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Crystal structures of AKR1C3 containing an *N*-(aryl)amino-benzoate inhibitor and a bifunctional AKR1C3 inhibitor and androgen receptor antagonist. Therapeutic leads for castrate resistant prostate cancer

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Abstract

Castrate resistant prostate cancer (CRPC) is associated with increased androgen receptor (AR) signaling often brought about by elevated intratumoral androgen biosynthesis and AR amplification. Inhibition of androgen biosynthesis and/or AR antagonism should be efficacious in the treatment of CRPC. AKR1C3 catalyzes the formation of potent AR ligands from inactive precursors and is one of the most upregulated genes in CRPC. AKR1C3 inhibitors should not inhibit the related isoforms, AKR1C1 and AKR1C2 that are involved in 5 α -dihydrotestosterone inactivation in the prostate. We have previously developed a series of flufenamic acid analogs as potent and selective AKR1C3 inhibitors (Adeniji A.O. et al., *J Med Chem.* **2012**, *55*, 2311). Here we report the X-ray crystal structures of one lead compound 3-((4'-(trifluoromethyl)phenyl)amino)benzoic acid (**1**) in complex with AKR1C3. Compound **1** adopts a similar binding orientation as flufenamic acid, however, its phenylamino ring projects deeper into a subpocket and

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The steroid/inhibitor binding cavity of AKR1C3 is composed of five compartments as defined by Byrns et al. in reference 15: oxyanion site (OS; formed by Tyr55, His117, and NADP⁺), steroid channel (SC; Trp227 and Leu54), and three subpockets, SP1 (Ser118, Asn167, Phe306, Phe311, and Tyr319), SP2 (Trp86, Leu122, Ser129, and Phe311), and SP3 (Tyr24, Glu192, Ser221, and Tyr305).

Atomic coordinates and structure factors for the AKR1C3•NADP⁺•**2** complex (code 4DBS) and the AKR1C3•NADP⁺•BMT-1 complex (code 4DBU) have been deposited with the RCSB Protein Data Bank.

CONFLICT OF INTEREST

The authors declare no conflict of interest

EDITORS NOTE

A provisional patent application based on these compounds has been submitted to the US patent office. US provisional patent applications no 61/4,754,091 filed April 13, 2011.

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confers selectivity over the other AKR1C isoforms. We exploited the observation that some flufenamic acid analogs also act as AR antagonists and synthesized a second generation inhibitor, 3-[4'-(nitronaphthalen-1-amino))benzoic acid (**2**). Compound **2** retained nanomolar potency and selective inhibition of AKR1C3 but also acted as an AR antagonist. It inhibited 5 α -dihydrotestosterone stimulated AR reporter gene activity with an IC₅₀ = 4.7 μ M and produced a concentration dependent reduction in androgen receptor levels in prostate cancer cells. The *in vitro* and cell-based effects of compound **2** make it a promising lead for development of dual acting agent for CRPC. To illuminate the structural basis of AKR1C3 inhibition, we also report the crystal structure of the AKR1C3•NADP⁺•**2** complex, which shows that compound **2** forms a unique double decker structure with AKR1C3.

Keywords

Aldo-keto reductase; non-steroidal anti-inflammatory drugs (NSAIDs); *N*-phenylanthranilic acids; adaptive androgen biosynthesis; androgen receptor antagonist; competitive inhibition

Aldo-keto reductase 1C3 (AKR1C3), also known as type 5 17 β -hydroxysteroid dehydrogenase, is a critical enzyme involved in the pre-receptor regulation of androgen action in the prostate and has been implicated in the pathogenesis of castrate resistant prostate cancer (CRPC). CRPC often develops in prostate cancer patients that have undergone androgen deprivation therapy either through orchiectomy or chemical castration. CRPC is characterized by reactivation of the androgen axis due to changes in androgen receptor (AR) signaling and/or adaptive intratumoral androgen biosynthesis.^{1–3} The elevated intratumoral androgen levels occurs despite castrate levels of circulating androgens and arises due to the upregulation of key enzymes involved in androgen biosynthesis.^{1, 4, 5} Blockade of the androgen axis either by targeting the enzymes involved in androgen biosynthesis, the androgen receptor, or both should be beneficial in CRPC. New agents such as abiraterone acetate block the production of the androgen precursor dehydroepiandrosterone in the adrenal, and MDV3100 targets the amplified AR. Both agents have proved effective in treating CRPC in clinical trials.^{6–12}

AKR1C3 is among the most upregulated genes in CRPC and catalyzes the reduction of weak androgen precursors, Δ^4 -androstene-3,17-dione and 5 α -androstane-3,17-dione, to give the potent androgens, testosterone and 5 α -dihydrotestosterone (DHT), respectively.^{5, 13, 14} The critical role played by AKR1C3 in androgen biosynthesis and its localization within the tumor make AKR1C3 an important target for the treatment of CRPC. However, the presence of the closely related AKR1C isoforms AKR1C1 and AKR1C2, both of which are involved in DHT inactivation in the prostate, makes it imperative that AKR1C3 be inhibited selectively.^{15–17} The high sequence identity (> 86%) between the AKR1C isoforms makes the discovery of selective AKR1C3 inhibitors challenging.

Non-steroidal anti-inflammatory drugs (NSAIDs) used clinically for their cyclooxygenase (COX) inhibitory properties are known to inhibit AKR1C3 at therapeutically relevant concentrations.^{18, 19} In particular, the *N*-phenylanthranilic acids (*N*-PA) typified by flufenamic acid (FLU) are among the most potent AKR1C3 inhibitors known. However, these compounds also display activity against AKR1C1 and AKR1C2. It will be equally important to strip these compounds of their COX inhibitory properties since chronic COX inhibition has been associated with adverse effects. Using FLU as a lead compound, we previously conducted structure activity relationship (SAR) studies and identified a pharmacophore that resulted in potent and selective AKR1C3 inhibitors with nanomolar potency and greater than 100-fold selectivity for AKR1C3 over other AKR1C enzymes. These compounds were also devoid of COX inhibitory properties. Our SAR revealed that an

unsubstituted *N*-benzoic acid (A) ring with the carboxylic acid at the *meta*-position relative to the bridge amine and the presence of an electron withdrawing group at the *para*-position of the phenylamino (B) ring were optimal for AKR1C3 inhibition.^{20, 21} One of most promising leads from the studies was a FLU isomer, 3-((4'-(trifluoromethyl)phenyl) amino)benzoic acid (**1**). (Figure 1 and Table 1).

To elucidate the basis of the observed AKR1C3 selectivity conferred by the new pharmacophore, compound **1** was submitted for crystallographic studies with AKR1C3 (Detailed experimental procedures are described in the Supplementary Data. Data collection and refinement statistics are listed in Table 2.). The structure of the AKR1C3•NADP⁺•**1** complex shows that compound **1** adopts a similar binding pose to that of FLU (PDB ID: 1S2C). The carboxylate group of the *N*-benzoic acid ring is anchored to the oxyanion site through hydrogen bonds to Tyr55 and His117 (Figure 2). The phenylamino ring extends into the SP1 subpocket defined by Ser118, Asn167, Phe306, Phe311, and Tyr319 (See the Footnote for a full definition of AKR1C3 subpockets.).²² However, due to the *meta*-substitution in the benzoic acid ring, the bridge amine in compound **1** no longer forms a hydrogen bond to the carbonyl group on the cofactor nicotinamide head. The phenylamino ring is tilted about 30° relative to the conformation seen with FLU. To accommodate this change, Phe311, which stacks against the phenylamino ring of FLU, is moved 3 Å sideways by a backbone shift within the C-terminal loop (Figure 3). Meanwhile, Phe306 is rotated 60° towards the phenylamino ring of compound **1** to form a partially overlapped π - π stacking. The trifluoromethyl group of compound **1** penetrates about 1 Å deeper into the SP1 pocket than FLU. The penetration is likely the basis of the observed selectivity of this agent on AKR1C3 over the other AKR1C isoforms since the other AKR1C isoforms have shallower SP1 pockets compacted by Phe118, Leu/Val306, and Leu/Met308. This verifies our hypothesis that our lead *N*-PA analogs attain selectivity by targeting the SP1 binding pocket.²¹

N-PA analogs were recently reported to act as AR antagonists.²⁷ Key features of these analogs are the inclusion of an additional ring on the phenylamino ring. This finding raised the possibility of a compound that will target both AKR1C3 and AR. With this in mind, we synthesized **3-((4-nitronaphthalen-1-yl)amino)benzoic acid**, (**2**) (Figure 4) which contains a naphthylamino group instead of the phenylamino group (Details of synthesis and characterization of compound **2** and AR antagonism assays are included in the supplementary Information).

Using the NADP⁺ dependent oxidation of *S*-tetralol to monitor AKR1C activity,^{20, 21} compound **2** retained potent AKR1C3 inhibition with an IC₅₀ value of 80 nM and 100-fold selectivity for AKR1C3 over AKR1C1, AKR1C2, and AKR1C4 (Table 1 and Figure 5). Relative to the corresponding 4-nitrophenyl derivative, 3-[(4'-nitrophenyl) amino]benzoic acid (**3**), which only offers a 100-fold selectivity over AKR1C2, replacing the phenyl ring with a naphthyl ring in compound **2** increased the selectivity by 150% due to a 4-fold loss of AKR1C2 potency. In LNCaP cells stably overexpressing AKR1C3 (LNCaP-1C3 cells),²⁸ addition of 10 μ M compound **2** led to a 75% inhibition of testosterone formation in cells treated with 100 nM [¹⁴C] - Δ^4 -androstene-3,17-dione as substrate (data not shown). Furthermore, unlike the the potent COX inhibitor FLU and compound **3**, compound **2** exhibited no significant inhibition of COX activity at 100 μ M.

The effect of compound **2** on androgen receptor signaling was evaluated using a luciferase AR reporter gene assay (see supplementary materials for method details). Incubation of 10 μ M of compound **2** in the presence of an increasing concentration of DHT shifted the concentration response curve of DHT in HeLa-AR3A-PSA-(ARE)₄-Luc13 cells (a kind gift from Dr. Elizabeth Wilson) to the right and increased the EC₅₀ value by four fold,

suggesting competitive inhibition of AR transcriptional activity (Figure 6A). No significant change in the EC₅₀ value of DHT was observed when other *N*-PA compounds were tested at 10 μ M (Data not shown). When the same cells were treated with 0.1 nM DHT (equivalent to the EC₅₀ value for AR obtained from the same assay) in the presence of increasing concentrations of compound **2**, we observed a concentration dependent reduction in AR transcriptional activity as measured by the luciferase expression with an IC₅₀ value of 4.7 μ M (Figure 6B). Treatment of the HeLa cells with 0.1 nM DHT led to an increase in AR levels, consistent with agonist induced AR stabilization.²⁹ The DHT induced increase in AR levels was inhibited in a concentration dependent manner by compound **2** (Figure 7). Compound **2** also reduced AR levels in the absence of DHT (Figure 7). This data indicates that the reduction in luciferase expression, observed in the AR reporter gene assay following treatment with compound **2** was due to inhibition of AR signaling and not due to toxicity of the compound or a direct inhibition of the luciferase enzyme activity. This decrease in AR levels could either be as a result of the elimination of the agonist- AR interaction, direct down regulation of AR expression, or both. The exact mechanism will need to be determined.^{29, 30}

To determine whether the bifunctional analog compound **2** targets the same binding subpocket as compound **1** to achieve AKR1C3 selectivity, compound **2** was co-crystallized in complex with AKR1C3 (Figures 2). To our surprise, two molecules of compound **2** were found per active site of AKR1C3. The first molecule of compound **2** is tethered to the oxyanion site similarly to compound **1**, but its naphthyl ring is inserted into the SP1 subpocket perpendicular to the phenylamino ring of compound **1**. This binding pose allows space for the naphthyl ring of the second inhibitor molecule to fit inside the SP1 pocket forming a double decker arrangement. The unexpected arrangement allows Phe306 to swing back to its original position in the AKR1C3•NADP⁺•FLU complex (Figure 3). However, to accommodate the second molecule of compound **2**, Phe311 is pushed even further away from the SP1 pocket leading to a 90° rotation of its phenyl ring. The double decker binding of inhibitors has been reported previously for other aldo-keto reductases. In the complex of Alrestatin bound to aldose-reductase (AKR1B1), two molecules of Alrestatin occupy the active site forming face-to-face π - π stacking.³¹ Our inhibition dose-response curve of compound **2** also supports the multisite binding of the inhibitor to AKR1C3 by showing a slope factor of approximately two, indicating that the second molecule of compound **2** binds tightly to the enzyme and may play an important role in the inhibition. The Alrestatin double-decker structure has been suggested to confer the inhibitory selectivity for aldose-reductase over aldehyde reductase (AKR1A1) by specific hydrogen bonding between the second molecule of Alrestatin and the C-terminal loop of AKR1B1. In our AKR1C3•NADP⁺•**2** complex, two AKR1C3 specific hydrogen bonds between the second compound **2** molecule and Ser118 and Ser129 (Phe118 and Ile/Leu129 in the other AKR1C isoforms) likely contribute to inhibitor selectivity.

We report the crystal structures of two potent and selective inhibitors in complex with AKR1C3. These structures add to the understanding of inhibitor interaction with AKR1C3 and can serve as tools in the rational design of potent and selective AKR1C3 Inhibitors. We also report modification of *N*-phenylaminobenzoates to *N*-naphthylaminobenzoates to generate a bifunctional analog that inhibits AKR1C3 and antagonizes the AR. This new compound retained potency and selectivity for AKR1C3, was devoid of activity on COX enzymes, and displayed antagonist properties on the AR leading to a loss of receptor expression. The difference in IC₅₀ values for the inhibition of AKR1C3 by compound **2** = 80 nM and the IC₅₀ value for AR antagonism = 4.7 μ M suggests that to hit both targets the selectivity for AKR1C3 over other AKR1C enzymes may be lost. However, this assumes that the IC₅₀ values in the *in vitro* screen are directly comparable to IC₅₀ values generated in the cell-based assays. Regardless, compound **2** will require optimization for AR antagonism.

The AKR1C3 structure with the bifunctional analog bound shows a unique double-decker structure that can be exploited in the design and optimization of second generation AKR1C3 inhibitors. The development of a bifunctional agent that inhibits AKR1C3 and AR should provide therapeutic benefit in CRPC. Such compounds could be superior to drugs that act on a single target e.g., CYP17A1 (abiraterone) or AR (MDV3100) and may have less adverse effects. These compounds are interesting leads for drug development CRPC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AKR	aldo-keto reductase
AKR1C3	type 5 17 β -hydroxysteroid dehydrogenase
AR	androgen receptor
COX	cyclooxygenase
CRPC	castrate resistant prostate cancer
DHT	5 α -dihydrotestosterone
FLU	flufenamic acid
NSAIDs	non-steroidal anti-inflammatory drugs
N-PA	<i>N</i> -phenylanthranilic acids
SAR	structure activity relationship

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Figure 1.
N-phenylanthranilic acid based AKR1C3 Inhibitors. The *N*-benzoic acid is designated the A-ring and the Phenylamino ring as the B-ring

Figure 2.

Schematics illustrate the occupancy of the AKR1C3 steroid binding subpockets (SP1, SP2, and SP3) by compound **1** (A), FLU (B), and compound **2** (C). Compound **1**, FLU, and the first molecule of compound **2** are tethered to the oxanion site (OS) by hydrogen bonds between the carboxylate group and Tyr55 and His117. The *N*-phenylamino/naphthylamino rings of the inhibitors project to the SP1 pocket. The second molecule of compound **2** stacks against the first one in the SP1 pocket by overlapping the *N*-naphthylamino ring but its *N*-benzoic acid ring extends to the steroid channel (SC) that opens to the solvent. The simulated-annealing omit map of compound **1** is contoured at 2.5σ and the map of compound **2** is contoured at 2.3σ . The atoms are colored as follow: carbon = black, oxygen = red, nitrogen = blue, fluorine = light blue, sulfur = orange. Water molecules are shown as red spheres. Hydrogen bonds are indicated by red dashes.

Figure 3.

Wall-eyed stereoviews of compound **1** (A, protein carbons are shown in magenta), FLU (B, yellow), and compound **2** (C, marine) in AKR1C3 active site. Inhibitors are shown in ball-and-stick representation with carbon atoms colored in black. Significant movement of Phe306 and Phe311 is seen in the three structures to accommodate different inhibitors. The non-carbon atoms are colored: oxygen = red, nitrogen = blue, fluorine = light blue, sulfur = yellow orange, phosphor = orange. Water molecules are shown as red spheres. Hydrogen bonds are indicated by red dashes. Not all active site residues are shown. The hydrogen bonds that exist between the FLU bridge amine and the carbonyl group on the cofactor nicotinamide head and between the second molecule of **2** and N167 in panel B are blocked in the view shown.

Figure 4. Synthesis of compound **2** using a Buchwald-Hartwig C-N coupling^{24–26} followed by methyl ester saponification. (a) $\text{Pd}(\text{OAc})_2$, 2,2'-bis(diphenylphosphino)-1,1'-binaphthyl (BINAP), Cs_2CO_3 , toluene, 120 °C. (b) KOH , EtOH , H_2O , 100 °C.

Figure 5. Inhibition dose response curve of compound **2** for AKR1C1-4. The compound exhibits substantial AKR1C3 selectivity over the other AKR1C isoforms.

A)

B.

Figure 6.

A) Concentration response curve of DHT in HeLa-AR3A-PSA-(ARE)₄-Luc13 cells in the presence and absence of 10 μM compound **2**. B) Inhibition of 0.1 nM DHT induced AR transcriptional activity in the same cells by compound **2**.

Figure 7. Western blotting analysis of the AR in cell lysates from treated HeLa-AR3A-PSA-(ARE)₄-Luc13 cells. Compound **2** reduces the level of AR in the presence and absence of DHT. (LE, long exposure, SE short exposure). The level of GAPDH is shown as loading control.

Table 1

Inhibitory properties of compounds on human AKR1C and COX enzymes.

Compds	IC ₅₀ Values (μM)					
	AKR1C3	AKR1C1	AKR1C2	AKR1C4	COX-1	COX-2
FLU ^a	0.05	ND	0.37	ND	2.23	0.02
1 ^a	0.06	22.7	15.4	62.7	> 100	> 100
2	0.08	11.1	11.7	8.17	> 100	> 100
3 ^a	0.03	6.74	3.38	32.7	30.76	0.74

^a ref. 20, 21

Inhibitory activity of the compounds on AKR1C enzymes was determined by the S-tetralol assay^{20, 21}

Table 2

Data collection and refinement statistics.

Structure	AKR1C3•NADP ⁺ •1 (PDB ID: 4DBU)	AKR1C3•NADP ⁺ •2 (PDB ID: 4DBS)
Data collection		
Resolution range (Å)	50.0-2.53	50.0-1.85
Cell parameters (Å, °)	47.27 × 49.14 × 84.73 $\alpha = 71.90, \beta = 81.58, \gamma = 70.26$	47.17 × 49.00 × 83.56 $\alpha = 74.42, \beta = 87.37, \gamma = 70.18$
Unique reflections measured	22331 (2242) ^b	56673 (5206) ^b
R_{merge}^a	0.093 (0.32) ^b	0.084 (0.38) ^b
$\langle I \sigma(I) \rangle$	9.3 (2.8) ^b	16.7 (3.1) ^b
Completeness (%)	98.6 (97.9) ^b	98.6 (90.3) ^b
Refinement statistics		
Reflections used in refinement/test set	21058/1027	53065/2676
R/R_{free}^c	0.230/0.293	0.181/0.222
Protein atoms ^d	5079	5079
Water molecules ^d	163	442
NADP ⁺ molecules ^d	2	2
Inhibitor molecules ^d	2	4
r.m.s. deviations		
Bond lengths (Å)	0.003	0.006
Bond angles (°)	0.62	0.98
Overall <i>B</i> -factor (Å ²)	29	33
Cofactor <i>B</i> -factor (Å ²)	21	26
Inhibitor <i>B</i> -factor (Å ²)	30	35
Ramachandran statistics ^e		
Allowed (%)	87.6	91.3
Additionally allowed (%)	11.7	8.3
Generously allowed (%)	0.4	0.2
Disallowed (%)	0.4	0.2

^a $R_{\text{merge}} = \sum |I - \langle I \rangle| / \sum I$, where I is the observed intensity and $\langle I \rangle$ is the average intensity calculated for replicate data.

^bThe number in parentheses refers to the outer 0.1-Å shell of data.

^cCrystallographic *R*-factor, $R = \sum (|F_o| - |F_c|) / \sum |F_o|$ for reflections contained in the working set. Free *R*-factor, $R_{\text{free}} = \sum (|F_o| - |F_c|) / \sum |F_o|$ for reflections contained in the test set excluded from refinement. $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively.

^dPer asymmetric unit.

^eRamachandran statistics were calculated with PROCHECK ²³.