bonds with two key active site residues—Arg752 and amide of Gln711. This group occupies the same position as the keto group in the endogenous substrate dihydrotestosterone (DHT). The amide oxygen is involved in an H-bond with Asn705. We also observed that the methyl amide part of enzalutamide points towards Helix12 (Fig. 1b) of the LBD, which was crucial for our design strategy moving forward.

In order to synthesize the proposed compounds, we prepared two key intermediates 18 and 19. The synthesis of compound 18 has been reported; a similar process was used to synthesize the bromophenyl compound 19. The routes for the synthesis of the target molecules are shown in Scheme 1. The details of the syntheses can be found in SI.

Our evaluation of the synthesized compounds began with binding assays for AR and HDAC family proteins (Table 1). To identify activity against HDAC family proteins we screened for inhibition of several Class I HDACs (HDAC1, 2, and 3) and our target, the Class IIb HDAC6 using a fluorogenic readout of de-acetylation of target peptides to quantitate de-acetylation activity. While pan–HDAC inhibitors have shown therapeutic promise, we focused on the described synergy of HDAC6 specific inhibition with an AR
antagonist to minimize toxicity issues and maximize the therapeutic benefit. The results are listed in Table 1 with Trichostatin A used as a positive control as a pan–HDAC inhibitor. The hybrid conjugates with hydroxamic acids as zinc binding groups (compounds 8–10) showed HDAC6 specific inhibition while retaining AR antagonistic activity. Replacing the linker amide or ester in 8 and 9 with methylene groups as in 13, tends to reduce HDAC6 binding activity. Among the other zinc chelating moieties tested, the sulfamide (6) may also provide an interesting starting point for optimization of a dual inhibitor of AR and HDAC6. Focusing on the more potent hydroxamic acid leads, we recognized that compound 8 (an ester) may not be metabolically stable in vivo. While it provided an interesting SAR point, we decided to follow-up on the amide analog 10 to gain further insights about the potential of such dual-acting compounds.

We tested for AR binding using a radio-ligand binding assay, where compounds were examined for the ability to inhibit

![Figure 2. Compound designs incorporating enzalutamide for AR binding and a Zn-chelating group to bind HDAC.](image)

![Figure 3. Structures of compounds to target AR and HDAC6.](image)
The binding of [3H]-mibolerone to AR purified from membrane fractions was measured. All the compounds have sub-micromolar AR binding affinity, as shown in Table 1. This was not surprising, considering our design had maintained most of the enzalutamide structure intact. Our lead HDAC6 selective compounds (8–10) also show potent AR binding affinity (Table 1). Compound 8 has a binding affinity to AR that is comparable to that published for enzalutamide at 21 nM.

In order to understand the binding and HDAC6 selectivity of our lead compounds, the recently determined crystal structure of HDAC6 (pdb code: 5G0J) was used as a starting point for docking calculations. The raw X-ray coordinates for the structure of HDAC6

<table>
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<th>Compound ID</th>
<th>HDAC1 IC50 (μM)</th>
<th>HDAC2 IC50 (μM)</th>
<th>HDAC3 IC50 (μM)</th>
<th>HDAC6 IC50 (μM)</th>
<th>AR binding IC50 (μM)</th>
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was subjected to additional refinement steps using the protein preparation wizard as discussed in SI. The ligands were prepared using the LigPrep workflow with a metal binding state for the ligands. Default IFD workflow was used for docking. Docking of the ligands. Default IFD 27 workflow was used for docking. Docking of the ligands was carried out using Prime MM-GBSA method. Calculations were done on the free ligand and on the ligand bound in the geometry it adopts in the HDAC6 complex, both with implicit solvent present. The strain energy is the difference between the two energies. Qualitatively, we observe a good correlation between ligand strain and measured IC50 values. For example, compound 8 (IC50 = 0.002 μM) has a ligand strain of 3.4 kcal/mol as compared to a weaker binder such as compound 13 (IC50 = 0.17 μM), which has a ligand strain of 9.6 kcal/mol. The increased ligand strain of compound 13 can be explained by the flexibility in the linker in compound 13, compared to the amide linker of compound 8. Based on the predicted binding mode, compound 9 (IC50 = 0.5 μM) has a hydrophilic O which may not be ideal to fit in the hydrophobic channel lined by residues-F583 and F643, which may result in lower potency. We proceeded to evaluate the cell-based activity of selected compounds in two key assays. First, we tested the potential AR antagonist activity by using a cell line, MDA-kb2, that stably expresses an androgen-responsive firefly luciferase reporter. To this end, we co-incubated cells with DHT and different concentrations of our compounds (Fig. 5), suggesting that the inherent antagonist activity associated with the AR-targeted portion of the molecule remains intact. To further determine the effects of AR antagonists and differential inhibition of HDAC activity, we then tested several agents for the effects of a 24 h treatment on the LNCaP prostate cancer cell line stimulated with DHT (Fig. 6a). Treatment with enzalutamide alone did not alter AR or acetylation levels of tubulin, as expected. The benzamide HDAC-1 and -3 inhibitor MS-275 did not affect AR levels, and mildly increased tubulin acetylation. The broad spectrum HDACi SAHA resulted in a lowering of AR levels, possibly due to inhibition of general transcriptional activity, and a dramatic increase in alpha-tubulin acetylation. The HDAC6 specific inhibitor, Tubastatin A, demonstrated a decrease in AR protein levels concomitantly with an increase in tubulin acetylation, supporting the reported AR–HDAC6 link.

We tested the effects of the dual AR–HDAC6 inhibitor molecules (8 and 10) as well and observed a reduction in the steady state AR protein level compared to the controls (Fig. 6b). Likewise, we observed hyperacetylation of tubulin compared to control, indicating that compounds 8 and 10 also have HDAC inhibitory activity in a cell-based assay. In these cell based assays the levels of AR was normalized using the ubiquitously expressed cytoskeletal protein tubulin.

Figure 4. Glide docking of compound 10 to HDAC6 X-ray structure. The hydroxamate forms bi-dentate interaction with Zn\(^2+\). The linker occupies a rather hydrophobic channel lined by residues-F583 and F643. An additional H-bond with the backbone amide of G582 is seen as well. The cap region forms a hydrogen bond with H462 from the loop L1.
Microsomal stability and glucuronidation potential of compound 10

<table>
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<th>Compound #</th>
<th>Average human LM (%) rem</th>
<th>Average mouse LM (%) rem</th>
<th>Average hUGT (%) rem</th>
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</table>

* Methods are described in Supporting information.

Systemic clearance greater than hepatic blood flow was observed for compound 10 in mice which was approximately consistent with its high in vitro metabolic turnover in the mouse microsomal stability assay. Similar systemic exposure was achieved by the IP and SC routes of administration, suggesting that either route of administration would be acceptable for preclinical testing.

We have described efforts that have led to the discovery and in vitro and in vivo evaluation of compound 10, a dual inhibitor of AR and HDAC6. The hydroxamic acid, which is key to HDAC inhibition is a known liability; such compounds are associated with poor pharmacokinetics and lack of selectivity among the HDAC isozymes. Compound 10 may be a useful tool compound in pharmacology studies to evaluate the value of dual inhibition of AR and HDAC6. It would have to be dosed via SC or IP route to enable sufficient exposure in vivo. Recognizing the value of non-hydroxamic containing HDAC inhibitors, we will focus future efforts to develop the hit compound 6. Compound 6 embodies a sulfamide as a Zn binding moiety, replacing the hydroxamate group. Docking of compound 6 into HDAC6 shows the sulfamide group engaging in a mono-dentate chelation of Zn, in addition to other interactions with the protein (Fig. S1 in SI). Compounds containing sulfamide have been previously shown to be active against Zn containing enzymes, such as human carbonic anhydrase II (for reference binding mode, see PDB code: 4FUS). Compound 6 is active as a dual inhibitor (HDAC6 IC50 = 1 µM, AR IC50 = 0.1 µM) and is selective for HDAC6 (>100 fold over the other isozymes tested), providing a good starting point for optimization to a lead compound.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at [http://dx.doi.org/10.1016/j.bmc.2016.09.058](http://dx.doi.org/10.1016/j.bmc.2016.09.058).

References and notes


