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Small-molecule androgen receptor downregulators as an approach to treatment of advanced prostate cancer

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ABSTRACT

Chemical starting points were investigated for downregulation of the androgen receptor as an approach to treatment of advanced prostate cancer. Although prototypic steroidal downregulators such as **6a** designed for intramuscular administration showed insufficient cellular potency, a medicinal chemistry program derived from a novel androgen receptor ligand **8a** led to 6-[4-(4-cyanobenzyl)piperazin-1-yl]-3-(trifluoromethyl)[1,2,4]triazolo[4,3-b]pyridazine (**10b**), for which high plasma levels following oral administration in a preclinical model compensate for moderate cellular potency.

Prostate cancer is the second leading cause of death from cancer among men in developed countries, and was projected to account for 28% of newly-diagnosed cases and 11% of deaths due to cancer in the USA in 2010.[1] The androgen receptor (AR), a ligand binding transcription factor in the nuclear hormone receptor super family, is a key molecular target in the etiology and progression of prostate cancer.[2–4] Binding of the endogenous AR ligand dihydrotestosterone (DHT, **1**) (Fig. 1) stabilizes and protects the AR from rapid proteolytic degradation. The early stages of prostate cancer tumor growth are androgen dependent and respond well to androgen ablation,[2–4] either via surgical castration or by chemical castration with a luteinizing hormone releasing hormone agonist in combination with an AR antagonist, such as hydroxyflutamide (**2a**) or bicalutamide (**2b**).

Although introduction of androgen deprivation therapy represented a major advance in prostate cancer treatment, recurrence within 1–2 years typically marks transition to the so-called castrate-resistant state, in which the tumor continues to grow in the presence of low circulating endogenous ligand and is no longer responsive to classical AR antagonists.[2–4] Castrate-resistant prostate cancer is a largely unmet medical need with a 5 year survival rate of less than 15%, and docetaxel is currently the only treatment shown to provide even minimal survival benefit.[5]

Recent evidence from both preclinical and clinical studies is consistent with the importance of reactivation of AR signaling in a majority of castrate-resistant prostate tumors.[3,4] It is also well established that the functional AR in castrate-resistant tumors is frequently mutated or amplified, and that over-expression can convert hormone responsive cell lines to hormone refractory.[2–4] Recent second-generation AR antagonists have been designed that retain antagonism in over-expressing cell lines, and among these agents MDV3100 has now progressed to late-stage clinical trials in patients with advanced prostate cancer.[6–8]

By analogy with fulvestrant (**3**),[9] an estrogen receptor (ER) downregulator approved by the FDA in 2002 for treatment of advanced breast cancer and initially characterized as a pure ER antagonist, a ligand which downregulates the AR represents one

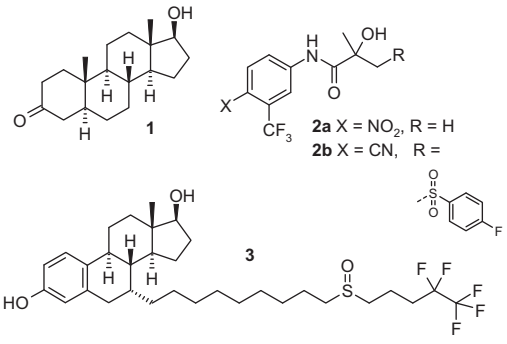

Figure 1. Chemical structures of DHT (**1**, endogenous AR ligand), hydroxyflutamide (**2a**) and bicalutamide (**2b**) (AR antagonists) and fulvestrant (**3**, ER receptor antagonist and downregulator).

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of a number of potential approaches[3,4] to treatment of hormone-refractory prostate cancer via a sustained reduction in tumor AR content. In contrast with direct intervention at the AR, several of these approaches involve indirect mechanisms such as HDAC or HSP90 inhibition.[3,4] At the outset of our work, we were aware of publications and patent applications describing steroidal[10,11] and non-steroidal[12] derivatives as AR destabilizers or downregulators, but to our knowledge no definitive data have been published that characterize the mode of action of these compounds. More recently, androgen receptor inactivation has been shown to contribute to the antitumor efficacy of steroidal derivative VN/124-1, a potent inhibitor of steroidal biosynthesis.[13]

Fulvestrant is a highly potent downregulator of the ER, formally derived from attachment of a long side chain containing a terminal pentafluoropentyl sulfoxide at the 7α-position of the endogenous ER ligand estradiol. The low oral bioavailability and high presystemic metabolism of fulvestrant preclude conventional routes of administration, and a long-acting intramuscular depot formulation was developed that provides the sustained exposure required for clinical efficacy.[14]

In anticipation that AR downregulators of comparable potency to fulvestrant could be identified, we initially sought a steroidal derivative that would be suitable for intramuscular administration. In this letter we describe how the modest downregulatory potency of prototype steroidal compounds led us to seek a chemical starting point commensurate with high oral exposure in order to compensate for compromised potency.

Illustrative compounds prepared during the course of this work are listed in Table 1, and synthetic routes are outlined in Schemes 1 and 2.[15] Prototype steroidal compounds **6a–b** derived from the potent AR ligands nortestosterone[16] and testosterone were obtained as outlined in Scheme 1, via a sequence involving as the key step copper-catalyzed conjugate 1,6-addition to precursor dienones **4a–b** of the Grignard reagent derived from the side-chain building block used in the process synthesis of fulvestrant.[17] Oxidation and deprotection of the intermediate thioethers then gave **6a–b**.

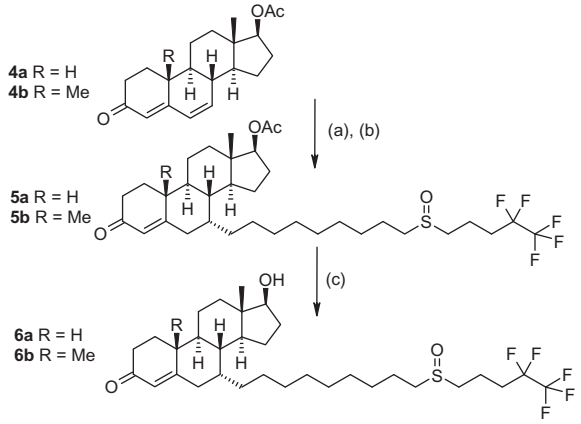
Novel AR binder **8a** and the corresponding piperazine derivative **8c** were obtained (Scheme 2) from 6-chloro-3-(trifluoromethyl)[1,2,4]triazolo[4,3-b]pyridazine **7**.[18] As part of the synthesis of wider compound libraries, AR downregulators **9a–d** and **10a–b** were also obtained from **7** by direct displacement with the appropriate secondary amine.

Binding of compounds listed in Table 1 to the isolated ligand-binding domain of recombinant rat AR was determined using a fluorescence polarization assay.[15,19] Also included as standards in Table 1 are functionally active AR ligands DHT and bicalutamide. Central to evaluation of compounds listed in Table 1 as AR downregulators was development of a novel and innovative microtitre plate-based mode of action assay.[15] By use of a fluorescent goat anti-mouse IgG secondary antibody to detect the immunoreactivity of a mouse anti-human AR monoclonal antibody (clone 441) that is specific for nuclear AR,[20] levels of AR in human LNCaP prostate cancer cells in response to compound were quantified by immunofluorescence detected by a TTP Acumen Explorer HTS Reader.

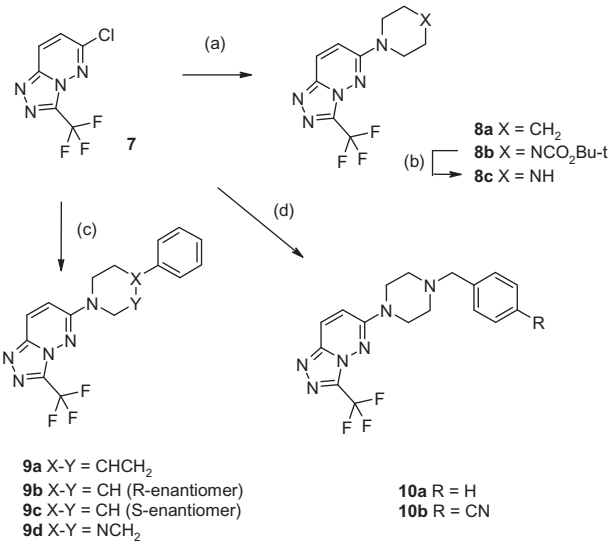
Table 1
AR binding[15,19] and cellular downregulation[15] data for **1, 2b, 6a–b, 8a, 8c, 9a–d, 10a–b**

Entry	AR binding pIC_{50}[a]	AR downregulation pIC_{50}[a]
1 (DHT)	8.39	<3.5
2b (Bicalutamide)	6.55	<3.5
6a	5.1	6.02
6b	4.8	5.66
8a	6.20	<3.5
8c	<4.1	<4.3
9a	4.9	5.95
9b	4.48	6.22
9c	4.88	<4.5
9d	<4.2	5.36
10a	<4.2	5.72
10b	<4.1	5.82

[a] $n \geqslant 3$, SEM values are available in the Supplementary data.



Scheme 1. Synthesis of compounds **6a–b**. Reagents and conditions: (a) $Br(CH_2)_9S(CH_2)_3CF_2CF_3$,[17] Mg, CuCl, THF, −30 °C; (b) $NaIO_4$, $MeOH/H_2O$, 20 °C; (c) NaOH, $MeOH/H_2O$, 20 °C.



Scheme 2. Synthesis of compounds **8a, c, 9a–d, 10a–b**. Reagents and conditions: (a) Piperidine or *t*-butyl piperazine-1-carboxylate, DIPEA, EtOH, 70 °C; (b) TFA, DCM, 20 °C; (c) 4-Phenylpiperidine, *R* or *S* 3-phenylpyrrolidine or 1-phenylpiperazine, DIPEA, EtOH, 70 °C; (d) *N*-Benzyl piperazine or N-(4-cyanobenzyl)piperazine, DIPEA, DMF, 70 °C.

In contrast with binding of fulvestrant to the ER,[9] prototypic steroidal derivatives **6a–b** were significantly weaker AR binders than the endogenous ligand DHT, and these compounds were only moderately potent AR downregulators in LNCaP cells. As mentioned later for compound **10b**, more detailed studies with **6a** were consistent with a mechanism of action involving downregulation of the AR. We anticipated that variation of the length and fluorination of the pendant 7α-sulfoxide substituent might lead to a significant improvement in downregulatory potency, but an initial set of approximately 20 compounds derived from testosterone and nortestosterone gave flat cellular SAR (data not shown).

Before progression to anti-tumor models, efficacy in vivo was assessed using the Hershberger assay,[21] a longstanding model used in the discovery of the AR antagonist bicalutamide,[22] in which effects on accessory sex organ weight in immature castrated

rats stimulated with testosterone propionate serve as a marker for intervention via the AR. On intramuscular administration for 4 days at the highest dose compatible with formulation (82 μmol/kg/day), compound **6a** gave no response in a Hershberger model in which bicalutamide (4.6 μmol/kg) was used as positive control, an observation consistent with analysis of plasma samples that showed a mean steady state concentration of **6a** (0.4 μM) below the cellular downregulatory IC_{50}.

By way of comparison, the ER downregulator fulvestrant is typically active in preclinical rodent models at doses of 0.4–4 μmol kg/day,[23] and monthly 412 μmol intramuscular depot administration in breast cancer patients gives sustained exposure that exceeds the minimum efficacious concentration for 28 days.[14] We concluded that the cellular potency of compound **6a** was approximately 2–3 orders of magnitude lower than required for intramuscular administration to patients, and that flat SAR implied the feasibility of attaining the required potency was low.

Although bicalutamide is only a moderately potent AR binder and antagonist, high plasma levels following oral administration in preclinical models and patients compensate for modest potency.[24] We therefore initiated a new medicinal chemistry program to seek downregulators of comparable potency to prototype **6a**, but with ADME properties commensurate with high exposure after oral dosing.

In order to identify novel, drug-like AR binding cores, a directed high-throughput screening set was generated. A computational approach was used that focused on covering a drug-like productive subspace with low complexity compounds identified via a two-dimensional pharmacophore model chosen to correspond to known AR ligands such as DHT and bicalutamide, in which a terminal hydrogen bond acceptor is located in or adjacent to a ring.

A hit rate of 1.7% was obtained[25] when the resulting set of 100,000 compounds was evaluated at a concentration of 10 μM in a directed high-throughput screen that measured affinity for the rat AR-ligand binding domain by fluorescence polarization.[15,19] Determination of IC_{50} values identified 6-(1-piperidinyl)-3-(trifluoromethyl)-[1,2,4]triazolo[4,3-b]pyridazine **8a** as a novel and moderately potent AR binder (pIC_{50} 6.2) that appeared an attractive chemical starting point (MWt 271, measured LogD 3.0). Excellent selectivity was seen versus a core panel of nuclear hormone receptor binding assays (pIC_{50} <4 vs ERα, PR and GR).

A plausible binding mode for novel AR binder **8a** is obtained by overlaying the hydrogen bond acceptor of the fused 1,2,4-triazole moiety and the trifluoromethyl substituent with the corresponding nitro and trifluoromethyl groups in the published structure of hydroxyflutamide **2a** bound to the AR (Fig. 2).[26]

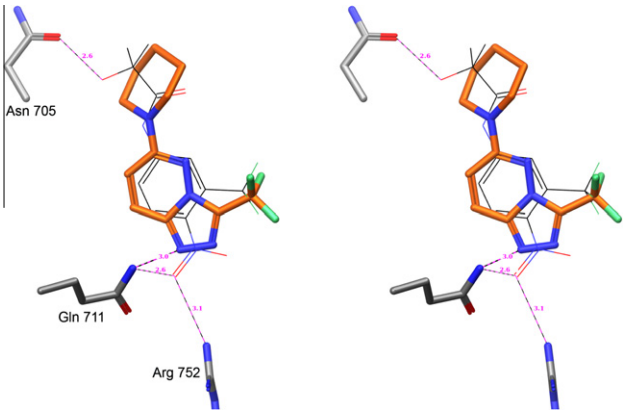


Figure 2. Stereo-diagram of potential binding mode for compound **8a** (thick orange lines) superimposed on reference structure of hydroxyflutamide **2a** bound to the AR ligand binding domain (thin black lines, pdb code 2ax6[26]). Potential hydrogen bonds are shown in dotted magenta lines.

To explore the effect on AR downregulation, libraries comprising several hundred compounds in total were synthesized, either from 6-chloro-3-(trifluoromethyl)[1,2,4]triazolo[4,3-b]pyridazine **7** and available cyclic secondary amines, or via reductive amination of piperazine **8c** (data not shown). Appending a phenyl substituent in the piperidine ring (**9a**) reduced AR binding but gave moderately potent downregulation. A significant difference in downregulation potency was seen for the corresponding pair of enantiomeric phenyl pyrrolidine dervatives (**9b–c**), implying the directionality of the aryl group is crucial for the interaction that leads to downregulation of the AR.

Although the unsubstituted piperazine (**8c**) showed no detectable AR binding, appending a phenyl substituent (**9d**) again converted the motif to a downregulator. From a further synthesis iteration, benzyl piperazine derivatives (**10a–b**) were obtained that are comparable in downregulatory potency to prototype steroid **6a**. Although binding of **10a–b** could not be detected in the fluorescence polarization assay, data determined from an assay using the full length AR binding domain[27] were consistent with weak but reproducible binding (66% inhibition at 10 μM concentration for **10b**). Data from human PC3 cell reporter assays[28] showed that whereas the novel AR binder **8a** is a functional AR agonist (pIC_{50} 5.0), downregulators such as **10b** are devoid of agonist activity. A proposed binding mode for **10b** is similar to that of **8a** (Fig. 2), but with the cyanobenzyl moiety orientated towards the Helix 12 region of the AR ligand binding domain, an interaction that is believed to play a key role in modulating receptor function.[26,29]

Compound **10b** showed low clearance and high oral bioavailability in a low dose rat pharmacokinetic study (Table 2). Exposure scaled well to higher doses and the compound binds only moderately to plasma proteins. As illustrated in Figure 3, compound **10b** dosed orally at 258 μmol/kg twice daily in the Hershberger model for 7 days caused a significant inhibition of testosterone-induced growth of rat seminal vesicles, the magnitude of effect being comparable to that seen with bicalutamide dosed at 4.6 μmol/kg.

Analysis of plasma samples 18 h subsequent to administration of the final dose of **10b** in the Hershberger model showed a concentration of compound **10b** (17 μM) that significantly exceeds the steady state exposure seen after intramuscular administration of prototypic steroidal downregulator **6a**, and corresponds to a terminal free plasma concentration of **10b** that is comparable to the IC_{50} for AR downregulation. Pharmacological studies confirming the mechanism of action of compound **10b** will be detailed in a future publication, along with data from rodent tumor models of human prostate cancer. We believe that although compounds like **10b** bind only weakly to the AR, the binding interaction promotes modulation of AR levels.

In summary, we have investigated chemical starting points for downregulation of the AR, an approach to treatment of advanced prostate cancer analogous to the clinically precedented use of the ER downregulator fulvestrant to combat advanced breast cancer. Prototypic steroidal downregulators such as **6a** designed for intramuscular administration showed insufficient cellular potency. However, a medicinal chemistry program derived from a novel AR ligand led to 6-[4-(4-cyanobenzyl)piperazin-1-yl]-3-(trifluoromethyl)[1,2,4]triazolo[4,3-b]pyridazine (**10b**), in which high plasma levels following oral administration in the Hershberger in vivo model compensate for moderate cellular potency. Further work leading to a clinical candidate[30] will be described in due course.

Table 2
Rat pharmacokinetic parameters for **10b**[a]

Species	% Free	Vdss l/kg	Cl ml/min/kg	Bioavailability %
Rat[a]	9.2	2.4	9.0	100

[a] Male Alderley Park Han Wistar rats dosed at 4 μmol/kg i.v. and 10 μmol/kg p.o.

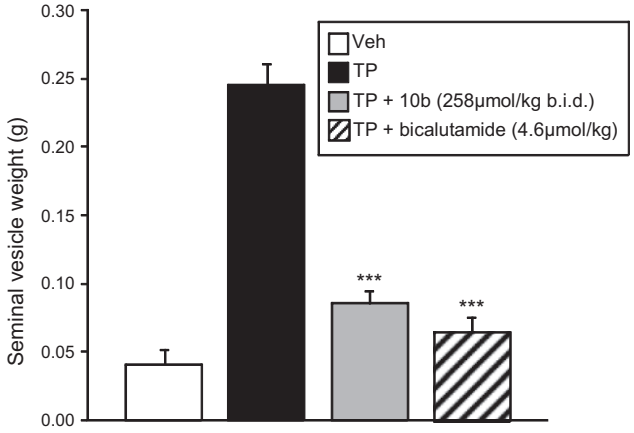


Figure 3. Inhibition of seminal vesicle weight by **10b** dosed at 258 μmol/kg po bid for 7 days to immature castrated rats stimulated with testosterone propionate (TP) 1.2 μmol/kg sc. ***P <0.001 compared to TP group.

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Supplementary data

Supplementary data (Protocols for the AR binding and cellular downregulation assays, along with experimental procedures and characterization data for key compounds **6a, 8a, 9a** and **10b**) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.06.122.

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