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TITLE PAGE

Development of non-steroidal anti-inflammatory drug (NSAID) analogs and steroid carboxylates selective for human aldo-keto reductase isoforms: Potential antineoplastic agents that work independently of cyclooxygenase isozymes

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RUNNING TITLE PAGE

Running title:

NSAID analogs that target human AKR1Cs and not COX.

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Abbreviations: aldo-keto reductase (AKR), cyclooxygenase (COX), non-steroidal anti-inflammatory drug (NSAID), peroxisome proliferator-activated receptor-gamma (PPARγ), prostaglandin (PG), hydroxysteroid dehydrogenase (HSD), selective intracrine modulators (SIMs), androgen receptor (AR), estrogen receptor (ER), progesterone receptor (PR), structure activity relationships (SARs).

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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 (AR, ER, and PR) and the orphan receptor, PPARγ. In human myeloid leukemia (HL-60) cells ligand access to PPARγ is regulated by AKR1C3, which diverts PGD_2 metabolism away from J-series prostanoids (Desmond, 2003). Inhibition of AKR1C3 by indomethacin, an NSAID, caused PPARγ mediated terminal differentiation of the HL-60 cells. To discriminate between anti-neoplastic effects of NSAIDs that are mediated by either AKR1C or 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 while not inhibiting recombinant COX-1 or COX-2. The inhibition constants, IC_{50}, K_I 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-phenylanthranilic acid analogs and most steroid carboxylates, exhibited IC_{50} values that had greater than 500-fold selectivity for AKR1C isozymes as compared to COX-1 and COX-2. Crystallographic and molecular modeling studies showed that the carboxylic acid of the inhibitor ligand was tethered by the catalytic Tyr55-OH_2^+ and explained why A-ring substituted N-phenylanthranilates inhibited only AKR1C enzymes. These compounds can be used to dissect the role of the AKR1C isozymes in neoplastic diseases and may have cancer chemopreventive roles independent of COX inhibition.

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INTRODUCTION

The human AKR1C isozymes are hydroxysteroid dehydrogenases (HSDs) and are involved in the pre-receptor regulation of steroid hormone action (Penning, 1997; Dufort, 1999; Dufort, 2001; Rizner, 2003). AKR1C isozymes 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, 1999; Penning, 2000; Dufort, 2001). Therefore AKR1C isozymes regulate the ligand occupancy and *trans*-activation of the nuclear steroid hormone receptors, which include the androgen receptor (AR), estrogen receptor (ER), and progesterone receptor (PR). Consequently, they maybe important in regulating steroid hormone concentrations in target tissues by modulating their intracrine formation (Labrie, 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 isozymes involved in regulating the local concentration of steroid hormones include AKR1C1 (20α-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, 2000). Despite their high sequence identity they display different substrate preferences, inhibition profiles, and tissue specific expression patterns (Matsuura, 1997; Dufort, 1999; Penning, 2000; Dufort, 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

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considered a peripheral 17β-HSD that will form testosterone and 17β-estradiol from their less active precursors (Figure 1) (Dufort, 1999; Penning, 2000; Dufort 2001; Rizner, 2003). Several of these isoforms will also transform prostaglandins, for example AKR1C3 is also known as prostaglandin $F_{2\alpha}$ synthase (Matsuura, 1998). Consequently, AKR1C isozymes may be further involved in the pre-receptor regulation of a large group of nuclear receptors (e.g. steroid receptors and nuclear orphan receptors).

Recently, Desmond et al. showed that inhibition of AKR1C3 by the non-steroidal anti-inflammatory drug (NSAID) indomethacin prevented the proliferation of human myeloid leukemia cells (HL-60) (Desmond, 2003). AKR1C3 prevented the conversion of PGD_2 to 15-$\Delta^{12,14}$-PGJ_2 (a ligand for the nuclear orphan peroxisome proliferator-activated receptor gamma (PPARγ)). By converting PGD_2 to $PGF_{2\alpha}$ AKR1C3 deprived PPARγ of its ligand and prevented terminal differentiation of the myeloid leukemia cells (Figure 2). This phenotypic change suggests a role for AKR1C3 in regulating myeloid leukemia cell differentiation and indicated that NSAIDs can have anti-neoplastic 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 mefenamic acid were potent inhibitors of AKR1C9 at pharmacologically relevant concentrations (Penning and Talalay, 1983). AKR1C9 is a model for the human AKR1C isozymes as it has 69% sequence identity at the amino acid level with the human AKR1C

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isozymes. This suggests that the human enzymes may be important targets for NSAIDs and their inhibition may contribute to the anti-neoplastic effects of these drugs.

Non-selective NSAIDs do not discriminate between COX-1 and COX-2 (Laneuville, 1994). Consequently, 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, 2002; Keller and Giardiello, 2003; Sanchez-Alcazar, 2003; Leng, 2003; Reid, 2003). These include the multidrug resistance proteins (MRPs), transcription factors, MAPK kinases, cell cycle regulatory proteins and the focus of this article the human AKR1C isozymes (Penning and Talalay, 1983; Askonas, 1991; Tegeder, 2001; Wick, 2002; Desmond, 2003; Keller and Giardiello, 2003; Leng, 2003; Reid, 2003; Sanchez-Alcazar, 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.

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, which specifically inhibit AKR1C isozymes but not COX, were identified. Since AKR1C isozymes are involved in pre-receptor 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. Consequently, the AKR1C selective inhibitors described will be able to discriminate between the effects of celecoxib on COX-2 verses those effects on AKR1C isozymes.

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MATERIALS AND METHODS

Materials

2-Chloro-5-methyl-benzoic acid was purchased form Pfaltz and Bauer Chemicals (Waterbury, CT) and 2-chloro-4-nitro-benzoic acid was purchased form Acros Organics (Pittsburg, PA). Chemicals tested in this study included glycocholic acid, taurodeoxycholic acid, 4-carboxy-2',4'-dinitrodiphenylamine, 4-chloro-N-phenylanthranilic acid, 4-chloro-N-(4'-toly)-anthranilic acid, 3-amino-4-phenylamino-benzoic acid, and 4-benzoyl-benzoic acid were purchased from Sigma-Aldrich Corp (St. Louis, MO). Mefenamic acid and urseodeoxycholic acid were purchased from ICN Biomedical Inc. (Aurora, OH). 5β-Cholanic acid-3-one, 5β-cholanic acid-3α-7α-diol, and 5β-cholanic acid-3α-ol (lithocholic acid) were purchased from Steraloids (Wilton, NH). Celecoxib was purchased from ChemPacific USA (Baltimore, MD). Arachidonic acid was purchased from Cayman Chemicals (Ann Arbor, MI).

Synthesis of Non-Steroidal Inhibitors: N-phenylanthranilic Acid Derivatives

The synthesis of the N-phenylanthranilic acid derivatives utilized the Ullmann-Goldberg reaction that couples benzoic acid and aniline derivatives as previously described (Ozaki, 1985). The general method was as follows: the halogenated benzoic acid derivative (0.04 moles), aniline (0.08 moles), potassium carbonate (0.05 moles), copper powder (0.003 moles) and pyridine (15%w/w) were refluxed in amyl alcohol (25 ml) for 18 h. The amyl alcohol was removed by rotary evaporation and a mixture of potassium carbonate and water were added to the resultant slurry. The mixture was acidified with HCl (1:1) to pH (4-5). The resultant solid was filtered, washed with cold

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H_2O, extracted in $CHCl_3$ and recrystallized from EtOH. The structure was confirmed by ^{1}H-NMR (500 mHz) in $CDCl_3$ and by M.S. All chemical shifts are reported as ppm relevant to a TMS internal standard. The high-resolution M.S. data was obtained on a Micromass AutoSpec instrument (Waters/Micromass) carried out using electrospray in either the positive or negative ionization mode.

5-Methyl-N-phenylanthranilic acid ($C_{14}NO_2H_{13}$). This was synthesized using 2-chloro-5-methyl-benzoic acid and aniline with a 46% yield, purified, and recrystallized as described above. [^{1}H]-NMR (500 mHz) indicated δ ppm 2.3 (3H, -CH_3, s), δ ppm 4.3 (1H, NH, s), and δ ppm 7.5-8.2 (8H, aromatic). MS (ES^+) gave a parent ion MH^+ m/z = 286.14 (+Na, +Cl), predicted m/z = 227.26.

4-Nitro-N-phenylanthranilic acid ($C_{13}N_2O_4H_{10}$). This was synthesized using 2-chloro-4-nitro-benzoic acid and aniline with an 11% yield, purified, and recrystallized as described above. [^{1}H]-NMR (500 mHz) indicated δ ppm 4.3 (1H, NH, s), δ ppm 7.5-8.2 (8H, 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 previously described (Barton, 1989). A solution of lithocholic acid (200 mg) in 90% ethanol (15 mL) containing HCl (0.6 g) 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 [^{1}H]-NMR and M.S. [^{1}H]-NMR (500 mHz) indicated a loss of the carboxylic acid proton at δ ppm 12.0

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(1H, -COOH, s) and a gain in the ethyl group at δ ppm 4.1 and 3.6 (2H, -CH_2, q and 3H, -CH_3, t). The MS (ES^+) gave a parent ion MH^+ m/z = 427.32 (+Na), predicted m/z = 403.31.

Purification of AKR1C Isoforms and COX Isoforms

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

Steady-state kinetic assays

A continuous assay was used to monitor AKR1C activity. This assay monitored the oxidation of 1-acenaphthenol by measuring the increase in absorbance of NADH at 340 nm E = 6,270 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-acenaphthenol ranging form 5 μM to 500 μM, and 4% methanol. 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-acenaphthenol (Burczynski, 1998; Penning, 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 is used as co-reductant to reduce PGG_2 to PGH_2 starting with arachidonic acid (Benedetto, 1987). The assay monitors the formation of *N,N,N',N'*-tetramethyl-1,4-phenylenediimine (E = 12,000 M^{-1} cm^{-1}) at 610 nm. Reactions

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were performed in 1.0 mL systems containing 100 mM Tris-HCl (pH 8.0), 80 μM TMPD, 2 μM FePPIX, 60 μM arachidonic acid, and 5% DMSO. Using this assay, the specific activities were determined to be 25 and 16.8 μmol of TMPD oxidized/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 verses substrate concentration were hyperbolic and could be fit to the Michaelis-Menten equation ($v = (V_{max} * S)/(K_M + S)$) to yield values (± standard deviation) for V_{max}, k_{cat} and K_M for a given substrate.

Reversible Inhibition Studies

IC_{50} values for AKR1C enzymes were measured by varying the inhibitor concentrations while holding the 1-acenaphthenol concentration equal to the K_M for AKR1C1 and AKR1C2. Due to the solubility constraints of 1-acenaphthenol, the K_M for AKR1C3 could not be accurately determined, but estimates approach 2 mM and the resulting IC_{50} experiments were performed at a concentration of 1-acenaphthenol equal to 100 μM (AKR1C2 K_M). Inhibitors were dissolved in DMSO and the final concentration of co-solvent was 4% (2% DMSO and 2% methanol) for AKR1C isozymes. IC_{50} values were measured for COX isozymes by varying inhibitor concentrations while holding the arachidonic acid concentration equal to 60 μM (Benedetto, 1987). Inhibitors were dissolved in DMSO and the final concentration of co-solvent was 5% for the COX measurements. The presence of the DMSO in the assay had no effect on initial velocities. Inhibition data was fit using Grafit 5.0 ($y = (\text{Range})/(1 + (I/IC_{50})^S) +$

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Background) yielding the IC_{50} value. Reversible inhibition patterns for the AKR1C and COX isozymes were determined by varying the concentration of inhibitor (0.125-3 x IC_{50}) over fixed substrate concentrations (0.2-5 x K_M) and the reactions were initiated with enzyme. Initial velocity data were globally fit to the competitive, non-competitive, 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. Utilizing 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 Chang-Prusoff relationship, [$K_I = (IC_{50})/(1+S/K_M)$] 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 pre-incubated with inhibitor for 10 minutes and the reaction was initiated with substrate (Gierse, 1996). Pre-incubation of COX-1 and COX-2 with celecoxib for 10 minutes is sufficient for this slow tight-binding inhibitor to cause maximal loss of enzyme activity (Copeland, 1994; Gierse, 1996).

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RESULTS

Inhibitors that will Discriminate Between AKR1C Isozymes and COX Isozymes

There are several major chemical classes of classical 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 (Figure 3B) while retaining 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 (Figure 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, 1997; Matsuura, 1998). This selectivity is impressive considering 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

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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 as K_I values were obtainable for most of the compounds tested (Figure 4). However, when comparing inhibition potency between the AKR1C isozymes and COX isozymes, IC_{50} values were used since K_I values were unobtainable for most of the compounds tested for the COX isozymes.

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 mefenamic 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, Figure 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 relationship, K_I values were calculated for the remainder of the compounds in each class as described in the Materials and Methods see Table 1 and Table 2.

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

The parent compound mefenamic acid (**1**) inhibited both AKR1C and COX isozymes, Figure 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 (IC_{50} = 0.39 μM), AKR1C2 (IC_{50}

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= 0.96 μM), AKR1C1 (IC_{50} = 4.0 μM), COX-1 (IC_{50} = 33.3 μM), and finally COX-2 (IC_{50} = 225 μM). Representative inhibition patterns with the NSAID analogs showed that the pattern of inhibition was competitive. Thus the IC_{50} values obtained with each of the AKR1C isozymes were directly comparable. In addition because of the relationship that exists between IC_{50} values and K_M under competitive inhibition conditions, K_I values could be computed for each of the remaining inhibitors. By contrast IC_{50} values for COX-1 and COX-2 inhibition were obtained at 60 μM arachidonic acid as previously described (Gierse, 1996). Our COX data are consistent with previous findings which showed that the N-phenylanthranilic acids are more potent against COX-1 (Gierse, 1996). Although the IC_{50} values for the COX isozymes were not determined at K_M, the N-phenylanthranilic acid analogs can be directly compared to the values obtained for mefenamic acid, the parent compound (**1**).

N-Phenylanthranilic acid derivates were examined for their selectivity to inhibit AKR1C isozymes verses 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 **4** which remained a potent inhibitor for the AKR1C isozymes, however, **4** did not inhibit the COX isozymes, Figure 7. Direct comparison of **1** and **4** indicated that halogenation of the A-ring led to a decrease in inhibition potency by more than 30-fold for COX-1 and five-fold for COX-2. The rank order for enzyme inhibition with **4** was AKR1C2; IC_{50} = 1.15 μM > AKR1C3; IC_{50} = 3.01 μM > AKR1C1; IC_{50} = 8.2 μM > COX-1 and COX-2; IC_{50} > 1,000 μM. The

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selectivity for the N-phenylanthranilic acid analogs ranged from 30-fold, (AKR1C1 IC_{50} = 8.3 μM and COX-1 IC_{50} = 240 μM), for compound **7** which contained substitution on both rings to greater than 1,200-fold (AKR1C3 IC_{50} = 0.40 μM and COX-1 IC_{50} >> 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 IC_{50} >> 1,000 μM and COX-2 IC_{50} >> 1,000 μM) when compared to the parent compound (**1**), which inhibited both COX isozymes, but remained a potent inhibitor for the AKR1C isozymes (AKR1C3 IC_{50} = 2.04 μM, AKR1C1 IC_{50} = 33.2 μM, and AKR1C2 IC_{50} = 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 IC_{50} values of > 1,000 μM for COX-1 and COX-2 and remained a potent inhibitor for the AKR1C isozymes (AKR1C3 IC_{50} = 10.7 μM, AKR1C2 IC_{50} = 26.2 μM, and AKR1C1 IC_{50} = 49.4 μM), while compound **1** gave IC_{50} values of 33.3 μM and 225 μM for COX-1 and COX-2, respectively. However, substituents 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 four-fold by **7** (IC_{50} = 240 μM) in comparison to **4** (IC_{50} > 1,000 μM). By contrast, substitution on the A-ring also substantially reduced potency against the COX isozymes while

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retaining AKR1C inhibition. For example, **2** was 13-fold and five-fold less potent against COX-1 and COX-2, respectively as compared to **1**.

Steroid Carboxylates - Isoform Selectivity, Potency, and Trends

C24-cholanic acids are known inhibitors of AKR1C2 but their inhibition potencies towards 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 while the COX isozymes were not inhibited. For example, 5β-cholanic acid-3-one (**9**) potently inhibited the AKR1C isozymes while the COX isozymes were unaffected at all concentrations tested, Figure 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 potently inhibited by the bile acids in comparison to the other AKR1C isozymes tested, while the COX isozymes were not inhibited. The selectivity for the AKR1C isozymes verses COX isozymes ranged from five-fold for **13** to greater than 30,000 for **12**.

Comparison of Inhibitors for AKR1C Isoform Selectivity

N-Phenylanthranilic Acid – Isoform Selectivity, Potency, and Trends

The N-phenylanthranilic acid analogs were analyzed for AKR1C isozyme selectivity. In general, the selectivity among the individual AKR1C isozymes was not as dramatic as the selectivity between AKR1C and COX isozymes. However, compounds **4**,

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7, and **8** were more than six-fold more selective for different AKR1C isozymes. Further analysis indicated potential trends that could be exploited to increase isozyme selectivity. Movement of the carboxylic acid from the *ortho* to the *para*-position on the A-ring increased the selectivity for AKR1C3 in comparison to AKR1C1 or AKR1C2. For example, **3** and **8** were more selective for AKR1C3 (K_I = 0.38 μM and K_I = 1.9 μM) than AKR1C1 (K_I = 2.66 μM and K_I = 12.5 μM) or AKR1C2 (K_I = 1.32 μM and K_I = 13.3 μM), respectively. Interestingly, substitution on the A-ring with the carboxylic acid at the *para* position increased the potency for AKR1C3 more than five-fold, compare **6** and **8**. Replacement of the heteroatom appears to favor selectivity for AKR1C3 over AKR1C1 and AKR1C2, compare **8** and **6**. This increase in selectivity for AKR1C3 might be due to 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 appear to favor AKR1C isozyme potency as **1** and **3** are very potent inhibitors for all AKR1C isozymes in comparison to 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 seven-fold and four-fold more potent for AKR1C1 than compounds **6** and **8**. Compound **3** is seven-fold and nine-fold more potent for AKR1C2 than compounds **6** and **8**, and it is more than 26-fold and five-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 as **4** and **7** are more than seven-fold more selective for AKR1C2 (K_I = 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).

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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 AKR1C3, 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-phenylanthranilic acids can introduce selectivity for the different AKR1C isozymes.

When comparing the inhibition of the N-phenylanthranilic acid analogs the electronegativity of the carboxylic acid is determined by the different substituents on the A-ring thus altering the p*Ka* 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 two-fold for all the AKR1C isozymes, when comparing **1** and **4** (AKR1C1 K_I = 0.81 μM for **1** compared to K_I = 3.1 μM for **4**, AKR1C2 K_I = 0.22 μM for **1** compared to K_I = 0.41 μM for **4**, and AKR1C3 K_I = 0.3 μM for **1** compared to 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 maybe 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 verses AKR1C1 and AKR1C3 ranges from greater than 48-fold for **11** to greater than 570-fold for **10** when K_I values are compared.

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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 seven-fold more potent than **13** and two-fold more potent than **14**), for the inhibition of AKR1C2 (**10** is 41-fold more potent than **13** and 11-fold more potent than **14**), and for the inhibition of AKR1C3 (**10** is 20-fold more potent than **13** and nine-fold more potent than **14**). The SAR with steroid carboxylates indicates that replacement of the carboxylic acid (**13**) with a more electronegative acid, like sulfonic acid (**14**), increases the potency by more than two-fold for all the AKR1C isozymes (AKR1C1 K_I = 23 μM for **14** compared to K_I = 66 μM for **13**, AKR1C2 K_I = 0.2 μM for **14** compared to K_I = 0.72 μM for **13**, and AKR1C3 K_I = 99 μM for **14** compared to K_I = 206 μM for **13**). This finding was further 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 nine-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 suggests that the COX-2 selective inhibitor celecoxib may have other targets at physiological concentrations. Consequently, celecoxib (**16**) was tested against

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the AKR1C isozymes and was found to be a potent inhibitor for all the AKR1C isozymes tested (Table 1) yielding the following rank order AKR1C3 (K_I = 5.2 μM) > AKR1C2 (K_I = 8.6 μM) > AKR1C1 (K_I = 14.9 μM). Interestingly when celecoxib was tested as a reversible inhibitor there was no difference in the inhibition potency between COX-1 (IC_{50} = 23.5 μM) and COX-2 (IC_{50} = 25.0 μM) (data not shown). However, when celecoxib was tested as a time-dependent inhibitor, inhibition on COX-2 was more than 600-fold more selective (COX-2 IC_{50} = 0.04 μM verses COX-1 IC_{50} = 23.7 μM), which has been previously reported (Gierse, 1996), Table 1. No time-dependence was noticed for the inhibition of the AKR1C isozymes (data not shown).

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DISCUSSION

Classical 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, 2004). To discern the cancer chemopreventive properties of these drugs we report NSAID analogs and steroid carboxylates that inhibit the AKR1C enzymes while not inhibiting COX-isozymes. Small changes in the structures of N-phenylanthranilic acid derivatives resulted in a loss of COX-inhibition while 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 while 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 $IC_{50} \geq 1{,}000$ 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 isoform selectivity.

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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, 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, PDB entry 1IHI) and NSAIDs (AKR1C3•$NADP^+$•Flufenamic acid, PDB entry 1S2C) (Jin, 2001; Lovering, 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 (Figure 9). The A-ring of the anthranilic acid (containing the carboxylate) can tolerate bulky substitutions since the substituent projects into the larger steroid-binding pocket. Consequently, AKR1C isoform selectivity may not be achieved by only modifying the A-ring.

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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, as variability exists in the size of this pocket. Structural data indicates 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 isozymes (Selinsky, 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, 2000; Wick, 2002; Sanchez-Alcazar, 2003). It is believed that these beneficial effects are mediated through inhibition of COX-2. Human AKR1C isozymes are also potently inhibited by both classical and COX-2 selective NSAIDs and may be an alternate target for their anti-neoplastic effects.

Desmond et al showed that AKR1C3 prevented the differentiation of HL-60 cells by preventing the PPARγ receptor from being activated by its ligand, 15-$\Delta^{12,14}$-PGJ_2.

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This indicated that the AKR1C isozymes 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 anti-proliferative, and results in apoptosis in many cell types and cancers (Wick, 2002; Desmond, 2003). Consequently, the beneficial effects of NSAIDs as cancer chemopreventives could be through the inhibition of the AKR1C isozymes rather than by COX.

COX-2 selective inhibitors, like celecoxib, are also effective in decreasing the proliferation of breast cancer (Marnett, 1995; Harris, 2000; Wick, 2002; Davies, 2003; Sanchez-Alcazar, 2003; DuBois, 2004). Non-selective and selective NSAIDs are believed to act beneficially in the breast by inhibiting COX-2 and decreasing PGE_2 levels (Davies, 2003; DuBois, 2004). PGE_2 induces the transcription of cyp19-aromatase, which converts testosterone to the potent estrogen 17β-estradiol, thereby increasing active estrogens in the breast (Zhao, 1996; Davies, 2003; DuBois, 2004). Consequently inhibition of COX-2 leads to a decrease in the production of 17β-estradiol. An alternate mechanism may involve the inhibition of AKR1C3, which is localized in the secretory epithelial cells in both the acini and terminal ducts of the mammary gland (Pelletier, 2001). AKR1C3 not only reduces estrone (a weak estrogen) to 17β-estradiol (a potent estrogen), but also reduces androstenedione to testosterone which can be aromatized to 17β-estradiol. AKR1C3 inhibition by NSAIDs would deprive the ER of its ligand and provide a mechanism for their antineoplastic effects on hormone dependent breast cancer.

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FOOTNOTES

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LEGENDS FOR FIGURES

FIG 1: **Regulation of ligand concentrations for nuclear receptors by AKR1C isozymes.** All AKR isozymes are shown working in the reduction direction only.

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.** PGG_2 – prostaglandin G_2, PGD_2 – prostaglandin D_2, $PGF_{2\alpha}$ - 9α, 11β-Prostaglandin $F_{2\alpha}$, 15Δ-PGJ_2 – 15-$\Delta^{12,14}$- prostaglandin J_2, RXR - retinoic acid receptor, PPARγ - peroxisome proliferator-activated receptor gamma, COX-1 – cyclooxygenase-1.

FIG 3: **The Ullman-Goldberg coupling reaction (A); and SAR trends in N-phenylanthranilic acids that eliminates COX-1 inhibition (B).**

FIG 4: **Inhibitors screened.**

FIG 5: **Representative inhibition patterns for AKR1C2 using 1-acenaphthenol as substrate: A. Competitive inhibition pattern for AKR1C2 by mefenamic acid. B. Competitive inhibition pattern for AKR1C2 by 5-methyl-N-phenylanthranilic acid. C. Competitive inhibition pattern for AKR1C2 by 5β-cholanic acid-3-one.** V – initial velocity, S – substrate (1-acenaphthenol). K_I values were determined as described under "Materials and Methods" and each point was performed in triplicate.

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FIG 6: **Inhibition of AKR1C isozymes and COX isozymes by mefenamic acid.** *IC_{50}* values were determined as described under "Materials and Methods" and performed in triplicate.

FIG 7: **Inhibition of AKR1C isozymes and COX isozymes by 4-chloro-N-phenylanthranilic acid.** *IC_{50}* values were determined as described under "Materials and Methods" and performed in triplicate.

FIG 8: **Inhibition of AKR1C isozymes and COX isozymes by 5β-cholanic acid-3-one.** *IC_{50}* values were determined as described under "Materials and Methods" and performed in triplicate.

FIG 9: **Positions of flufenamic acid (FLU) and ursodeoxycholate (URS) in AKR1C2 (blue) and AKR1C3 (gold).** Ursodeoxycholate and flufenamic acid are in ball and stick representation with the carboxylic acid highlighted as red and positioned near tyrosine 55. These two structures were superimposed using the Cα chain as a reference with an rmsd of 1.0 Å.

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Table 1[a]: Inhibition of AKR1C and COX Isozymes by N-phenylanthranilic Acid Analogs and Celecoxib

Compound	AKR1C1	AKR1C2	AKR1C3	COX-1	COX-2	SR[b]	SR[c]	SR[d]
1	4.00±0.5	0.96±0.11	0.39±0.09	33.3	225	3.7	2.7	1.4
	K_I = 0.81	K_I = 0.22	K_I = 0.30[e]	K_I = 3.90				
2	3.2±0.13	1.23±0.06	10.8±0.43	434	>> 1,000	1.7	8.3	14
	K_I = 0.88	K_I = 0.53	K_I = 7.27					
3	7.1±0.80	3.69±0.20	0.40±0.06	>> 500	ND[f]	2.0	6.9	3.5
	K_I = 2.66	K_I = 1.32	K_I = 0.38					
4	8.2±0.90	1.15±0.06	3.01±0.22	> 1,000	>> 1,000	7.5	1.1	7.0
	K_I = 3.10	K_I = 0.41	K_I = 2.90					
5	9.2±0.50	2.09±0.40	1.98±0.28	>> 250	ND	4.6	1.8	2.5
	K_I = 3.50	K_I = 0.75	K_I = 1.90					
6	49.4±8.90	26.2±3.24	10.7±1.50	~ 1,000	>> 1,000	2.0	1.8	1.1
	K_I = 18.5	K_I = 9.40	K_I = 10.2					
7	8.30±0.50	0.56±0.17	1.47±0.37	240	>> 1,000	15	2.2	7.0
	K_I = 3.10	K_I = 0.20	K_I = 1.40					
8	33.2±4.40	36.9±2.45	2.04±0.19	>> 1,000	>> 1,000	1.1	6.4	6.8
	K_I = 12.5	K_I = 13.3	K_I = 1.9					
16	39.6±3.0	23.9±1.6	5.45±0.4	23.7±2.5	0.04±0.00	1.7	2.9	1.7
	K_I = 14.9	K_I = 8.6	K_I = 5.2					

[a] – Values are in μM
[b] – Selectivity ratio between AKR1C1 and AKR1C2 comparing K_I values
[c] – Selectivity ratio between AKR1C1 and AKR1C3 comparing K_I values
[d] – Selectivity ratio between AKR1C2 and AKR1C3 comparing K_I values
[e] – K_I values for AKR1C3 were computed assuming a K_M of 2 mM for 1-acenapthenol
[f] – ND – Not Determined

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Table 2[a]: Inhibition of AKR1C and COX Isozymes by Steroid Carboxylates

Compound	AKR1C1	AKR1C2	AKR1C3	COX-1	COX-2	SR[b]	SR[c]	SR[d]
9	14.0±2.80	0.04±0.00	1.02±0.10	>> 250	>> 250	63	3.5	18
	K_I = 1.30	K_I = 0.021	K_I = 0.37[e]					
10	22.2±2.50	0.05±0.00	10.5±2.70	>> 1,000	>> 1,000	483	1.2	578
	K_I = 8.30	K_I = 0.017	K_I = 10.0					
11	9.20±0.60	0.09±0.00	1.57±0.18	>> 500	>> 500	110	2.3	48
	K_I = 3.45	K_I = 0.032	K_I = 1.50					
12	13.1±1.10	0.03±0.00	1.38±0.13	>> 1,000	ND[f]	450	3.7	120
	K_I = 4.90	K_I = 0.011	K_I = 1.30					
13	177±27.0	2.00±0.30	216±40.0	>> 1,000	>> 1,000	92	3.1	287
	K_I = 66.0	K_I = 0.72	K_I = 206					
14	61.2±5.90	0.56±0.06	104±19.0	>> 1,000	>> 1,000	114	4.3	493
	K_I = 23.0	K_I = 0.20	K_I = 99.0					
15	>> 100	2.50±0.25	> 100	>> 250	ND	42	2.5	106
		K_I = 0.90						

[a] – Values are in μM
[b] – Selectivity ratio between AKR1C1 and AKR1C2 comparing K_I values
[c] – Selectivity ratio between AKR1C1 and AKR1C3 comparing K_I values
[d] – Selectivity ratio between AKR1C2 and AKR1C3 comparing K_I values
[e] – K_I values for AKR1C3 were computed assuming a K_M of 2 mM for 1-acenapthenol
[f] – ND – Not Determined

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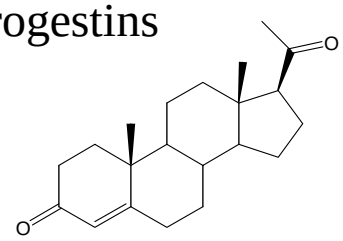
Figure 1

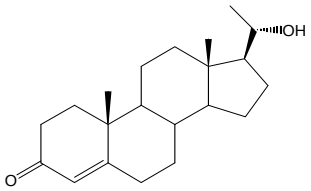
Progestins



Progesterone

Potent Progestin

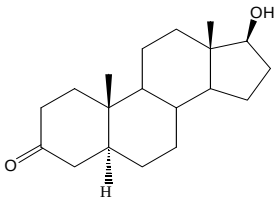
AKR1C1



20α-Hydroxyprogesterone

Weak Progestin

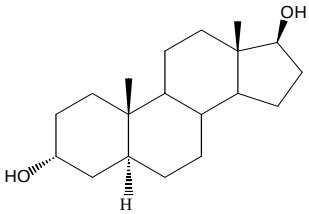
Androgens



5α-Dihydrotestosterone

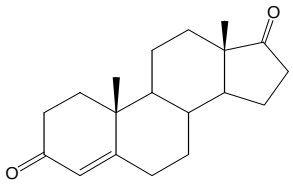
Potent Androgen

AKR1C2



3α-Androstanediol

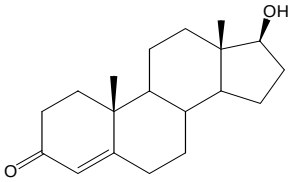
Weak Androgen



Androstenedione

Weak Androgen

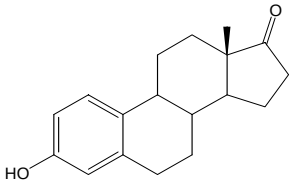
AKR1C3



Testosterone

Potent Androgen

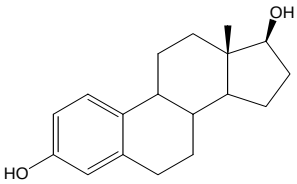
Estrogens



Estrone

Weak Estrogen

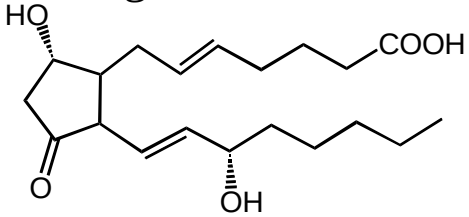
AKR1C3



17β-Estradiol

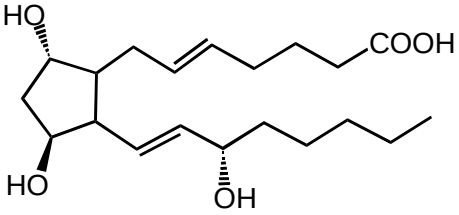
Potent Estrogen

Prostaglandins



Prostaglandin D2

AKR1C3



9α, 11β-Prostaglandin $F_{2\alpha}$

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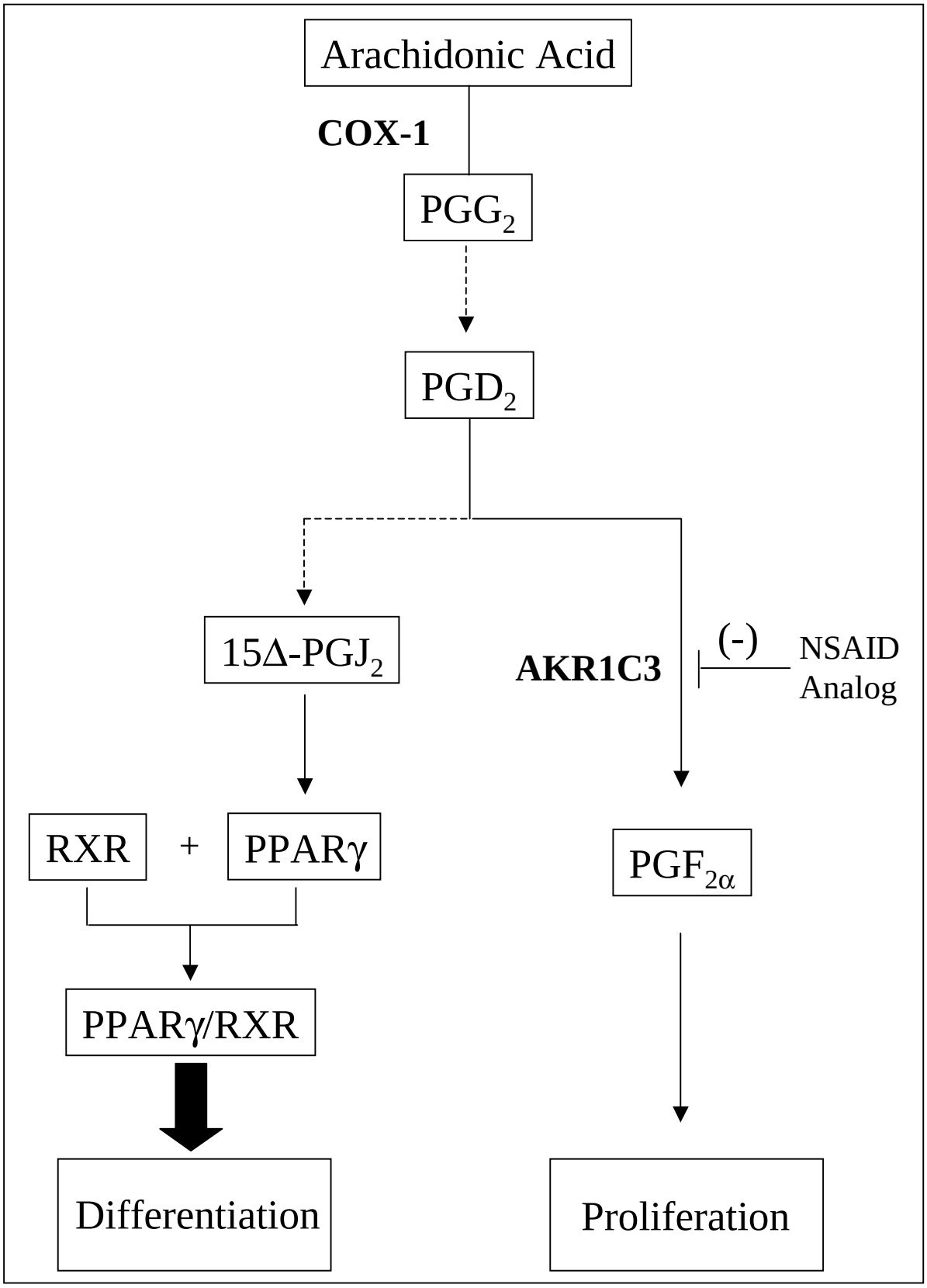
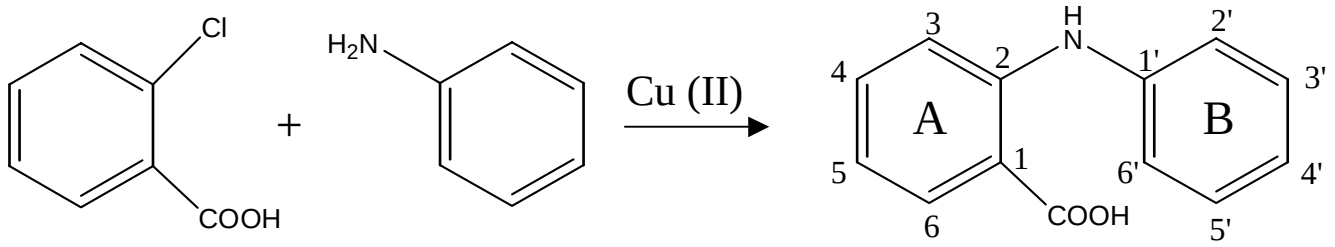
Figure 2



Adapted from Desmond et al, Cancer Res. 63: 505, 2003

Figure 3

A



B Substitution	Compound	Activity
2',3'-Dimethyl	Mefenamic Acid	COX (PGH_2) Inhibitor
3'-CF_3	Flufenamic Acid	COX (PGH_2) Inhibitor
A-Ring	---	No COX Inhibition Predicted
Movement of COOH	---	No COX Inhibition Predicted
Substitution on Amine	---	No COX Inhibition Predicted
Replacement of Heteroatom	---	No COX Inhibition Predicted

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Figure 4

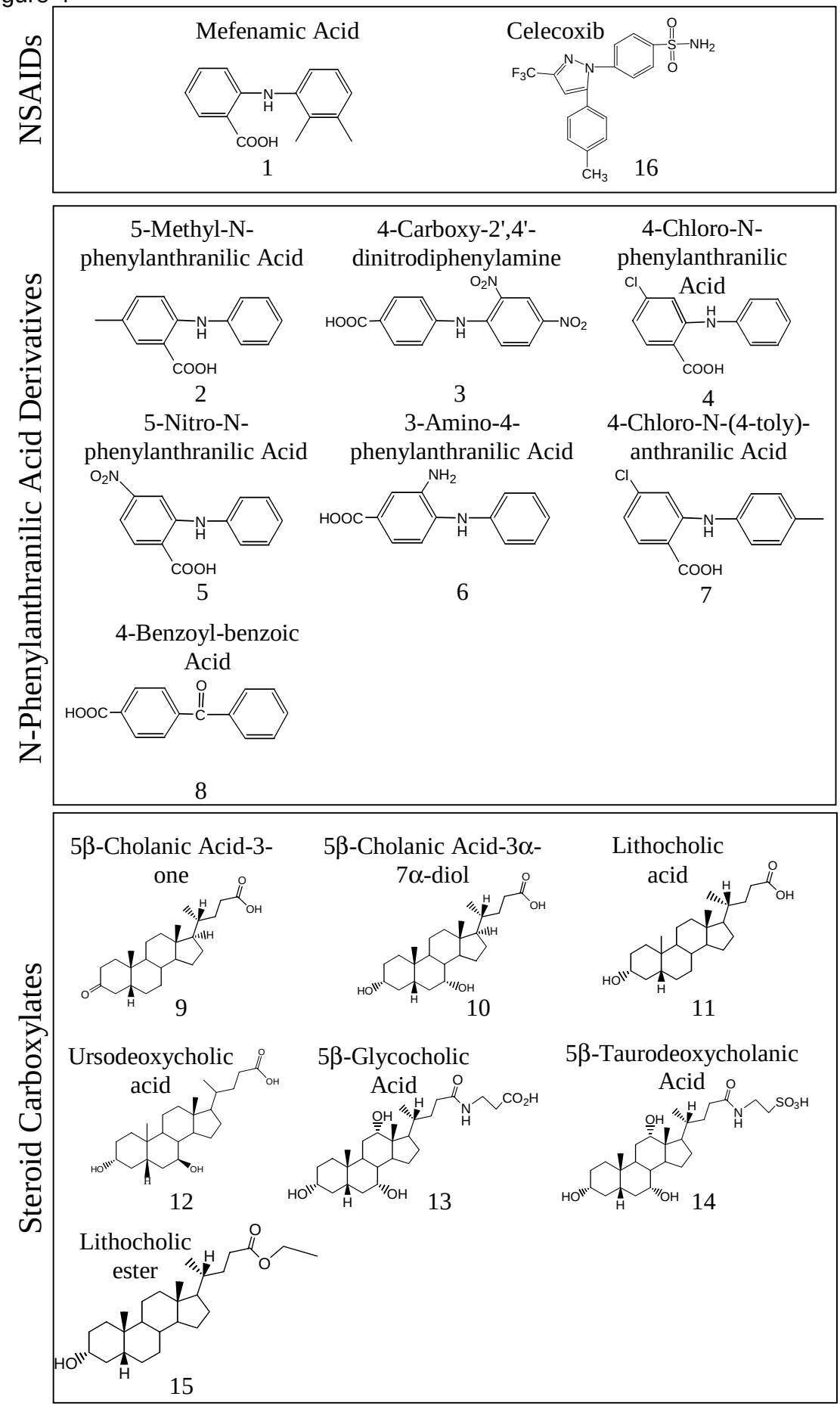

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Figure 5

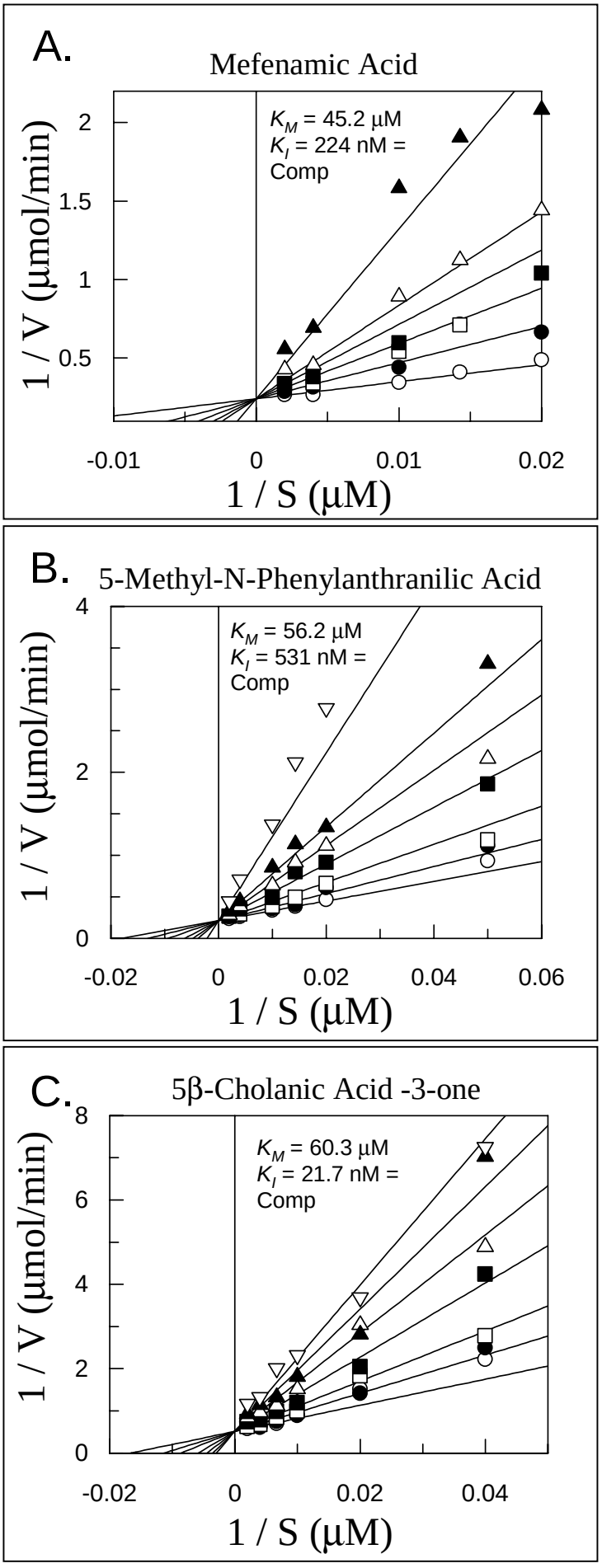

Figure 6

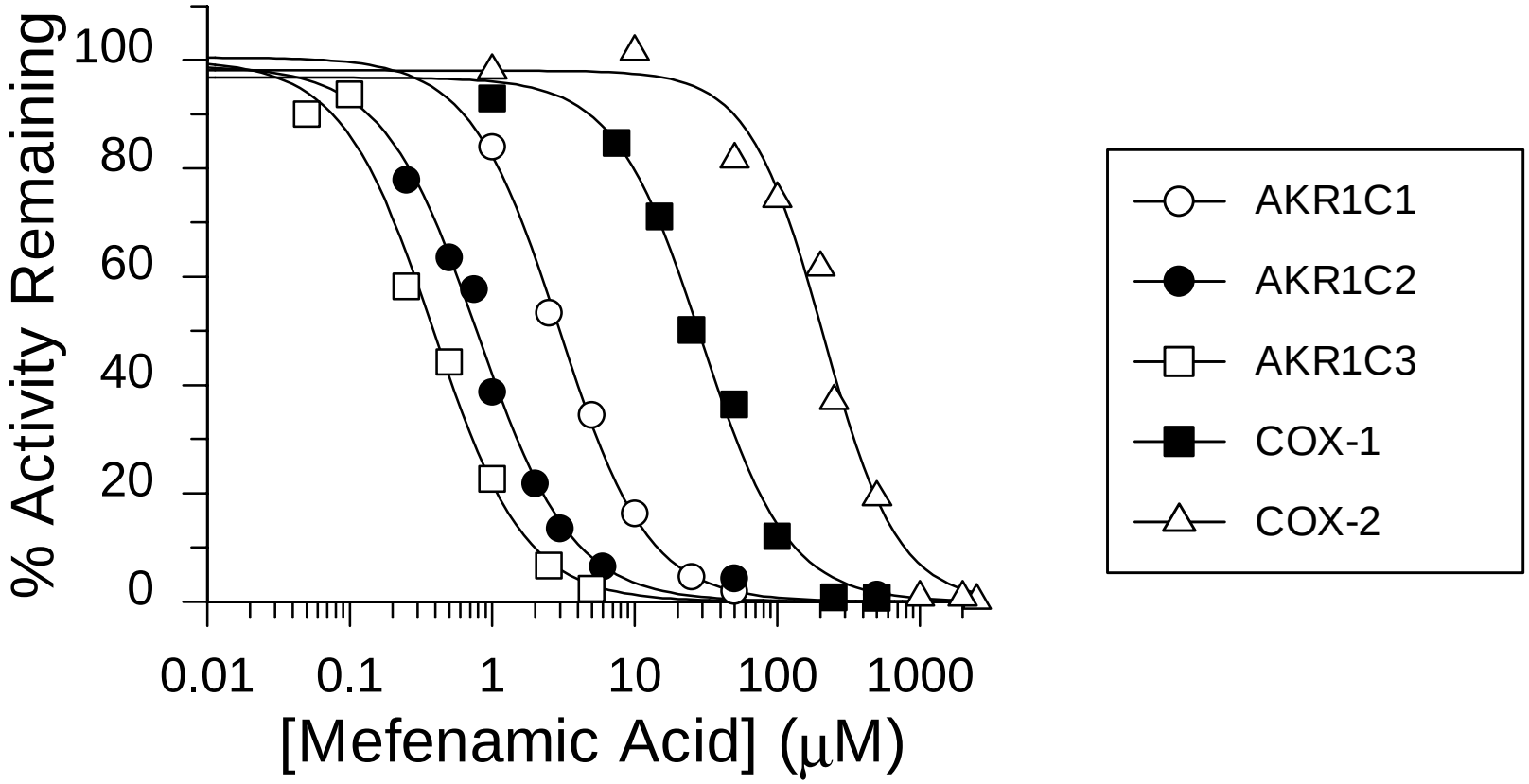

Figure 7

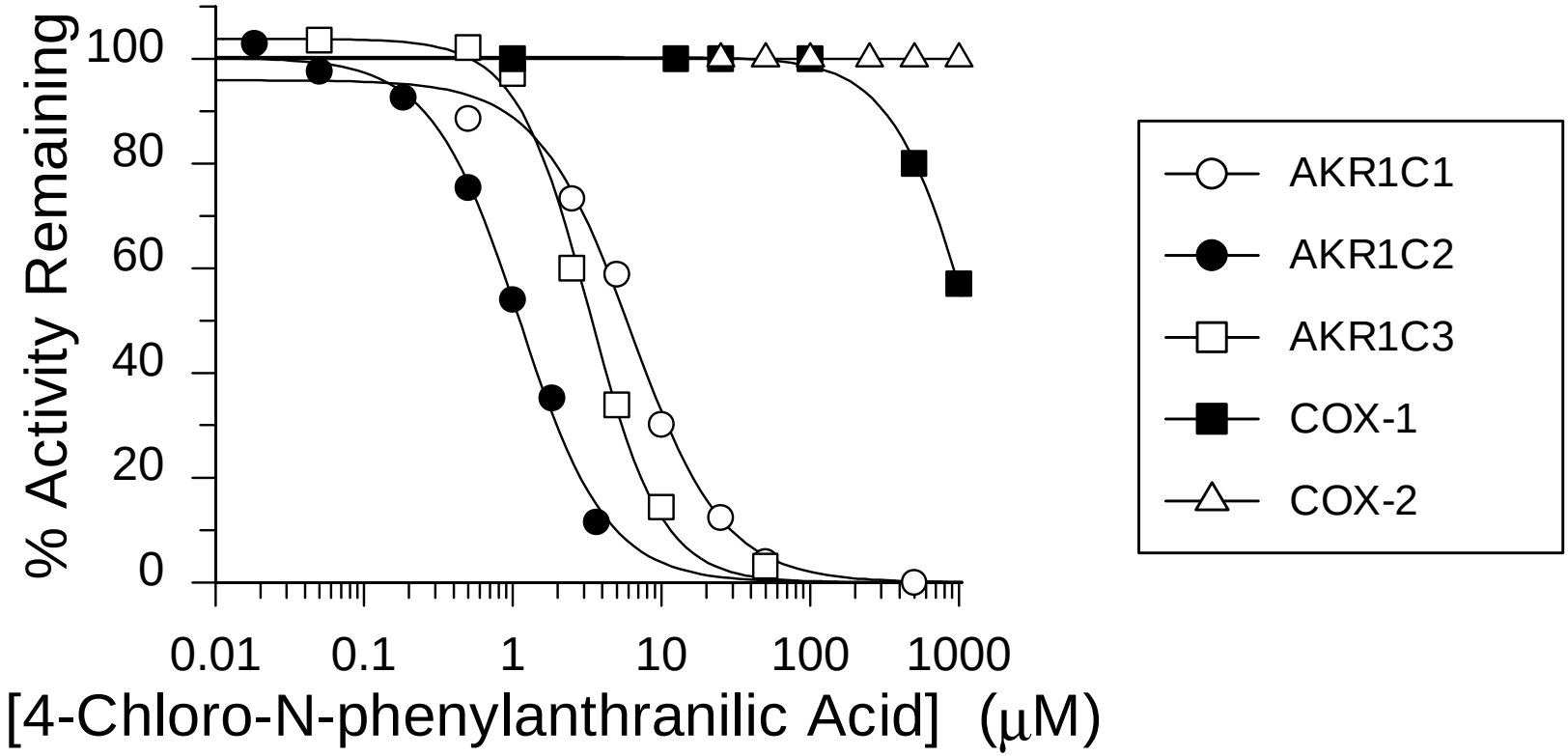

Figure 8

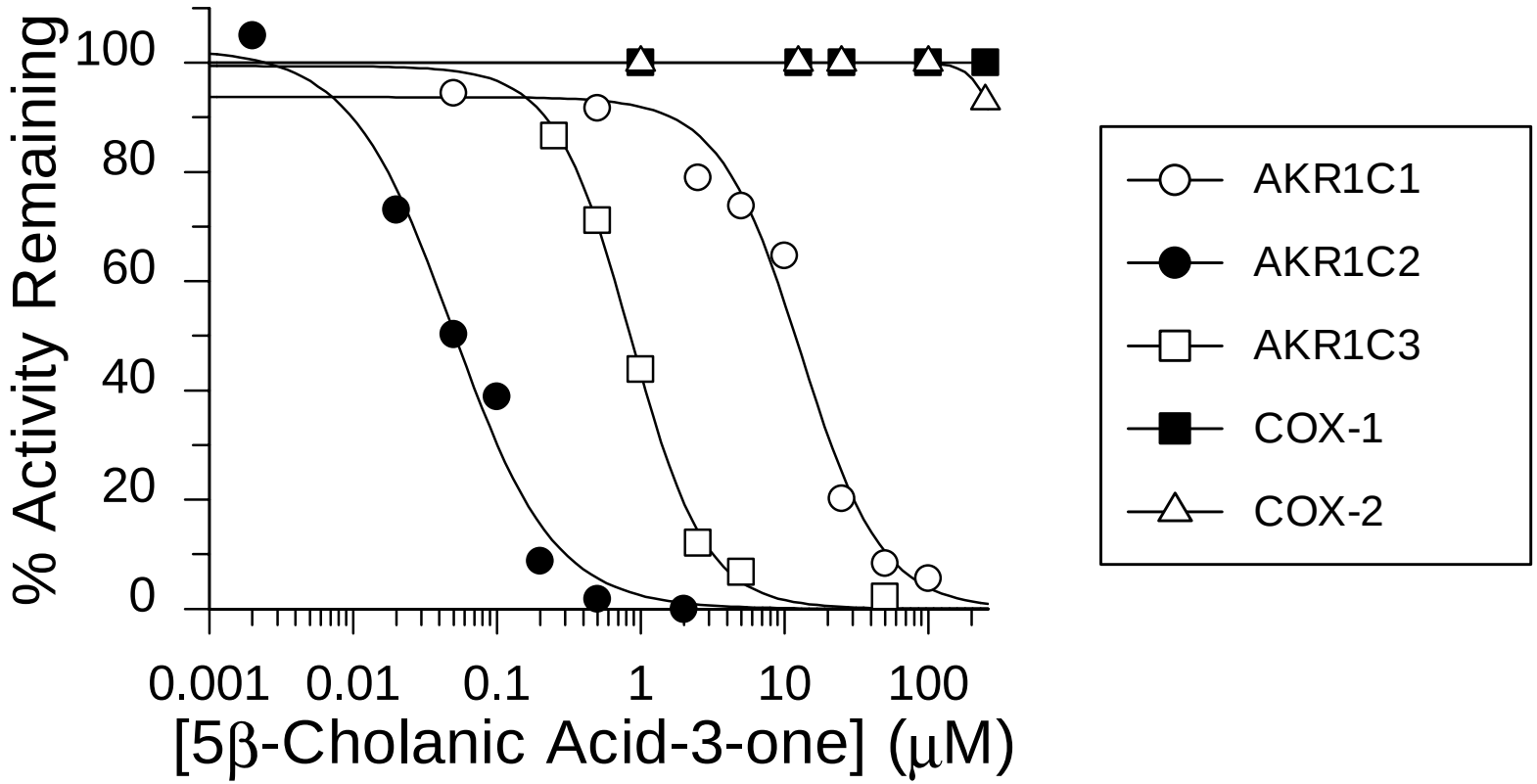

Figure 9