Development of Nonsteroidal Anti-Inflammatory Drug Analogs and Steroid Carboxylates Selective for Human Aldo-Keto Reductase Isoforms: Potential Antineoplastic Agents That Work Independently of Cyclooxygenase Isozymes

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ABSTRACT

Human aldo-keto reductases (AKRs) regulate nuclear receptors by controlling ligand availability. Enzymes implicated in regulating ligand occupancy and trans-activation of the nuclear receptors belong to the AKR1C family (AKR1C1-AKR1C3). Nuclear receptors regulated by AKR1C members include the steroid hormone receptors (androgen, estrogen, and progesterone receptors) and the orphan peroxisome proliferator-activated receptor (PPARγ). In human myeloid leukemia (HL-60) cells, ligand access to PPARγ is regulated by AKR1C3, which diverts PGD2 metabolism away from J-series prostanoids (Desmond et al., 2003). Inhibition of AKR1C3 by indomethacin, a nonsteroidal anti-inflammatory drug (NSAID), caused PPARγ-mediated terminal differentiation of the HL-60 cells. To discriminate between antineoplastic effects of NSAIDs that are mediated by either AKR1C or cyclooxygenase (COX) isozymes, selective inhibitors are required. We report a structural series of N-phenylanthranilic acid derivatives and steroid carboxylates that selectively inhibit recombinant AKR1C isoforms but do not inhibit recombinant COX-1 or COX-2. The inhibition constants, IC50, KI values, and inhibition patterns were determined for the NSAID analogs and steroid carboxylates against AKR1C and COX isozymes. Lead compounds, 4-chloro-N-phenylanthranilic acid and 4-benzoyl-benzoic acid for the N-phenylanthranilates and steroid carboxylates, exhibited IC50 values that had greater than 500-fold selectivity for AKR1C enzymes. These compounds can be used to dissect the role of the AKR1C isoforms in neoplastic diseases and may have cancer chemopreventive roles independent of COX inhibition.

The human AKR1C isoforms are hydroxysteroid dehydrogenases (HSDs) and are involved in the prereceptor regulation of steroid hormone action (Penning, 1997; Dufort et al., 1999; Dufort et al., 2001; Rizner et al., 2003). AKR1C isoforms regulate the concentration of active and inactive androgens, estrogens, and progestins in target tissues by catalyzing the reduction of ketosteroids at the C3, C17, or C20 positions (Dufort et al., 1999; Penning et al., 2000; Dufort et al., 2001). Therefore, AKR1C isoforms regulate the ligand occupancy and trans-activation of the nuclear steroid hormone receptors, which include the androgen, estrogen (ER), and progesterone receptors. Therefore, they may be important in regulating steroid hormone concentrations in target tissues by modulating their intracrine formation (Labrie et al., 2001). Selective inhibitors of AKR1C isoforms provide an approach to obtain tissue specific effects of steroid hormones and are termed selective intracrine modulators (SIMs).

The human AKR1C isoforms involved in regulating the local concentration of steroid hormones include AKR1C1 (20a-HSD), AKR1C2 (Type 3 3α-HSD), and AKR1C3 (Type 5 17β-HSD, Type 2 3α-HSD) and share greater than 86% amino acid sequence identity (Penning et al., 2000). Despite
their high sequence identity, they display different substrate preferences, inhibition profiles, and tissue-specific expression patterns (Matsuura et al., 1997; Dufort et al., 1999; Penning et al., 2000; Dufort et al., 2001). AKR1C isozymes catalyze the reduction of 3-, 17- and 20-ketosteroids in different ratios so that AKR1C1 is considered a 20α-HSD that will inactivate progesterone, AKR1C2 is considered a peripheral 3α-HSD that will inactivate 5α-dihydrotestosterone, and AKR1C3 is considered a peripheral 17β-HSD that will form testosterone and 17β-estradiol from their less active precursors (Fig. 1) (Dufort et al., 1999; Penning et al., 2000; Dufort 2001; Rizner et al., 2003). Several of these isozymes will also transform prostaglandins; for example, AKR1C3 is also known as prostaglandin F$_2$α synthase (Matsuura et al., 1998). Therefore, AKR1C isozymes may be further involved in the prereceptor regulation of a large group of nuclear receptors (e.g., steroid receptors and nuclear orphan receptors).

Desmond et al. (2003) recently showed that inhibition of AKR1C3 by the nonsteroidal anti-inflammatory drug (NSAID) indomethacin prevented the proliferation of human myeloid leukemia HL-60 cells. AKR1C3 prevented the conversion of PGD$_2$ to 15-Δ$_{12,14}$-PGJ$_2$ [a ligand for the nuclear orphan peroxisome proliferator-activated receptor γ (PPARγ)]. By converting PGD$_2$ to PGF$_{2\alpha}$, AKR1C3 deprived PPARγ of its ligand and prevented terminal differentiation of the myeloid leukemia cells (Fig. 2). This phenotypic change suggests a role for AKR1C3 in regulating myeloid leukemia cell differentiation and indicated that NSAIDs can have antineoplastic properties via a non-cyclooxygenase (COX)-2 pathway. This result not only validated the concept of SIMs, whereby targeting a specific AKR1C isozyme has beneficial therapeutic effects, but also indicated a need to develop NSAID analogs that discriminate between AKR1C isozymes and their traditional targets, COX-1 and COX-2. In 1983, Penning and Talalay showed that the commonly prescribed NSAIDs indomethacin and mfenamic acid were potent inhibitors of AKR1C9 at pharmacologically relevant concentrations. AKR1C9 is a model for the human AKR1C isozymes because it has 69% sequence identity at the amino acid level with the human AKR1C isozymes. This suggests that the human enzymes may be important targets for NSAIDs, and their inhibition may contribute to the antineoplastic effects of these drugs.

Nonselective NSAIDs do not discriminate between COX-1 and COX-2 (Laneuville et al., 1994). Therefore, selective COX-2 inhibitors were developed to reduce the side effects of classic NSAIDs. However, mounting evidence suggests non-selective and selective NSAID inhibitors have other targets (Wick et al., 2002; Keller and Giardiello, 2003; Leng et al., 2003; Reid et al., 2003; Sanchez-Alcazar et al., 2003). These include the multidrug resistance proteins, transcription factors, mitogen-activated protein kinase kinases, cell cycle regulatory proteins, and the human AKR1C isozymes, which are the focus of this article (Penning and Talalay, 1983; Askonas et al., 1991; Tegeder et al., 2001; Wick et al., 2002; Desmond et al., 2003; Keller and Giardiello, 2003; Leng et al., 2003; Reid et al., 2003; Sanchez-Alcazar et al., 2003). To test the hypothesis that AKR1C isozymes are involved in COX-independent neoplasias, compounds are needed that are selective for AKR1C isozymes but not for COX-1 or COX-2.

![Fig. 1. Regulation of ligand concentrations for nuclear receptors by AKR1C isozymes. All AKR1C isozymes are shown working in the reduction direction only.](https://molpharm.aspetjournals.org/)

![Fig. 2. Potential therapeutic benefit of inhibiting AKR1C3 using an NSAID analog that does not inhibit COX-1 leading to the activation of the PPARγ receptor and ultimately cellular differentiation.](https://molpharm.aspetjournals.org/)
We report a set of AKR1C inhibitors based on N-phenylanthranilic acids and cholanic acids that can discriminate between AKR1C and COX isozymes. Lead compounds that specifically inhibit AKR1C isozymes but not COX were identified. Because AKR1C isozymes are involved in prereceptor regulation of nuclear receptors, these new compounds may lead to cancer chemopreventive agents that exert their effects independently of COX. The selective COX-2 inhibitor celecoxib (Celebrex) was also found to inhibit the AKR1C isozymes. Therefore, the AKR1C selective inhibitors described will be able to discriminate between the effects of celecoxib on COX-2 versus those effects on AKR1C isozymes.

Materials and Methods

2-Chloro-5-methyl-benzoic acid was purchased from Pfaltz and Bauer Chemicals (Waterbury, CT), and 2-chloro-4-nitro-benzoic acid was purchased from Acros Organics (Pittsburgh, PAI). Glycocolic acid, tauroenecolic acid, 4-carboxy-2, 4-dinitrodiphenylamine, 4-chloro-N-phenylanthranilic acid, 4-chloro-N(’-toly)-anthranilic acid, 3-amino-4-phenylamino-benzoic acid, and 4-benzoyl-benzoic acid were purchased from Sigma-Aldrich (St. Louis, MO). Mafenamic acid, 3-amino-4-phenylamino-benzoic acid, and 4-benzoyl-benzoic acid, taurodeoxycholic acid, 4-carboxy-2, 4-dinitrodiphenylamine, 4-chloro-N-phenylanthranilic acid, 4-chloro-N(’-toly)-anthranilic acid, 3-amino-4-phenylamino-benzoic acid, and 4-benzoyl-benzoic acid were purchased from Acros Organics (Pittsburgh, PA). Glycocholic acid was purchased from Wilton, NH). Celecoxib was purchased from ChemPacific USA (Baltimore, MD). Arachidonic acid was purchased from Cayman Chemicals (Ann Arbor, MI).

Synthesis of Nonsteroidal Inhibitors: N-Phenylanthranilic Acid Derivatives

The synthesis of the N-phenylanthranilic acid derivatives used the Ullmann-Goldberg reaction that couples benzoic acid and aniline derivatives as described previously (Ozaki et al., 1985). The general method was as follows: 0.04 mol of the halogenated benzoic acid derivative, 0.08 mol of aniline, 0.05 mol of potassium carbonate, 0.003 mol of copper(II) powder, and 15% (w/w) pyridine were refluxed in 25 ml of amyl alcohol for 18 h. The amyl alcohol was removed by rotary evaporation, and a mixture of potassium carbonate and water was added to the resultant slurry. The mixture was acidified with HCl (1:1) to pH 4 to 5. The resultant solid was filtered, washed with cold H2O, extracted in CHCl3, and recrystallized from EtOH. The structure was confirmed by 1H NMR (500 MHz) in CDCl3 and by mass spectroscopy (MS). All chemical shifts are reported as parts per million relevant to a tetramethylsilane internal standard. The high-resolution MS data were obtained on a Micromass AutoSpec instrument (Waters, Milford, MA) carried out using electrospray in either the positive or negative ionization mode.

5-Methyl-N-phenylanthranilic Acid (C14H14NO2). This was synthesized using 2-chloro-5-methyl-benzoic acid and aniline with a 46% yield, purified, and recrystallized as described above (I). H NMR (500 MHz) indicated δ ppm 2.3 (3H, -CH3, s), δ ppm 4.3 (1H, NH, s), and δ ppm 7.5 to 8.2 (2H, aromatic). MS (ES+) gave a parent ion MH+ m/z = 286.14 (+Na+, Cl), predicted m/z = 227.26. 4-Nitro-N-phenylanthranilic Acid (C14H12N2O2). This was synthesized using 2-chloro-4-nitro-benzoic acid and aniline with an 11% yield, purified, and recrystallized as described above. 1H NMR (500 MHz) indicated δ ppm 4.3 (1H, NH, s), δ ppm 7.5 to 8.2 (2H, aromatic), and δ ppm 9.5 (1H, -COOH, s). The MS (ES+) gave a parent ion M+ -H m/z = 257.14, predicted m/z = 258.06.

Synthesis of Steroidal Inhibitors: Formation of Ethyl Esters

The lithocholic ester was synthesized as described previously (Barton et al., 1989). A solution of 200 mg of lithocholic acid in 15 ml of 90% ethanol containing 0.6 g of HCl was stirred at room temperature for 8 h. After partial concentration, the residue was extracted with dichloromethane. The organic phase was washed with water until neutral and then with saturated brine, dried over sodium sulfate, and evaporated under reduced pressure. The overall yield was 190 mg (95%) and the structure was confirmed by 1H NMR and MS. 1H NMR (500 MHz) indicated a loss of the carboxylic acid proton at δ ppm 12.0 (1H, -COOH, s) and a gain in the ethyl group at δ ppm 2.3 ppm 4.3 (1H, NH, s), and δ ppm 7.5 to 8.2 (2H, aromatic). The MS (ES+) gave a parent ion MH+ m/z = 427.32 (+Na, Cl), predicted m/z = 403.31.

Purification of AKR1C Isoforms and COX Isoforms

Recombinant homogenous AKR1C isozymes were purified according to published procedures (Burczynski et al., 1998; Penning et al., 2000). Purification to homogeneity of COX-1 from ram seminal vesicles and COX-2 from baculovirus-infected SF-21 cells was performed as described previously (George et al., 1996, and Smith et al., 2000, respectively).

Steady-State Kinetic Assays

A continuous assay was used to monitor AKR1C activity. This assay monitored the oxidation of 1-acephanthalenol by measuring the increase in absorbance of NADH at 340 nm (molar extinction coefficient) = 6270 M–1 cm–1. Reactions were performed in 1.0-ml systems containing 100 mM potassium phosphate, pH 7.0, 1% BSA, 2.3 mM NAD+, increasing concentrations of 1-acephanthalenol ranging from 5 to 500 μM, and 4% methanol. By using this assay, the specific activities were determined to be 2.1 μmol/min/mg for AKR1C1, 2.5 μmol/min/mg for AKR1C2, and 2.8 μmol/min/mg for AKR1C3 at 1 mM 1-acephanthalenol (Burczynski et al., 1998; Penning et al., 2000). A continuous assay was also used to monitor COX-activity. This assay monitored the oxidation of N,N,N,N’-tetramethyl-1,4-phenylenediamine (TMPD) when it was used as co-reductant to reduce PGG2 to PGH2 starting with arachidonic acid (Benedetto et al., 1987). The assay monitors the formation of N,N,N,N’-tetramethyl-1,4-phenylenediamine (ε = 12,000 M–3 cm–1) at 610 nm. Reactions were performed in 1.0-ml systems containing 100 mM Tris-HCl, pH 8.0, 80 μM TMPD, 2 μM ferriprotoporphyrin IX, 60 μM arachidonic acid, and 5% DMSO. Using this assay, the specific activities were determined to be 25 and 16.8 μmol/min/mg of enzyme at 150 μM arachidonic acid for COX-1 and COX-2, respectively.

Initial velocities were obtained by linear regression to reaction progress curves. Plots of velocity versus substrate concentration were hyperbolic and could be fit to the Michaelis-Menten equation (V0 = (Vmax × S)/Km + S) to yield values (± S.D.) for Vmax, km, and Km for a given substrate.

Reversible Inhibition Studies

IC50 values for AKR1C enzymes were measured by varying the inhibitor concentrations while holding the 1-acephanthalenol concentration equal to the Km for AKR1C1 and AKR1C2. Because of the solubility constraints of 1-acephanthalenol, the Km for AKR1C3 could not be accurately determined, but estimates approached 2 mM, and the resulting IC50 experiments were performed at a concentration of 1-acephanthalenol equal to 100 μM (Km of AKR1C2). Inhibitors were dissolved in DMSO, and the final concentration of cosolvent was 4% (2% DMSO and 2% methanol) for AKR1C isozymes. IC50 values were measured for COX isozymes by varying inhibitor concentrations while holding the arachidonic acid concentration equal to 60 μM (Benedetto et al., 1987). Inhibitors were dissolved in DMSO, and the final concentration of cosolvent was 5% for the COX measurements. The presence of the DMSO in the assay had no effect on initial velocities. Inhibition data were fit using Grafit 5.0 [y = {range}/[1 + (IC50)power] + background] yielding the IC50 value. Reversible inhibition patterns for the AKR1C and COX isozymes were determined by varying the concentration of inhibitor (0.125–3 × IC50).
over fixed substrate concentrations (0.2–5 × $K_M$), and the reactions were initiated with enzyme. Initial velocity data were globally fit to the competitive, noncompetitive, and uncompetitive inhibition models and the results presented represent the best fit as judged by standard deviation using Grafit 5.0 to determine the inhibition constant $K_I$. Using the association that exists between the IC$_{50}$ value, $K_M$, and the substrate concentration, the $K_I$ values were calculated for many inhibitors using the Cheng-Prusoff (1973) relationship, \[ [K_I] = \frac{IC_{50}}{S/K_M}, \] where the lead compound in that class displayed competitive inhibition kinetics.

Time-Dependent Inhibition of Celecoxib for COX and AKR1C Isozymes

The inhibition potency of celecoxib for COX and AKR1C isozymes was determined by two methods. First, celecoxib was added directly to the enzyme assays as described above under conditions in which it would compete for substrate. Second, the time-dependent inhibition of COX and AKR1C isozymes by celecoxib (0.0005–50 μM for COX-2, 0.005–200 μM for COX-1, and 0.005–100 μM for AKR1C isozymes) was investigated. In this method, the AKR1C enzymes (1.5 μg for AKR1C1, 1.5 μg for AKR1C2, and 11.6 μg for AKR1C3) and COX enzymes (10.7 μg for COX-1 and 7.6 μg for COX-2) were preincubated with inhibitor for 10 min and the reaction was initiated with substrate (Gierse et al., 1996). Preincubation of COX-1 and COX-2 with celecoxib for 10 min is sufficient for this slow tight-binding inhibitor to cause maximal loss of enzyme activity (Copeland et al., 1994; Gierse et al., 1996).

Results

Inhibitors That Will Discriminate between AKR1C Isozymes and COX Isozymes

There are several major chemical classes of classic NSAIDs that target COX and AKR1C isozymes. Of these, the two most potent are the indole acetic acids (e.g., indomethacin) and the $N$-phenylanthranilic acids (e.g., mefenamic acid and meclofenamic acid). In this study, we concentrated on the $N$-phenylanthranilic acids for several reasons. First, their known structure-activity relationships (SAR) points to subtle changes that could be exploited to eliminate COX inhibition (Fig. 3B) but retain AKR1C inhibition (Scherrer, 1974; Lombardino, 1985). These structural alterations include changing the heteroatom, substitution on the amine, substitution on the A-ring, and varying the ortho relationship that exists between the amine and the carboxylic acid in the A-ring. Second, $N$-phenylanthranilic acids can be synthesized via a single-pot reaction using the Ullmann-Goldberg coupling reaction in which any halogenated benzoic acid is coupled to any aniline derivative, providing a route to a combinatorial library (Fig. 3A).

Another structural lead to AKR1C inhibitors exists in the C24-cholanic acids (bile acids). AKR1C2 is also known as the human bile acid binding protein and binds bile acids with nanomolar affinity, whereas AKR1C1 and AKR1C3 bind bile acids with micromolar affinity (Matsuura et al., 1997, 1998). This selectivity is impressive considering that these enzymes share greater than 86% amino acid identity. More impressive is that AKR1C1 and AKR1C2 are greater than 97% similar at the amino acid level and differ by only one amino acid in the active site, yet bile acids are more than 100-fold selective for AKR1C2 than AKR1C1. This result indicates that selective inhibitors maybe attainable for each AKR1C isozyme.

$K_I$ values are more representative of the inhibition potency and will be used to compare inhibition potency between AKR1C isozymes because $K_I$ values were obtainable for most of the compounds tested (Fig. 4). However, in a comparison of inhibition potency between the AKR1C isozymes and COX isozymes, IC$_{50}$ values were used because $K_I$ values were unobtainable for most of the compounds tested for the COX isozymes.

**Results**

Fig. 3. A, the Ullmann-Goldberg coupling reaction. B, SAR trends in $N$-phenylanthranilic acids that eliminate COX-1 inhibition.

Fig. 4. Inhibitors screened.
Inhibition Patterns for the AKR1C Isozymes and the COX Isozymes

Inhibition patterns for the AKR1C isozymes and COX-1 were determined for the N-phenylanthranilic acid parent compound mfenamic acid (1), 5-methyl-N-phenylanthranilic acid (2), and 5β-cholanic-3-one (9). Representative competitive inhibition patterns for AKR1C2 with compounds 1, 2, and 9 were obtained (Fig. 5). The representative inhibition patterns indicate a competitive mode of inhibition for each chemical class, whereby the substrate and inhibitor compete for the same binary complex (AKR1C2-NAD+). Using the Chang-Prusoff (1973) relationship, $K_i$ values were calculated for the remainder of the compounds in each class as described under Materials and Methods (Tables 1 and 2).

N-Phenylanthranilic Acids—Enzyme Selectivity, Potency, and Trends

The parent compound mfenamic acid (1) inhibited both AKR1C and COX isozymes (Fig. 6). Inhibition by 1 indicated that it is more selective for the AKR1C isozymes than the COX isozymes, but it was able to inhibit all enzymes tested. The rank order of potency against these enzymes was: AKR1C3 (IC50 = 0.39 μM), AKR1C2 (IC50 = 0.96 μM), AKR1C1 (IC50 = 4.0 μM), COX-1 (IC50 = 33.3 μM), and finally COX-2 (IC50 = 225 μM) (Table 1). Representative inhibition patterns with the NSAID analogs showed that the pattern of inhibition was competitive. Thus the IC50 values obtained with each of the AKR1C isozymes were directly comparable. In addition, because of the relationship that exists between IC50 values and $K_i$ under competitive inhibition conditions, $K_i$ values could be computed for each of the remaining inhibitors. By contrast, IC50 values for COX-1 and COX-2 inhibition were obtained at 60 μM arachidonic acid as described previously (Gierse et al., 1996). Our COX data are consistent with previous findings, which showed that the N-phenylanthranilic acids are more potent against COX-1 (Gierse et al., 1996). Although the IC50 values for the COX isozymes were not determined at $K_i$, the N-phenylanthranilic acid analogs can be directly compared with the values obtained for mfenamic acid, the parent compound (1).

N-Phenylanthranilic acid derivatives were examined for their selectivity to inhibit AKR1C isozymes versus COX isozymes (Table 1). The N-phenylanthranilic acid analogs were found to be potent inhibitors for the AKR1C isozymes but did not inhibit COX isozymes, as expected. The loss of COX inhibition by simple modifications to the A-ring was predicted by existing SAR; however, the maintenance of AKR1C inhibition was a unique finding. For example, halogenation of the A-ring led to a decrease in inhibition potency by more than 30-fold for COX-1 and 5-fold for COX-2. The rank order for enzyme inhibition with 4 was AKR1C2 (IC50 = 1.15 μM) > AKR1C3 (IC50 = 3.01 μM) > AKR1C1 (IC50 = 8.2 μM) > COX-1 and COX-2 (IC50 > 1000 μM). The selectivity for the N-phenylanthranilic acid analogs ranged from 30-fold (AKR1C1, IC50 = 8.3 μM; COX-1, IC50 = 240 μM) for compound 7, which contained substitution on both rings, to greater than 1200-fold (AKR1C3, IC50 = 0.40 μM; COX-1, IC50 > 500 μM) for compound 3, in which the ortho relationship between the carboxylate and amine was altered to para. No time dependence was noticed for the inhibition of AKR1C and COX isozymes by the N-phenylanthranilic acid derivatives (data not shown).

Analysis of the N-phenylanthranilic acid derivatives revealed potential trends in loss of COX isozyme inhibition. Replacement of the heteroatom almost completely abolished COX inhibition but did not affect AKR1C inhibition. For example, compound 8 did not inhibit COX-1 or COX-2 activity (COX-1, IC50 > 1000 μM; COX-2, IC50 > 1000 μM) compared with the parent compound (1), which inhibited both COX isozymes but remained a potent inhibitor for the AKR1C isozymes (AKR1C3, IC50 = 2.04 μM; AKR1C1, IC50 = 33.2 μM; and AKR1C2, IC50 = 36.9 μM). Movement of the carboxylic acid from the ortho to the para position on the A-ring decreased inhibition dramatically for COX-1 and COX-2 but did not affect AKR1C inhibition. For example, compound 6 gave IC50 values of > 1000 μM for COX-1 and COX-2 and remained a potent inhibitor for the AKR1C isozymes (AKR1C3, IC50 = 10.7 μM; AKR1C2, IC50 = 26.2 μM; and AKR1C1, IC50 = 49.4 μM), whereas compound 1 gave IC50 values of 33.3 and 225 μM for COX-1 and COX-2, respectively. However, substitutions on the B-ring resulted in a slight increase in COX inhibition potency. For example, addition of a para-methyl group to the B-ring increased the inhibition potency for COX-1 more than 4-fold by 7 (IC50 = 240...
μM) compared with 4 (IC_{50} > 1000 μM). By contrast, substitution on the A-ring also substantially reduced potency against the COX isozymes but retained AKR1C inhibition. For example, 2 was 13-fold and 5-fold less potent against COX-1 and COX-2, respectively, compared with 1.

Steroid Carboxylates—Isoform Selectivity, Potency, and Trends. C24-cholanic acids are known inhibitors of AKR1C2, but their inhibition potencies toward other AKR1C and COX isozymes are unknown. Screening the steroid carboxylates indicated that these compounds were both more potent and selective for the AKR1C isozymes than the COX isozymes (Table 2). The AKR1C isozymes were potently inhibited by the bile acids, whereas the COX isozymes were not inhibited. For example, 5β-cholanic acid-3-one (9) potently inhibited the AKR1C isozymes, whereas the COX isozymes were unaffected at all concentrations tested (Fig. 8). The rank order of enzyme inhibition with compound 9 was AKR1C2 (IC_{50} = 0.04 μM) > AKR1C3 (IC_{50} = 1.02 μM) > AKR1C1 (IC_{50} = 14.0 μM) > COX-1 and COX-2 (IC_{50} ≥ 250 μM). As expected, AKR1C2 was much more potent inhibited by the bile acids compared with the other AKR1C isozymes tested, whereas the COX isozymes were not inhibited. The selectivity for the AKR1C isozymes versus COX isozymes ranged from 5-fold for 13 to greater than 30,000 for 12.

### Table 2

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<td>AKR1C1</td>
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<tr>
<td>1</td>
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N.D., not determined.

### Table 3

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N.D., not determined.

**NSAID Analogs That Target Human AKR1Cs and Not COX**

*N-Phenylnanthranilic Acid—Isoform Selectivity, Potency, and Trends.* The *N*-phenylnanthranilic acid analogs were analyzed for AKR1C isoform selectivity. In general, the selectivity among the individual AKR1C isoforms was not as dramatic as the selectivity between AKR1C and COX isoforms. However, compounds 4, 7, and 8 were more than 6-fold more selective for different AKR1C isoforms. Further analysis indicated potential trends that could be exploited to increase isoform selectivity. Movement of the carboxylic acid from the ortho to the para position on the A-ring increased the selectivity for AKR1C3 compared with AKR1C1 or AKR1C2. For example, 3 and 8 were more selective for AKR1C3 (K_{i} = 0.38 μM and K_{i} = 1.9 μM, respectively) than AKR1C1 (K_{i} = 2.66 μM and K_{i} = 12.5 μM, respectively) or AKR1C2 (K_{i} = 1.32 μM and K_{i} = 13.3 μM, respectively).
respectively). It is interesting that substitution on the A-ring with the carboxylic acid at the para position increased the potency for AKR1C3 more than 5-fold (compare 6 and 8). Replacement of the heteroatom seems to favor selectivity for AKR1C3 over AKR1C1 and AKR1C2 (compare 8 and 6). This increase in selectivity for AKR1C3 might be a result of the position of the carboxylic acid on the A-ring, replacement of the heteroatom, or a combination of both. Multiple substitutions on the B-ring seem to favor AKR1C isozyme potency, in that 1 and 3 are very potent inhibitors for all AKR1C isozymes compared with COX isozymes. Furthermore, when compounds containing the carboxylic acid in the para position are compared (3, 6, and 8), it is apparent that multiple substitutions on the B-ring enhanced the inhibition potency for the AKR1C isozymes. For example, compound 3 is 7-fold and 4-fold more potent for AKR1C1 than compounds 6 and 8. Compound 3 is 7-fold and 9-fold more potent for AKR1C2 than compounds 6 and 8, and it is more than 26-fold and 5-fold more potent for AKR1C3 than compounds 6 and 8, respectively. Introduction of a chloride at the fourth position of the A-ring increases the selectivity for AKR1C2, given that 4 and 7 are more than 7-fold more selective for AKR1C2 (IC$_{50}$ = 0.41 μM, $K_I$ = 0.2 μM) than for AKR1C1 ($K_I$ = 3.1 μM, $K_I$ = 3.1 μM), and AKR1C3 ($K_I$ = 2.9 μM, $K_I$ = 1.4 μM). Furthermore, introduction of a methyl group at the fifth position of the A-ring produced a compound (2) that was 14-fold more selective for AKR1C2 than AKR1C9, but this compound did not discriminate between AKR1C2 and AKR1C1 (compare 1 and 2). It is clear that small changes on the A-ring or B-ring of the N-phenylantranilic acids can introduce selectivity for the different AKR1C isozymes.

When comparing the inhibition of the N-phenylantranilic acid analogs, the electronegativity of the carboxylic acid is determined by the different substituents on the A-ring, thus altering the $pK_a$, for the carboxylic acid. For example, the addition of a chloride group at the fourth position on the A-ring decreased the inhibition potency by more than 2-fold for all the AKR1C isozymes, whereas 1 and 4 (AKR1C1 $K_I$ = 0.81 μM for 1 compared with $K_I$ = 3.1 μM for 4, AKR1C2 $K_I$ = 0.22 μM for 1 compared with $K_I$ = 0.41 μM for 4, and AKR1C3 $K_I$ = 0.3 μM for 1 compared with $K_I$ = 2.9 μM for 4). The effect of the halogen on the A-ring decreases the $pK_a$ of the carboxylic acid, thereby increasing the strength of the carboxylic acid and consequently affecting the overall inhibition potency. The relative $pK_a$ may be an important factor that influences the overall inhibition potency by increasing or decreasing the propensity of the acid to be deprotonated, and is further influenced by the presence of the amine.

**Steroid Carboxylates—Isoform Selectivity, Potency, and Trends.** The steroid carboxylates tested were potent inhibitors for all the AKR1C isozymes; however, these compounds were more selective for AKR1C2 than AKR1C1 or AKR1C3. The selectivity for AKR1C2 versus AKR1C1 and AKR1C3 ranges from greater than 48-fold for 11 to greater than 570-fold for 10 when $K_I$ values are compared. Increasing the side-chain length and changing the acid at position C24 resulted in the decrease of inhibition potency for all the AKR1C isozymes. For example, by comparing the $K_I$ values for 10 with 13 and 14, a decrease in potency is seen for the inhibition of AKR1C1 (10 is 7-fold more potent than 13 and 2-fold more potent than 14), AKR1C2 (10 is 41-fold more potent than 13 and 11-fold more potent than 14), and AKR1C3 (10 is 20-fold more potent than 13 and 9-fold more potent than 14). The SAR with steroid carboxylates indicates that replacement of the carboxylic acid (13) with a more electronegative acid, such as sulfonic acid (14), increases the potency by more than 2-fold for all the AKR1C isozymes (AKR1C1 $K_I$ = 23 μM for 14 compared with $K_I$ = 66 μM for 13, AKR1C2 $K_I$ = 0.2 μM for 14 compared with $K_I$ = 0.72 μM for 13, and AKR1C3 $K_I$ = 99 μM for 14 compared with $K_I$ = 206 μM for 13). The importance of the acid was substantiated when the carboxylic acid for compound 11 was esterified with an ethyl group (15). This small change resulted in a severe decrease in inhibition potency for all the AKR1C isozymes (compare 11 and 15). The inhibition potency of 15 for AKR1C1 decreased by more than 9-fold (IC$_{50}$ = 9.2 μM for 11 and IC$_{50}$ > 100 μM for 15), the inhibition potency for AKR1C2 decreased by more than 28-fold (IC$_{50}$ = 0.09 μM for 11 and IC$_{50}$ = 2.5 μM for 15), and inhibition potency for AKR1C3 decreased by more than 64-fold (IC$_{50}$ = 1.57 μM for 11 and IC$_{50}$ > 100 μM for 15).

**Inhibition of COX and AKR1C Isozymes by the COX-2 Selective Inhibitor Celecoxib.**

Recent data suggest that the COX-2 selective inhibitor celecoxib may have other targets at physiological concentrations. Therefore, celecoxib (16) was tested against the AKR1C isozymes and was found to be a potent inhibitor for all the AKR1C isozymes tested (Table 1) yielding the following rank order AKR1C9 ($K_I$ = 5.2 μM) > AKR1C2 ($K_I$ = 8.6 μM) > AKR1C1 ($K_I$ = 14.9 μM). It was interesting that when celecoxib was tested as a reversible inhibitor, there was no
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**Discussion**

Classic NSAIDs and selective COX-2 inhibitors also potently inhibit AKR1C isozymes implicated in regulating ligand concentrations for nuclear receptors. We show that AKR1C isozymes are inhibited by celecoxib at concentrations similar to its peak plasma concentrations (Chow et al., 2004). To discern the cancer chemopreventive properties of these drugs, we report NSAID analogs and steroid carboxylates that inhibit the AKR1C enzymes but do not inhibit COX isozymes. Small changes in the structures of N-phenylanthranilic acid derivatives resulted in a loss of COX-inhibition, but AKR1C inhibition was retained. Using these compounds, the roles of AKR1C and COX-isozymes in malignancies affected by trans-activation of nuclear receptors can now be discerned.

**N-Phenylanthranilic Acid Derivatives.** Using the SAR criteria for N-phenylanthranilic acid inhibition of COX, we developed analogs that were selective for the AKR1C isozymes, whereas their effects on COX isozymes were minimal. Changes to the N-phenylanthranilic acid scaffold (A-ring substitution and heteroatom substitution) dramatically decreased COX isozyme inhibition in some instances by more than 30-fold (compounds with IC₅₀ ≥ 1000 μM), but potent inhibition of the AKR1C isozymes was retained.

The selectivity between the AKR1C isozymes was less dramatic than that observed between the AKR1C and COX isozymes. However, movement of the carboxylic acid from the ortho to the para position on the A-ring favored AKR1C3 inhibition, and/or additional substitution of the B-ring favored isozyme selectivity.

**Steroid Carboxylate Derivatives.** The steroid carboxylates, including the bile acids, were known selective inhibitors for the AKR1C isoforms with preference for AKR1C2. Bile acids show 100-fold selectivity for AKR1C2 over AKR1C1 yet differ by a single amino acid at the active site. The structural basis for this difference in inhibition potency is explained by the AKR1C2-NADP⁺-Ursodeoxycholate crystal structure, which indicated that replacement of Val54 in AKR1C2 to a leucine in AKR1C1 disrupts binding of the bile acids (Jin et al., 2001). The significant finding is that these steroid carboxylates do not inhibit COX isozymes. Further selectivity in these compounds for AKR1Cs may be achieved by restricting the rotation around the steroid side chain.

**Comparison of the Ligand Binding Sites of AKR1C Isozymes and COX.** Crystal structures exist for the AKR1C isozymes with steroid carboxylates (AKR1C2-NADP⁺-ursodeoxycholate, Protein Data Bank entry 1IHI) and NSAIDs (AKR1C3-NADP⁺-flufenamic acid, Protein Data Bank entry 1S2C) (Jin et al., 2001, and Lovering et al., 2004, respectively). Both ternary complex structures have the carboxylic acid of the inhibitor bound to the catalytic tyrosine and can provide starting points for inhibitor design. When the two active sites in these ternary complexes are overlaid, it is apparent that flufenamic acid and ursodeoxycholate bind differently (Fig. 9). The A-ring of the anthranilic acid (containing the carboxylate) can tolerate bulky substitutions because the substituent projects into the larger steroid-binding pocket. Therefore, AKR1C isoform selectivity may not be achieved by only modifying the A-ring.

Comparison of the flufenamic acid and ursodeoxycholate complex structures also indicated that the B-ring of the N-phenylanthranilic acid is bound in a smaller pocket formed from amino acids 118, 120, 167, 306, and 311 on the C-loop adjacent to the larger steroid binding pocket. This smaller binding pocket may be exploited to develop AKR1C isoform selective inhibitors, given that variability exists in the size of this pocket. Structural data indicate that this additional binding pocket is larger in AKR1C3 than in AKR1C2. Replacement of amino acids Phe319, Phe118, and Leu308 in AKR1C2 for Tyr319, Ser118, and Ser308 in AKR1C3 and the positioning of the main chain at Ser308 cause an increase in the pocket size for AKR1C3.

Inspection of COX crystal structures with bound NSAIDs explains the observed selectivity of the NSAID analogs for AKR1C isoforms (Selinsky et al., 2001). In these structures, the NSAID pocket is composed of a long, narrow hydrophobic channel with a constricted binding region defined by amino acids His90, Arg120, Tyr355, and Glu524, where the A-ring of a N-phenylanthranilic acid binds. Therefore, COX cannot tolerate substitutions on the A-ring as easily as AKR1C isozymes.

**AKR1C Isozymes and Neoplastic Diseases.** NSAIDs are effective in the treatment of breast, prostate, lung, and colon cancers (Marnett, 1995; Harris et al., 2000; Wick et al., 2002; Sanchez-Alcazar et al., 2003). It is believed that these beneficial effects are mediated through inhibition of COX-2. Human AKR1C isozymes are also potently inhibited by both classic and COX-2 selective NSAIDs and may be an alternate target for their antineoplastic effects.

Desmond et al. (2003) showed that AKR1C3 prevented the differentiation of HL-60 cells by preventing the PPARγ receptor from being activated by its ligand, 15-Δ₁₂,₁₄-PGJ₂. This indicated that the AKR1C isoforms are able to regulate ligand access to orphan nuclear receptors and may be involved in the progression of neoplastic diseases. Inhibition of AKR1C3 could lead to the activation of PPARγ receptor, which induces differentiation, is antiproliferative, and results in apoptosis in many cell types and cancers (Wick et al.,...


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