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Nonsteroidal anti-inflammatory drugs and their analogues as inhibitors of aldo-keto reductase AKR1C3: New lead compounds for the development of anticancer agents

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Abstract—Nonsteroidal anti-inflammatory drugs (NSAIDs) like indomethacin, flufenamic acid, and related compounds have been recently identified as potent inhibitors of AKR1C3. We report that some other NSAIDs (diclofenac and naproxen) also inhibit AKR1C3, with the IC_{50} values in the low micromolar range. In order to obtain more information about the structure–activity relationship and to identify new leads, a series of compounds designed on the basis of NSAIDs were synthesized and screened on AKR1C3. The most active compounds were 2-[(2,2-diphenylacetyl)amino]benzoic acid **4** (IC_{50} = 11 μM) and 3-phenoxybenzoic acid **10** (IC_{50} = 0.68 μM). These compounds represent promising starting points for the development of new anticancer agents.

Selective control of the biological activity of steroids, by inhibiting specific enzymes involved in their biosynthesis and metabolism, has been an attractive pharmaceutical target over the last two decades. In many cases, weak precursor hormones are converted into more potent metabolites by specific enzymes, known as molecular switches.[1,2] The active forms have a high affinity toward corresponding receptors and the inactive forms have a very low affinity. The enzymes that interconvert the active and inactive forms, and thus act as molecular switches are pre-receptor regulatory enzymes.[2–4] Of these, we recently focused our attention on the human enzyme AKR1C3, a member of the aldo-keto reductase superfamily.[5] AKR1C3 is a peripheral 17β-hydroxysteroid dehydrogenase (17β-HSD type 5) that reduces a weak androgen, androstenedione, to the potent androgen testosterone, and a weak estrogen estrone to the potent estrogen 17β-estradiol, using NADPH as a coenzyme (Fig. 1).[6] AKR1C3 is thus an interesting target for the development of agents for treating hormone dependent forms of cancer like prostate cancer, breast cancer, and endometrial cancer. As AKR1C3 also catalyzes the reduction of 3-keto- and 20-ketosteroids and prostaglandin D_2 (PGD_2), it has been known variously as

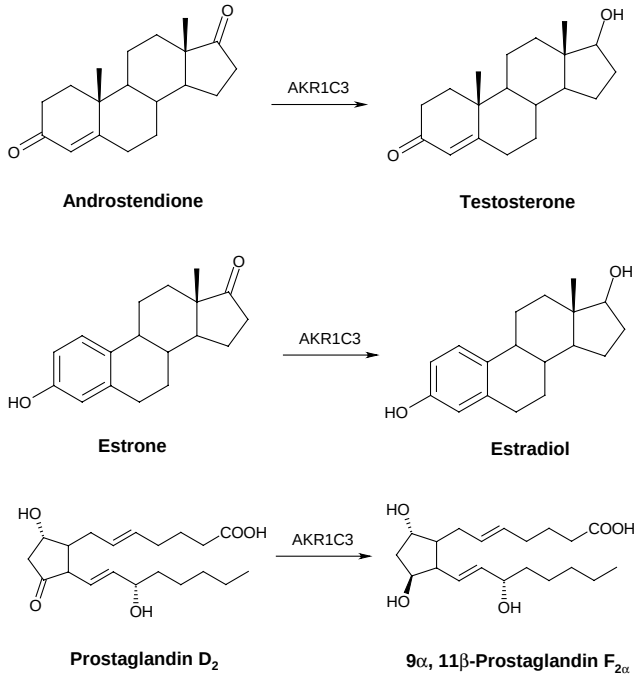

Figure 1. Reactions catalyzed by AKR1C3 in vivo.

Keywords: AKR1C3 inhibitors; Anti-inflammatory drugs; Anticancer agents; Cancer chemoprevention.
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3α-hydroxysteroid dehydrogenase type 2[7] and PGD_2 11-ketoreductase.[8]

Although AKR1C3 is a promising therapeutic target, only a few inhibitors have been reported so far.[9] Development of inhibitors that consist of nonsteroid core, and are thus devoid of residual steroidogenic activity, would be especially attractive. Dietary phytoestrogens (such as coumestrol, quercetin, and biochanin) and mycoestrogen zearalenon were reported to inhibit the enzyme in low micromolar concentrations,[10] as well as some other small molecule compounds like benzodiazepines,[11] benzofuranes, and phenolphthalein derivatives.[12] Indomethacin, flufenamic acid, and some related nonsteroidal anti-inflammatory drugs (NSAIDs, Fig. 2) are also very potent inhibitors, as is the cyclooxygenase (COX)-2 selective celecoxib[8,13,14] However, other NSAIDs (e.g., naproxen, diclofenac, salicylates, etc.) have been evaluated only on rat liver 3α-hydroxysteroid dehydrogenase (AKR1C9), a model for the human AKR1C isozymes.[15] Starting from mefenamic acid, a series of very active *N*-phenylanthranilic acid derivatives were synthesized (Fig. 2).[13] Recently, the X-ray crystal structures of AKR1C3 complexed with indomethacin, flufenamic acid, PGD_2, and rutin were reported, providing a structural basis for rational design of new and improved inhibitors.[14,16]

NSAIDs are drugs used to control inflammatory diseases by inhibiting COX and, in particular, COX-2 activity. It is generally accepted that NSAIDs also protect against the progression of gastrointestinal tumors and also other cancers like prostate carcinoma and leukemia.[17] NSAIDs are anti-proliferative against a broad spectrum of in vivo and in vitro models of human malignancies.[18] Recently, Desmond et al. suggested that NSAIDs could exert their anti-neoplastic activities via a non-COX-2 pathway.[19] They demonstrated that inhibition of AKR1C3 by indomethacin prevented the proliferation of human myeloid leukemia cells (HL-60). AKR1C3 converts PGD_2 into $PGF_{2\alpha}$, thereby preventing its conversion to 15-$\Delta^{12,14}$-PGJ_2, a natural ligand for the peroxisome proliferator-activated receptor-γ (PPARγ). Inhibitors of AKR1C3 are thus potential anti-neoplastic agents as they can indirectly activate PPARγ receptor by diverting PGD_2 catabolism to the generation of J-series prostanoids. Activation of PPARγ receptor induces differentiation, is anti-proliferative, and causes apoptosis in many cell types and cancers.[19,20] Since many of the frequently used NSAIDs have not been evaluated for inhibition of human AKR1C3, we have examined the inhibitory potencies of diclofenac, naproxen, ibuprofen, ketoprofen, acetylsalicylic acid, paracetamol, phenacetin, and etodolac (Table 1).

NSAIDs, like *N*-phenylanthranilic acid derivatives, are excellent starting points for the design of new inhibitors of AKR1C3. We postulated that the structurally related *N*-acylanthranilic acids could be potential inhibitors of this enzyme. In addition, Bauman et al. reported that,

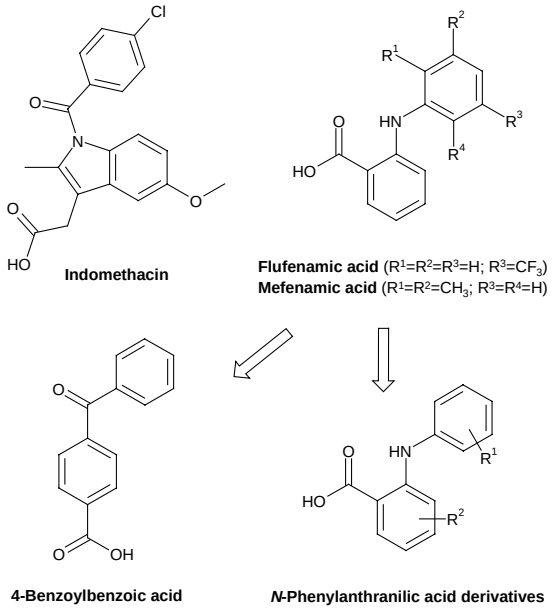
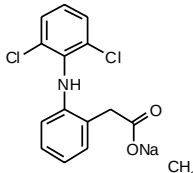
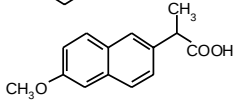
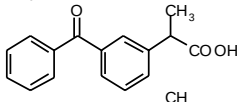
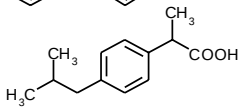
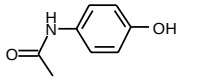
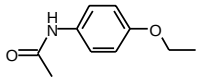
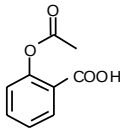
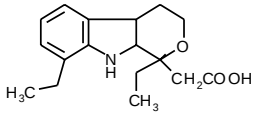


Figure 2. Nonsteroidal anti-inflammatory drugs and related compounds that inhibit AKR1C3.

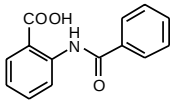
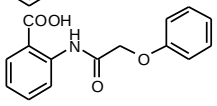
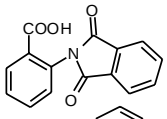
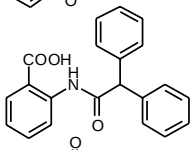
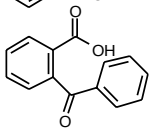
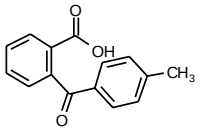
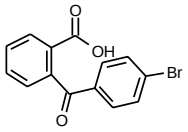
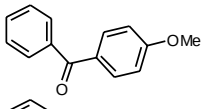
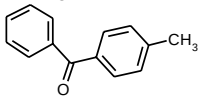
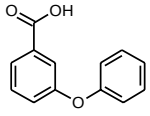
Table 1. Inhibition of AKR1C3 by nonsteroidal anti-inflammatory drugs

NSAID	Structure	IC_{50} (μM)
Diclofenac		2.6
Naproxen		0.48
Ibuprofen		33
Ketoprofen		(12%)[a]
Paracetamol		(8%)[a]
Phenacetin		(10%)[a]
Acetylsalicylic acid		NI[a]
Etodolac		NI[a]

[a] The values in parentheses are the % inhibition at 50 μM concn of inhibitor; NI, no inhibition observed.

besides *N*-phenylanthranilic acids, the benzophenone derivative 4-benzoylbenzoic acid (Fig. 2) potently inhibits AKR1C3.[13] Benzophenone derivatives and related compounds are also interesting because it is known that the replacement of the nitrogen atom between the two aromatic rings by a carbonyl group almost completely abolishes COX inhibition, while retaining AKR1C3 inhibition.[13] Due to serious, adverse cardiovascular effects of COX-2 inhibitors,[21] design of AKR1C3 inhibitors devoid of COX inhibitory activity would be an advantage. In order to prepare new inhibitors and to obtain more information about their structure–activity relationship, we synthesized a series of *N*-acylanthranilic acids, 2-benzoylbenzoic acids, benzophenones, and one phenoxybenzoic acid (Table 2), and screened their AKR1C3 inhibitory activities.

Table 2. New inhibitors of human recombinant AKR1C3

Compound	Structure	IC_{50} (μM)
1		(10%)[a]
2		(28%)[a]
3		NI[a]
4		11
5		70
6		44
7		39
8		180
9		(18%)[a]
10		0.68

[a] Values in parentheses are % inhibition at 50 μM concn of inhibitor; NI, no inhibition observed.

NSAIDs and related compounds synthesized in this study were tested for their inhibitory activity against human recombinant AKR1C3. AKR1C3 catalyzed reduction of 9,10-phenanthrenequinone in the presence of coenzyme NADPH.[15] The reaction was followed spectrophotometrically by measuring the change in NADPH absorbance ($\varepsilon_{\lambda 340} = 6270\ M^{-1}\ cm^{-1}$) in the absence and presence of inhibitor.[22] Initial velocities were calculated and IC_{50} values were determined (Tables 1 and 2).[23]

Of the NSAIDs evaluated, diclofenac ($IC_{50} = 2.6$ μM), naproxen ($IC_{50} = 0.48$ μM), and ibuprofen ($IC_{50} = 33$ μM) inhibited human AKR1C3 (Table 1).[22] Diclofenac is a 2-(2-anilinophenyl)acetic acid derivative which is closely related structurally to *N*-phenylanthranilic acids, the most promising inhibitors of AKR1C3. The good inhibitory properties of diclofenac imply that further investigation of a series of functionalized 2-(2-anilinophenyl)acetic acids could yield new and more potent inhibitors. The naphthalene derivative, naproxen, which belongs to the aryl-propanoic acid family of NSAIDs, is an even better inhibitor. The inhibitory activity of ibuprofen was in the low micromolar range while, in our hands, ketoprofene was almost inactive. Many members of the profene family of drugs, like suprofen, flubiprofen, ibuprofen, and ketoprofen, have been reported to be good inhibitors of the oxidative reaction catalyzed by AKR1C3.[8] Since AKR1C3 in vivo catalyzes the reduction of sex hormones, our method involving the inhibition of the reductive reaction is more appropriate for anticancer drug design and development purposes. The 4-aminophenol derivatives paracetamol and phenacetin were poor inhibitors, while acetylsalicylic acid and etodolac were devoid of any inhibitory activity.

The X-ray crystal structure of AKR1C3 reveals a substrate-binding site that consists mainly of hydrophobic aromatic amino acid side chains (Tyr24, Tyr55, Leu54, Trp227, and Phe306). The four conserved amino acids Asp50, Tyr55, Lys84, and His117 have been proposed to form a catalytic tetrad involved in the oxidation of alcohol or reduction of ketone functional groups via a 'push-pull' mechanism.[25] An oxyanion hole, which is located at the bottom of the hydrophobic pocket, is formed by active site tyrosine (Tyr55), histidine (His117), and the coenzyme's nicotinamide ring. A potent AKR1C3 inhibitor, flufenamic acid, binds at the active site of the enzyme in the vicinity of the nicotinamide ring, with the carboxylate oxygen occupying the oxyanion hole.[14] A similar binding mode, where the carboxylate group is bound next to the conserved tyrosine and histidine residues, is found in crystal structures of many AKR enzymes that have been complexed with carboxylic acid-containing compounds.[13] Another NSAID, indomethacin, also contains a carboxylate, but surprisingly, this does not bind toward the oxyanion hole. Instead, it H-bonds to the $NADP^+$ diphosphate moiety located at the opposite side of the active site.[14]

In order to investigate the possible binding mode, our best inhibitor, naproxen, was docked into the AKR1C3 active site (pdb code 1S2A), using AutoDock 3.0 with

the Lamarckian genetic algorithm.[26] AutoDock calculated that naproxen occupies a similar position of active site as indomethacin (Fig. 3). It binds to the bottom of the active site's hydrophobic pocket and on top of the coenzyme's nicotinamide moiety. Also, naproxen's carboxylate could form H-bonds with the oxygen atoms of the $NADP^+$ diphosphate moiety. This binding mode suggests that potential inhibitors, consisting of two carboxylic acid groups appropriately attached to the opposite sides of a lipophilic aromatic system, should be designed and synthesized. These hypothetical inhibitors could utilize the aromatic fragment (e.g., naphthalene ring) to interact with the active site hydrophobic pocket while the first carboxylate may occupy the oxyanion hole and the second forms H-bonds with oxygens of coenzyme's diphosphate moiety.

Target anthranilamides **1**, **2**, and **4** were prepared from anthranilic acid and the appropriate carboxylic acids by the method of mixed anhydrides. For the synthesis of compound **3**, anthranilic acid was *N*-phthaloylated by phthalanhydride. 2-Benzoylbenzoic acids **5**–**7** were prepared by Friedel–Crafts acylation of benzene and its derivatives with phthalanhydride. Benzophenones **8** and **9** were synthesized by Friedel-Crafts acylation with benzoyl chloride of anisole and toluene, respectively. 3-Phenoxybenzoic acid **10** was obtained by Cr_2O_3 oxidation of 3-phenoxybenzyl alcohol.[24]

Of the anthranilic acid derivatives described in this paper *N*-benzoylanthranilic acid **1** and *N*-(2-phenoxyacetyl)anthranilic acid **2** were only weak inhibitors of human AKR1C3, while *N*-phthaloylanthranilic acid **3** was inactive. However, if the anthranilic acid was acylated with 2,2-diphenylacetic acid, the amide **4** was obtained and found to be a very promising inhibitor (IC_{50} = 11 μM). Compound **4** is thus an interesting lead compound for developing new inhibitors of human AKR1C3, since its aromatic rings can be substituted with a variety of substituents at different positions in order to optimize binding to the enzyme with further improvement of the inhibitory activity.

N-Phenylanthranilic acids and related compounds are so far the most thoroughly investigated inhibitors of AKR1C3 and isozymes. Bauman et al. recently reported that movement of the carboxylic acid from the *ortho* to the *para* position did not decrease the enzyme inhibition, nor did replacement of the nitrogen atom between the aromatic rings by a keto group.[13] In fact, 4-benzoylbenzoic acid (Fig. 2) was one of the best AKR1C3 inhibitors in the series. However, benzoylbenzoic acids with a carboxylic group at the *ortho* position have not yet been evaluated. We found that 2-benzoylbenzoic acids **5**–**7** were fair inhibitors of AKR1C3, with IC_{50} values between 39 and 70 μM. It appears that 4′-substitution with electron-donating substituents slightly improves the inhibitory activity. In order to investigate the influence of the carboxylic acid group, benzophenone derivatives **8** and **9**, which lack a 2-carboxylate, were prepared. 4-Methoxybenzophenone **8** is a poor inhibitor (IC_{50} = 180 μM) and 4-methylbenzophenone is almost devoid of any inhibitory activity, so we can conclude that, in this series of compounds, a carboxylic acid moiety is essential for good inhibition of AKR1C3.

Another compound structurally related to the *N*-phenylanthranilic acid inhibitors of AKR1C3 is 3-phenoxybenzoic acid **10**. Here, benzoic acid is substituted at the *meta*-position and, in addition, the two aromatic rings are linked together by an oxygen atom. These structural modifications resulted in a very active inhibitor, with IC_{50} in the submicromolar range. Docking studies of this new inhibitor revealed a binding mode which is very similar to that of flufenamic acid (pdb code 1S2C). Also, compound **10** binds to the active site of the enzyme in the vicinity of the coenzyme's nicotinamide ring, with the aromatic resi-

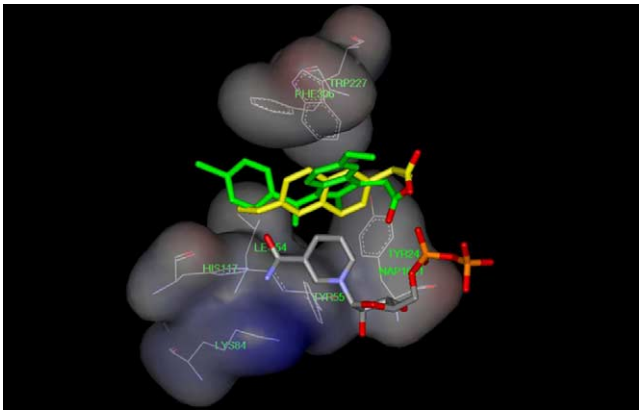


Figure 3. Superimposition of the computer model of naproxen (in yellow, carboxylate oxygens in red) on the X-ray structure of indomethacin (in green, carboxylate oxygens in red) bound to AKR1C3. The surfaces of the active site's most important amino acid residues and the nicotinamide diphosphate part of the coenzyme $NADP^+$ (NAP, in sticks) are shown.

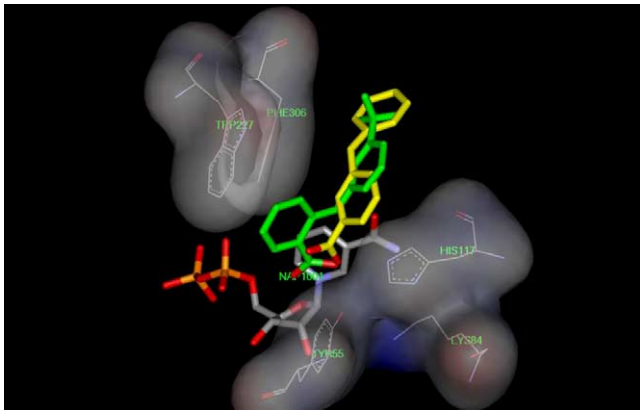


Figure 4. Superimposition of the computer model of compound **10** (in yellow, carboxylate oxygens in red) on the X-ray structure of flufenamic acid (in green, carboxylate oxygens in red) bound to AKR1C3. The surfaces of the active site's most important amino acid residues and the nicotinamide diphosphate part of the coenzyme $NADP^+$ (NAP, in sticks) are shown.

dues located in the hydrophobic pocket. The carboxylate oxygen is located in the oxyanion hole formed by active site Tyr55, His117, and the nicotinamide ring (Fig. 4).

We have identified some new, structurally diverse, inhibitors of human recombinant AKR1C3. The active compounds presented in this paper are promising lead compounds for the development of new anticancer agents. In principle, two different mechanisms of action are possible for their antitumor activities. Inhibitors of AKR1C3 are potential agents for treating hormone dependent forms of cancer, since AKR1C3 catalyzes the conversion of androstenedione and estrone to their active metabolites. On the other hand, AKR1C3 inhibitors can also have a cancer chemopreventive role, since inhibition of AKR1C3 can lead to diversion of prostaglandin catabolism toward the generation of J-series prostanoids and thereby to the activation of PPARγ receptor.

Acknowledgments

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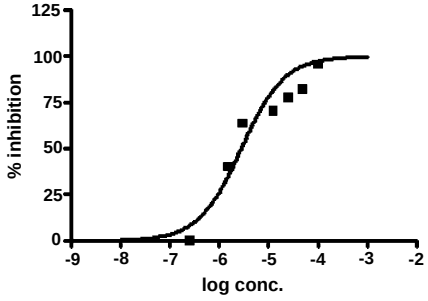
22. Assays were carried out in 0.6 mL aliquots of 100 mM phosphate buffer (pH 6.5) containing 0.9% DMF as co-solvent. The concentration of substrate was 5 μM, the coenzyme 200 μM, and the enzyme 0.5 μM. Concentrations of inhibitors were from 0.01 to 100 μM.
23. Representative example of IC_{50} determination for diclofenac. Initial velocities of enzymatic reactions in the absence (v_0) or presence of inhibitor (v_i) were calculated. Percentage inhibition (% inh.) was given by $100 - ((v_i/v_0) \times 100)$. IC_{50} values were determined graphically from a plot of % inh. versus log (inhibitor concn) using GraphPad Prism Version 4.00 (GraphPad Software, Inc.).



24. NSAIDs were obtained from Sigma. Spectroscopic data of all synthesized compounds were consistent with the literature (a) Lauer, W. M.; Benton, C. S. *J. Org. Chem.* **1959**, *24*, 804; (b) Sunders, S.; Peet, N. P. *J. Heterocycl. Chem.* **1979**, *15*, 1379; (c) Abramowitch, E.; Lamchen, M. *J. Chem. Soc.* **1965**, 2165; (d) Wolinski, J.; Buza, D.; Czerwinska-Fejgin, E.; Zamlynski, W. *Chemia Analityczna (Warsaw, Poland)* **1959**, *4*, 989; (e) Curtun, D. Y.; Quirk, R. P. *Tetrahedron* **1968**, *24*, 5791; (f) Mahmoodi, N. O.; Salehpour, M. *J. Heterocycl. Chem.* **2003**, *40*, 875; (g) Russel, T. *J. Chem. Soc.* **1962**, 3379; (h) Futami, Y.; Nishino, H.; Kurosava, K. *Bull. Chem. Soc. Jpn.* **1989**, *62*, 3567; (i) Bunyan, H. *J. Chem. Soc.* **1962**, 2771; (j) Chaintreau, A.; Adrian, G.; Couturier, D. *Synth. Commun.* **1981**, *11*, 439.
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