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Discovery of substituted 3-(phenylamino)benzoic acids as potent and selective inhibitors of type 5 17 β -hydroxysteroid dehydrogenase (AKR1C3)

Adegoke O. Adeniji^a, Barry M. Twenter^b, Michael C. Byrns^a, Yi Jin^a, Jeffrey D. Winkler^{b,*}, and Trevor M. Penning^{a,*}

^aDepartment of Pharmacology and Center for Excellence in Environmental Toxicology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6084

^bDepartment of Chemistry, University of Pennsylvania, Philadelphia, PA 19104

Abstract

Aldo-keto reductase 1C3 (AKR1C3) also known as type 5 17 β -hydroxysteroid dehydrogenase has been implicated as one of the key enzymes driving the elevated intratumoral androgen levels observed in castrate resistant prostate cancer (CRPC). AKR1C3 inhibition therefore presents a rational approach to managing CRPC. Inhibitors should be selective for AKR1C3 over other AKR1C enzymes involved in androgen metabolism. We have synthesized 2-, 3-, and 4-(phenylamino)benzoic acids and identified 3-(phenylamino)benzoic acids that have nanomolar affinity and exhibit over 200-fold selectivity for AKR1C3 versus other AKR1C isoforms. The AKR1C3 inhibitory potency of the 4'-substituted 3-(phenylamino)benzoic acids shows a linear correlation with both electronic effects of substituents and the pK_a of the carboxylic acid and secondary amine groups, which are interdependent. These compounds may be useful in treatment and/or prevention of CRPC as well as understanding the role of AKR1C3 in endocrinology.

Keywords

Aldo-keto reductase (AKR); Castrate resistant prostate cancer (CRPC); Non steroidal anti-inflammatory drugs (NSAIDs); *N*-Phenylanthranilic acids; Androgen biosynthesis

Prostate cancer (PC) is the second most common cancer in American men and is responsible for about 11% of all cancer related deaths.^{1, 2} PC is initially dependent on testicular androgens and responsive to androgen ablation. However, the therapeutic benefit of the androgen deprivation therapy is temporary as it is often followed by recurrence of a more aggressive metastatic disease, known as castrate resistant prostate cancer (CRPC). CRPC is characterized by elevated intratumoral androgen levels, increased androgen receptor (AR) signaling and expression of pro-survival genes despite castrate level circulating androgen concentrations.³⁻⁵ The source of intratumoral androgens is likely dehydroepiandrosterone

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*Corresponding Authors. penning@upenn.edu (T.M. Penning). Address: 130C John Morgan Bldg, 3620 Hamilton Walk Philadelphia, PA 19104-6084 Telephone: 1-215-898-9445; Fax: 1-215-573-2236; winkler@sas.upenn.edu (J.D. Winkler) Address: 231 S 34th Street Philadelphia, PA 19104-6323 Telephone: 1-215-898-0052; Fax: 1-215-573-6329.

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(DHEA) from the adrenal, which is subsequently metabolized to testosterone and 5 α -dihydrotestosterone (5 α -DHT) by the pathway shown in Figure 1.

Recently, there has been clinical success with the CYP17 α -hydroxylase/17,20-lyase (CYP17) inhibitor, abiraterone acetate in the treatment of CRPC, endorsing the concept that intratumoral androgen biosynthesis from DHEA plays a role.^{6, 7} Abiraterone acetate is generally given with a glucocorticoid to suppress the adrenal pituitary axes and prevent the undesirable build up of the mineralocorticoid desoxycorticosterone.^{6, 8}

This intratumoral conversion of DHEA to active androgens is driven primarily by elevated expression of key enzymes in the androgen biosynthetic pathway.^{5, 9, 10} In particular, Type 5, 17 β -hydroxysteroid dehydrogenase (HSD), also known as aldo-keto reductase 1C3 (AKR1C3) has been shown to be among the most highly upregulated enzymes in CRPC.¹⁰⁻¹² This enzyme catalyzes the reduction of the weak androgen, 4-androstene-3,17-dione to testosterone which can subsequently be reduced by 5 α -reductases to 5 α -DHT.^{13, 14} (Figure 1) It represents a superior drug target to CYP17 since it catalyzes the final step in testosterone production.

AKR1C3 is also involved in prostaglandin biosynthesis, as it catalyzes the NADPH dependent formation of pro-proliferative signaling molecules, prostaglandin (PG) F_{2 α} and 11 β -PGF_{2 α} from PGH₂ and PGD₂ respectively.¹⁵⁻¹⁷ The reduction of PGH₂ and PGD₂ by AKR1C3 also prevents the spontaneous conversion of PGD₂ to the anti-proliferative product, 15-deoxy $\Delta^{12,14}$ PGJ₂.^{18, 19} The net effect of increased AKR1C3 expression and activity as seen in CRPC, will be an increase in cellular proliferative signaling and tumor growth. AKR1C3 thus represents a rational therapeutic target for the management of CRPC irrespective of whether it is hormone dependent.

AKR1C1 (20 α -HSD) and AKR1C2 (Type 3 3 α -HSD) are closely related enzymes with > 86% sequence identity to AKR1C3, both of which can act on androgens.^{20, 21} The two enzymes catalyze the conversion of 5 α -DHT to 5 α -androstane-3 β ,17 β -diol (3 β -Adiol) and 5 α -androstane-3 α ,17 β -diol (3 α -Adiol) respectively (Figure 1).^{21, 22} 3 β -Adiol is a pro-apoptotic ligand for estrogen receptor β , while 3 α -Adiol is an inactive androgen.^{22, 23} Inhibition of AKR1C1 and AKR1C2 in the prostate can therefore be expected to increase androgen dependent proliferative signaling. This makes it imperative that inhibitors developed for AKR1C3 should have little or no effect on its closely related isoforms.

Non steroidal anti-inflammatory drugs (NSAIDs) are known to be inhibitors of the AKR1C enzymes.²⁴ NSAIDs based on *N*-phenylanthranilic acids (*N*-PA), in particular, are known to be potent and non-selective inhibitors of AKR1C enzymes.²⁵ Developing *N*-PA-based AKR1C3 inhibitors that are devoid of cyclooxygenase (COX) or other AKR1C enzyme inhibitory activity is desirable since much is known about the absorption, distribution, metabolism, excretion and toxicity (ADMET) of this class of drugs. Based on known structure-activity relationships for COX isozymes, substitution on the A-ring of an *N*-PA, or movement of the carboxylic acid from the *ortho*-position would eliminate COX inhibition.²⁵⁻²⁷

Also, the x-ray crystal structure of the AKR1C3-NADP⁺-flufenamic acid complex (PDB# 1S2C) provides insights on how the prototypical *N*-PA, flufenamic acid is bound. Its carboxylic acid group is anchored via hydrogen bond interaction at the oxyanion hole of the active site, which is formed by the cofactor and catalytic tetrad. The secondary amine of the drug also forms hydrogen bond interactions with the carboxamide oxygen of nicotinamide ring of the cofactor, while the B-ring containing the *para*-trifluoromethyl group substituent is bound in a distinct subpocket.^{28, 29} Analysis of this subpocket in AKR1C3 showed that it was larger, different in shape and lined by polar amino acids (S118, S308 and Y319) relative

to either AKR1C1 or AKR1C2, where the corresponding residues are F118, L308, and F319.^{25, 29} This suggested that appropriate substitution on the B-ring may provide the needed AKR1C3 selectivity.

In this study, we have systematically examined the effect of the movement of the carboxylic acid on the A-ring of an *N*-PA and concurrently measured the effect of substituents on the B-ring. This has led to a series of 3-(phenylamino)benzoic acid derivatives that are potent and selective inhibitors for AKR1C3 over AKR1C1 and AKR1C2. (Figure 2 and Table 1)

Compounds were synthesized according to the scheme in Figure 2. The reaction involved a Buchwald-Hartwig C-N coupling followed by methyl ester saponification.³⁰⁻³² In most cases, the product crystallizes out of the aqueous medium upon acidification allowing the product to be collected by a simple vacuum filtration. Compounds were characterized by NMR analysis and high resolution mass spectrometry (see Supplemental Material).³³

AKR1C1 and AKR1C2 share 98% sequence identity and differ in only one amino acid at their respective active sites.^{20, 34} It is therefore likely that an inhibitor of AKR1C2 will also inhibit AKR1C1. Based on this assumption, the screening strategy adopted was to determine the inhibitory potency of each compound against AKR1C2 and AKR1C3 and use the ratio of IC₅₀ value for AKR1C2: IC₅₀ value for AKR1C3 as an index of compound selectivity for AKR1C3. The inhibitory potency of compounds on AKR1C2 and AKR1C3 was determined by measuring the inhibition of the NADP⁺ dependent oxidation of S-tetralol.³⁵ All assays were performed at the K_M of the respective AKR1C isoforms so that IC₅₀ values were directly comparable.

Our lead compound was the prototypic *N*-PA, flufenamic acid (*o*-CO₂H, 3'-CF₃) **1**, which gave an IC₅₀ value of 50 nM for AKR1C3 and showed a modest 7-fold selectivity for the inhibition of AKR1C3 over AKR1C2, Table 1. We first synthesized compounds **2-4** to evaluate the effect of changing the position of the carboxylic acid group on the A-ring on inhibitory potency and selectivity for AKR1C3. The B-ring unsubstituted compounds were initially chosen to preclude substituent effects. It was found that movement of the CO₂H group had no remarkable effect on the inhibitory potency for AKR1C3 thus, *ortho*-, *meta*- and *para*-carboxylate gave IC₅₀ values of 1.5 μM, 0.94 μM and 2.8 μM for compounds **2**, **3**, and **4** respectively. These compounds offered two important clues to further inhibitor development. First, compound **3** showed a 14-fold selectivity for the inhibition of AKR1C3 over AKR1C2, indicating a 48-fold increase in AKR1C3 selectivity with movement of the carboxylic acid from *o*- to *m*- position (compare IC₅₀ value ratios for compounds **2** and **3**), suggesting that the *m*-CO₂H may be important for selectivity. Second, compound **2**, the B-ring unsubstituted analog of **1** was 30-fold less potent as an AKR1C3 inhibitor than **1**, suggesting that B-ring substitution was important for potency. This loss of potency is expected given the greater distance and consequently, reduced interactions between the B-ring and the subpocket in the absence of a substituent on the B-ring. Using compound **3** as the new lead (*m*-CO₂H on the A-ring), eight B-ring *para* substituted analogs of **3**, (**5-12**) were synthesized and evaluated to assess the effects of different substituents on AKR1C3 potency and selectivity. The *p*- substituents were selected since they should project furthest into the subpocket, increasing the potential for interaction with the relevant amino acid residues.

As shown in Table 1, the lowest IC₅₀ values for AKR1C3 inhibition were obtained with an electron withdrawing group (EWG) as the B-ring substituent, such as **5** (*p*-NO₂) which gave a value of 36 nM. Compound **6** (*p*-Ac) and **7** (*p*-CF₃) were the two most selective inhibitors and gave IC₅₀ values that were 360 and 250 fold selective for AKR1C3 over AKR1C2 respectively. However, all the B-ring *para* substituted analogs (both EWG and EDG)

exhibited significantly better inhibitory potency for AKR1C3 and were also more selective for AKR1C3 relative to AKR1C2 than the parent compound **3**.

Interestingly, analogs with EWG, i.e., compounds **5-9**, displayed relatively similar AKR1C3 inhibitory potency with flufenamic acid, **1**, while those with electron donating substituents (EDG), compounds **10-12** displayed weaker AKR1C3 inhibitory activity than **1**. In contrast, all B-ring *para*-substituted compounds bearing an A-ring *m*-CO₂H display significantly higher IC₅₀ values than flufenamic acid for AKR1C2, regardless of the electronic properties of the substituent. The increase in IC₅₀ values for AKR1C2 due to the presence of the *m*-CO₂H group results in increased AKR1C3 selectivity.

A regression analysis of the electronic effect of the varied substituent at the *para* position and the inhibitory potency for AKR1C2 and AKR1C3 was then performed. The plot revealed a significant correlation between the electronic effect and AKR1C3 potency for the *para*-substituted 3-(phenylamino)benzoic acid analogs ($R^2 = 0.81$, $p = 0.0009$) Figure 3. However, no significant correlation between the electronic effect and inhibitory potency for AKR1C2 ($R^2 = 0.37$, $p = 0.084$) was noted for these compounds.

Further analysis showed there was also a significant correlation between the AKR1C3 potency and the calculated pKa of the carboxylic acid group ($R^2 = 0.80$, $p = 0.0012$) and the pKa of the secondary amine ($R^2 = 0.79$, $p = 0.0014$), both of which are related to the electronic effect of the B ring substituents. Our data on the electronic effects seen with the AKR1C3 inhibitors is consistent with these compounds forming hydrogen bonds with AKR1C3 at the active site. EWG or EDG on the B-ring that alter the acidity of the carboxylic acid and basicity of the secondary amine group in particular would be expected to affect the inhibitory potency of the compounds. The electronic effect observed for AKR1C3 inhibition could also be as a result of discrete interactions between residues lining the subpocket and the B-ring substituents.

Hydrogen bond formation at the active site is also expected in AKR1C2 but the lack of correlation between inhibitory potency and the electronic effect suggests other factors might be more relevant for inhibitor binding. Most of the B-ring substituted compounds have relatively similar inhibitory potency on AKR1C2 and gave IC₅₀ values which were in the low micromolar range, it is likely that with A-ring *m*-CO₂H analogs, the B- ring is precluded from binding in the expected subpocket in this isoform, presumably as a result of the smaller size of this pocket. We speculate that it is likely that the B-ring of these inhibitors is forced to occupy the larger vacant steroid binding site in AKR1C2. Further x-ray crystallographic studies will be required to determine the precise mechanism by which these compounds interact with the two enzymes and account for the selectivity seen with *m*-COOH analogues.

Several AKR1C3 inhibitors of varied structural classes have been reported in the literature.³⁶⁻⁴² However, selectivity for AKR1C3 over AKR1C1-2 is often low or not indicated. We have developed 3-(phenylamino) benzoic acid analogs that are potent inhibitors of AKR1C3 with orders of magnitude selectivity for AKR1C3. These compounds highlight structural modifications on *N*-PA that are essential for AKR1C3 inhibition and confer selectivity over AKR1C1 and AKR1C2. A *meta* arrangement of the carboxylic acid and secondary amine moieties as well as a strong electron withdrawing group at the *para* position of the B-ring confers selectivity for AKR1C3 over AKR1C2.

N-PA are known to be competitive inhibitors of AKR1C3. The compounds in this study were also found to inhibit AKR1C3 in a competitive manner (data not shown). Also, they are predicted to have little or no inhibitory activity on COX enzymes since the *ortho* arrangement of the -CO₂H and -NH₂ on the A-ring has been shown to be critical for COX activity.^{26, 27, 43}

The success of abiraterone acetate, in clinical trials for management of CRPC makes the discovery of these AKR1C3 selective inhibitors promising. Because of the position of AKR1C3 in the androgen biosynthetic pathway in the prostate, these compounds are likely to be more specific and have less undesirable side effects as seen with CYP17 inhibitors.

In conclusion, we have discovered inhibitors with nanomolar potency for AKR1C3 that exhibit significant selectivity for AKR1C3 over other AKR1C enzymes. At the very least, they may be used as probes to elucidate the role of AKR1C3 in cell culture models. More importantly, these compounds may serve as useful therapeutic leads in the treatment and/or prevention of CRPC as well as other androgen/estrogen dependent malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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33. a.) To a solution of bromide (or triflate) (1 equiv) in toluene (0.1 M) was added aniline (1.2 equiv), Cs_2CO_3 (1.4 equiv) BINAP (0.08 equiv), and $\text{Pd}(\text{OAc})_2$ (0.05 equiv) at room temperature. The reaction mixture was allowed to stir at 120 °C for 4-48 h. Once the reaction appeared to be complete by consumption of the bromide (or triflate) by TLC analysis, the mixture was allowed to cool to room temperature, diluted with EtOAc, washed with 2M aq HCl (2x), brine, and dried over sodium sulfate. The solution was concentrated, loaded on silica gel, and purified by silica gel chromatography. b.) To a solution of methyl ester (1 equiv) in EtOH (0.2 M) was added KOH (2 equiv per ester) in water (0.2 M) at room temperature. The reaction mixture was allowed to stir at 100 °C for 1-6 h. Once the reaction appeared complete by TLC analysis, EtOH was evaporated from the reaction mixture; the resultant solution was cooled to 0 °C and acidified to pH 2 w 2M aq HCl. The resultant precipitated product was collected by vacuum filtration and washed with water.
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35. The inhibitory potency of these compounds was determined using purified homogenous recombinant enzymes. Full concentration response studies were conducted and IC_{50} values calculated using Grafit 5.0 software. The compounds were evaluated for effects on enzyme catalyzed oxidation of S-tetralol using a 96-well format. The reaction was fluorimetrically (exc/em; 340nm/460nm) monitored by the measurement of NADPH production on a BIOTEK Synergy 2 Multimode plate reader at 37 °C. Assay mixture consists of S-tetralol (in DMSO), inhibitor (in DMSO), 100 mM phosphate buffer, pH 7.0, 200 μM NADP^+ , and purified recombinant enzyme (30 μl) to give a total volume of 200 μl and 4% DMSO. The S-tetralol concentration used for AKR1C2 and AKR1C3 inhibition assay were 22.5 μM and 165 μM respectively, equal to the K_M obtained for the respective isoforms under the same experimental conditions. Initial velocity, obtained by linear regression of the progress curve, in the presence of varying concentration of inhibitor was compared to solvent control to give percent inhibition values. IC_{50} values were obtained from a single experiment with each concentration run in quadruplicate.
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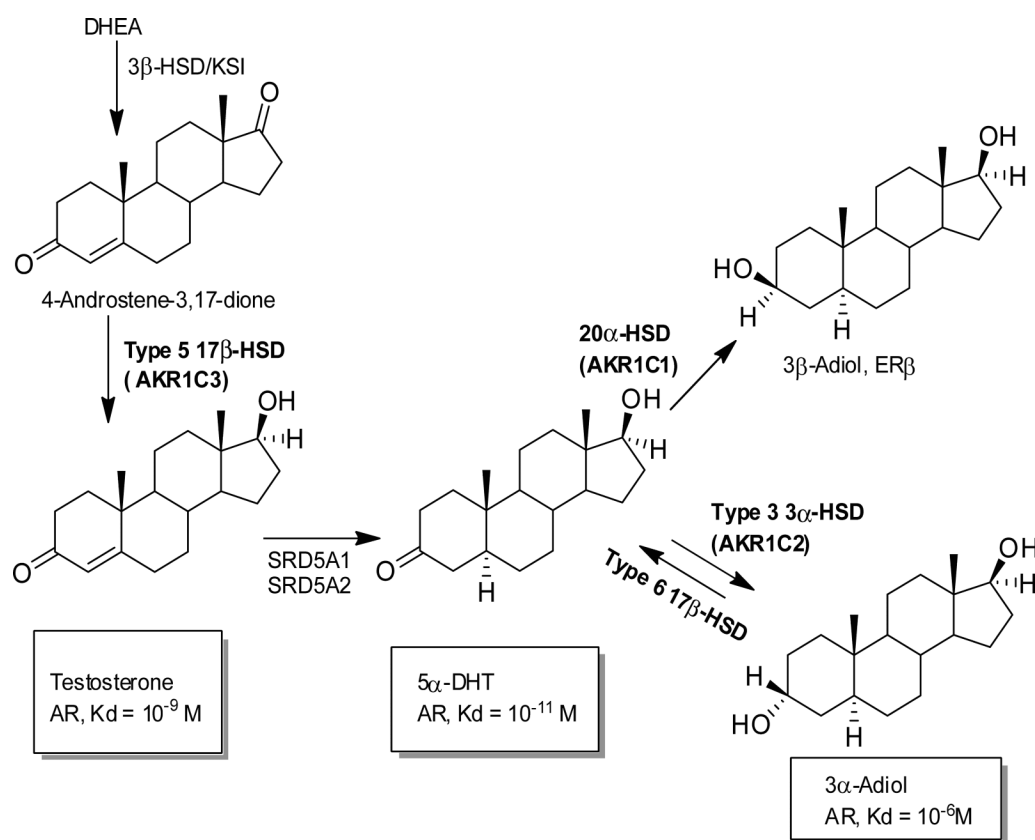


Figure 1. Role of AKR1C1-3 in androgen biosynthesis in the prostate

(SRD5A1 and SRD5A2 are Type 1 and 2 5α-reductases respectively; AR = androgen receptor, 3β-HSD/KSI: 3β-hydroxysteroid dehydrogenase/ketosteroid isomerase, 3β-Adiol = 5α-androstane-3β,17β-diol, 3α-Adiol = 5α-androstane-3α,17β-diol, ERβ = estrogen receptor β)

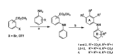


Figure 2. Synthetic scheme for 2-, 3-, and 4-(phenylamino)benzoic acid analogs, compounds 1-12, (see Table 1)

(a.) Pd(OAc)₂, 2,2'-bis(diphenylphosphino)-1,1'-binaphthyl (BINAP), Cs₂CO₃, toluene, 120 °C (b.) KOH, EtOH, H₂O, 100 °C

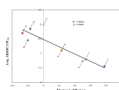
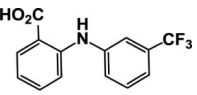
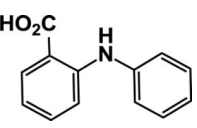
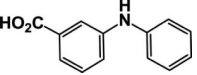
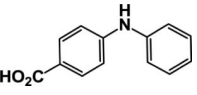
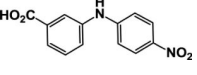
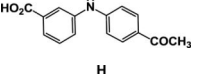
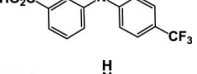
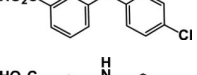
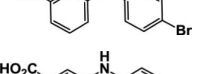
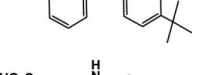
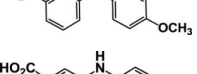
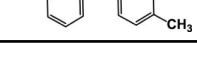


Figure 3.
Correlation of AKR1C3 inhibitory potency and electronic effect (σ) of substituents

Table 1

Compound structure and inhibitory potency on AKR1C2 and AKR1C3

	Compounds	AKR1C3 IC ₅₀ (μM)	AKR1C2 IC ₅₀ (μM)	Ratio IC ₅₀ values AKR1C2:AKR1C3
1	 Flufenamic acid	0.051	0.37	7.3
2		1.5	0.44	0.29
3		0.94	13	14
4		2.8	3.0	1.1
5		0.036	3.4	94
6		0.054	19	360
7		0.062	15	250
8		0.13	19	150
9		0.12	18	150
10		0.28	31	110
11		0.49	11	23
12		0.70	56	80