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An indomethacin analogue, N-(4-chlorobenzoyl)-melatonin, is a selective inhibitor of aldo-keto reductase 1C3 (type 2 3α-HSD, type 5 17β-HSD, and prostaglandin F synthase), a potential target for the treatment of hormone dependent and hormone independent malignancies

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ABSTRACT

Aldo-keto reductase (AKR) 1C3 (type 2 3α-HSD, type 5 17β-HSD, and prostaglandin F synthase) regulates ligand access to steroid hormone and prostaglandin receptors and may stimulate proliferation of prostate and breast cancer cells. NSAIDs are known inhibitors of AKR1C enzymes. An NSAID analogue that inhibits AKR1C3 but is inactive against the cyclooxygenases and the other AKR1C family members would provide an important tool to examine the role of AKR1C3 in proliferative signaling. We tested NSAIDs and NSAID analogues for inhibition of the reduction of 9,10-phenanthrenequinone (PQ) catalyzed by AKR1C3 and the closely related isoforms AKR1C1 and AKR1C2. Two of the compounds initially screened, indomethacin and its methyl ester, were specific for AKR1C3 versus the other AKR1C isoforms. Based on these results and the crystal structure of AKR1C3, we predicted that N-(4-chlorobenzoyl)-melatonin (CBM), an indomethacin analogue that does not inhibit the cyclooxygenases, would selectively inhibit AKR1C3. CBM inhibited the reduction of PQ by AKR1C3, but did not significantly inhibit AKR1C1 or AKR1C2. Indomethacin and CBM also inhibited the AKR1C3-catalyzed reduction of Δ^4-androstene-3,17-dione but did not significantly inhibit the reduction of steroid hormones catalyzed by AKR1C1 or AKR1C2. The pattern of inhibition of AKR1C3 by indomethacin and CBM was uncompetitive versus PQ, but competitive versus Δ^4-androstene-3,17-dione, indicating that two different inhibitory complexes form during the ordered bi bi reactions. The identification of CBM as a specific inhibitor of AKR1C3 will aid the investigation of its roles in steroid hormone and prostaglandin signaling and the resultant effects on cancer development.

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Abbreviations: AKR, aldo-keto reductase; HSD, hydroxysteroid dehydrogenase; CBM, N-(4-chlorobenzoyl)-melatonin; DHT, 5α-dihydrotestosterone; 3α-androstanediol, 5α-androstane-3α,17β-diol; PG, prostaglandin; PPARγ, peroxisome proliferator-activated receptor γ; COX, cyclooxygenase; PQ, 9,10-phenanthrenequinone; ABMI, N-(4-aminobenzyl)-6-methoxy-3-indole acetic acid.

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1. Introduction

Prostate and breast cancer are the second leading causes of cancer mortality in men and women, respectively [1]. While anti-hormonal therapies are often effective in the treatment of these diseases, they can also lead to the development of hormone-refractory cancer [2,3]. One approach that might prevent this outcome is to simultaneously inhibit hormone-dependent and hormone-independent proliferative pathways. Aldo-keto reductase (AKR) 1C3 (type 2 3α-HSD, type 5 17β-HSD, and prostaglandin F synthase) is one target that potentially stimulates proliferation of both hormone-dependent and hormone-refractory malignancies through independent pathways. AKR1C3 produces proliferative steroid hormones and prostaglandins and prevents formation of anti-proliferative prostaglandins, effects that likely contribute to the growth of prostate and breast cancers [4,5].

Despite its >86% sequence identity with the other human members of the AKR1C subfamily, AKR1C3 has unique catalytic activities that set it apart as a potential therapeutic target. Each of the AKR1C isoforms selectively reduces ketosteroids and contributes to the pre-receptor regulation of steroid hormone receptors [4–7]. AKR1C1 (20α-HSD) reduces progesterone to its less active metabolite 20α-hydroxyprogesterone, thereby limiting progestin binding to the progesterone receptor. AKR1C2 (type 3 3α-HSD) reduces 5α-dihydrotestosterone (DHT) to its less active metabolite 3α-androstanediol, preventing binding of androgen to the androgen receptor. AKR1C4 (type 1 3α-HSD) is a liver-specific 3-ketosteroid reductase involved in the clearance of steroid hormones from the circulation and the biosynthesis of bile acids. AKR1C3, which is expressed at high levels in the prostate and breast, catalyzes the activation of Δ^4-androstene-3,17-dione to testosterone and estrone to 17β-estradiol, as well as the inactivation of progesterone to 20α-hydroxyprogesterone [7,8]. In the prostate, AKR1C3-mediated testosterone production will enhance DHT formation and increase androgen receptor activity. In the breast, testosterone will subsequently be converted to 17β-estradiol by aromatase activity and the combined effect of the AKR1C3 activities will be to increase estrogen receptor *trans*-activation and decrease progesterone receptor *trans*-activation, resulting in a pro-estrogenic state in this tissue.

AKR1C3 also plays an important role in the biosynthesis of prostaglandins, catalyzing the formation of prostaglandin (PG) $F_{2\alpha}$ and 9α,11β-PGF_2 from PGH_2 and PGD_2, respectively [9,10]. Of its endogenously relevant substrates, AKR1C3 has the highest catalytic activity for the reduction of PGD_2 [9,10]. The PGF_2 isomers will activate the Gq-coupled F-prostanoid receptor and initiate protein kinase C and MAPK signaling cascades that stimulate proliferation through mechanisms that include inhibition of the peroxisome proliferator-activated receptor γ (PPARγ) and activation of NF-κB [11–14]. The AKR1C3-mediated depletion of PGD_2 will also prevent the formation of anti-proliferative PGJ_2 isomers, including 15-deoxy-$\Delta^{12,14}$-PGJ_2, which are natural PPARγ ligands and inhibitors of NF-κB signaling [15–18].

AKR1C enzymes are potently inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) [10,19]. Inhibition of AKR1C3 provides a potential mechanism for the COX-independent anticancer effects of NSAIDs [15,19]. NSAID analogues that inhibit AKR1C3 but not the COX enzymes would be predicted to be devoid of the gastrointestinal toxicity that results from inhibition of COX-1. A selective inhibitor of AKR1C3 would be an important tool for understanding its role in cancer development and other disorders. We have described analogues of the *N*-phenylanthranilic acid family of NSAIDs that inhibit the AKR1C enzymes but do not affect the cyclooxygenase (COX) isozymes [19]. However, none of these compounds were selective for AKR1C3 over the other AKR1C isoforms. Inhibitors based on the structure of indomethacin represent an alternative approach to a specific AKR1C3 inhibitor. The available crystal structure of the AKR1C3·$NADP^+$·indomethacin complex identifies structural determinants that could be exploited to develop an AKR1C3-selective inhibitor based on this drug [20].

In the present study, we examined the inhibition of the AKR1C enzymes by a series of NSAIDs and indomethacin analogues. Using the structure–activity relationships derived from these results and crystal structure data from the literature, we identified modifications to the structure of indomethacin that would prevent inhibition of COX-1, COX-2, AKR1C1, and AKR1C2, but retain the inhibition of AKR1C3. Based on these predictions, we synthesized a compound, *N*-(4-chlorobenzoyl)-melatonin (CBM), which had been previously shown not to inhibit COX-1 or COX-2 [21]. CBM inhibited AKR1C3 with similar affinity to that seen with indomethacin, but did not inhibit AKR1C1 or AKR1C2.

2. Materials and methods

2.1. Materials

Δ^4-Androstene-3,17-dione, testosterone, and progesterone were purchased from Steraloids (Newport, RI). [4-^{14}C] Δ^4-Androstene-3,17-dione (53.6 mCi/mmol), [4-^{14}C] progesterone (50.8 mCi/mmol), [4-^{14}C] DHT (53.5 mCi/mmol), and [4-^{14}C] testosterone (50.0 mCi/mmol) were purchased from Perkin-Elmer (Boston, MA). 9,10-Phenanthrenequinone (PQ), DHT, NSAIDs, and other reagents used were purchased from Sigma–Aldrich (St. Louis, MO). 1-Acenaphthenol was purchased from Acros (Morris Plains, NJ). Ethyl acetate, ether, and methylene chloride were of at least ACS grade and were purchased from Fisher Scientific (Fair Lawn, NJ). Indomethacin methyl ester, *N*-(4-aminobenzyl)-6-methoxy-3-indole acetic acid (ABMI), and CBM were synthesized as described previously and structures validated by [^{1}H] NMR [21,22].

Homogenous recombinant AKR1C1, AKR1C2, and AKR1C3 were purified according to published methods [5,23] and stored at −80 °C in 20 mM potassium phosphate buffer, pH 7.0, containing 30% glycerol, 1 mM EDTA, and 1 mM 2-mercaptoethanol. Enzyme activity was standardized using 1-acenaphthenol as substrate as described previously [19], specific activities for the enzymes were 1.8, 2.4, and 1.4 μmol of 1-acenaphthenol (1 mM) oxidized/min/mg for AKR1C1, AKR1C2, and AKR1C3, respectively.

2.2. Enzyme activity assays

The reduction of PQ was determined in systems containing 0.2–5.0 μM PQ, 170 μM NADPH, 0.02% BSA, and 4% ethanol in 100 μM potassium phosphate buffer (pH 7.0, 500 μL total volume) at 37 °C. Initial velocities were determined with a Beckman DU640 spectrophotometer (Fullerton, CA) by measuring the decrease in absorbance of the pyridine nucleotide at 340 nm (ε = 6270 M^{-1} cm^{-1}) after the initiation of the reaction by the addition of enzyme. The non-enzymatic reaction rates, determined by monitoring each sample for 3 min prior to addition of enzyme, were subtracted to give the enzymatic initial velocity. Non-enzymatic rates were typically less than 10% of the enzymatic rate. Reactions were performed with samples in duplicate, and the kinetic values reported are the average of at least six independent experiments.

Steroid hormone reduction reactions specific for each AKR1C isoform were monitored in systems containing 0.02 μCi [^{14}C]-steroid, varied unlabeled steroid to obtain the final concentration (2.5–40 μM Δ^4-androstene-3,17-dione for AKR1C3; 1.5–16.5 μM DHT for AKR1C2; 1.5–16 μM progesterone for AKR1C1), 200 μM NADPH, and 4% ethanol in 100 μM potassium phosphate buffer (pH 7.0, 200 μL total volume) at 37 °C. Reactions were initiated by addition of NADPH and were terminated by the addition of 1 mL of ice-cold, water-saturated ethyl acetate. Steroids were extracted by continuous mixing for 30 s, and the organic phases were evaporated to dryness. Samples were redissolved in 50 μL ethyl acetate, and applied to LK6D Silica TLC plates (Whatman Inc., Clifton, NJ). Chromatography was accomplished using 4:1 (v/v) methylene chloride/ethyl acetate, and plates were counted using a Bioscan System 200 plate reader (Washington, DC). The total radioactivity found in the product peak was converted to nmoles of steroid using the final specific radioactivity of the substrate. The formation of product was determined at three or four separate time points within the linear portion of the reaction (5–20% product formation). The slope of the line was used to determine the initial velocity of the reaction. Recovery of radioactivity in the combined substrate and product peaks was greater than 95%. Testosterone (5–42.5 μM) oxidation catalyzed by AKR1C3 was also monitored with this system, except that $NADP^+$ was used in place of NADPH. Reactions were performed with samples in duplicate, and the kinetic values reported are the average of at least three independent experiments.

2.3. Enzyme inhibition studies

Inhibition of the reduction of PQ catalyzed by AKR1C1, AKR1C2 and AKR1C3 was determined using increasing concentrations of each inhibitor with the substrate concentrations set to K_M of the corresponding enzyme, to obtain IC_{50} values. Inhibition of progesterone (5 μM) reduction catalyzed by AKR1C1 (K_M = 5.7 μM), DHT (3.5 μM) reduction catalyzed by AKR1C2 (K_M = 4.6 μM), and Δ^4-androstene-3,17-dione (5 μM) reduction catalyzed by AKR1C3 (K_M = 6.6 μM) by indomethacin and CBM were also measured. The reversibility of inhibition of AKR1C3 by indomethacin was measured by preincubating AKR1C3 with 5 μM indomethacin and 170 μM NADPH at 37 °C for time points up to 1 h prior to diluting a 10 μL aliquot into the PQ reduction assay (500 μL). The percentage of enzyme activity remaining was then determined. All inhibition data were obtained from single experiments, with samples run in duplicate.

Patterns of inhibition were determined by measuring velocities using a range of inhibitor concentrations at different fixed substrate concentrations. Inhibition data were fit using Grafit 5.0 to obtain the IC_{50} values. Initial velocity data were globally fit to competitive, noncompetitive, uncompetitive, and mixed inhibition models. The kinetic model demonstrating the best fit was used to determine the inhibition constant K_I.

3. Results

3.1. Determination of the kinetics of AKR1C-catalyzed reduction reactions

Steady state kinetic parameters for the reduction of PQ catalyzed by homogenous recombinant AKR1C1, AKR1C2, and AKR1C3 were determined spectrophotometrically at 37 °C, pH 7 (Table 1). Each AKR1C isoform reduced PQ with high catalytic efficiency (48,000–100,000 min^{-1} mM^{-1}). Steady state kinetic parameters for the reduction of progesterone, DHT, and Δ^4-androstene-3,17-dione catalyzed by AKR1C1, AKR1C2, and AKR1C3, respectively, were determined radiometrically at 37 °C, pH 7 (Table 1). Catalytic efficiencies of the AKR1C-catalyzed steroid hormone reduction reactions (24–1000 min^{-1} mM^{-1}) were substantially lower than those observed for the reduction of PQ.

3.2. Inhibition of AKR1C-mediated PQ reduction by NSAIDs and indomethacin analogues

We initially examined the inhibition of AKR1C1, AKR1C2, and AKR1C3 by a series of NSAIDs, as well as two previously synthesized indomethacin analogues, N-(4-aminobenzyl)-6-methoxy-3-indole acetic acid (ABMI) and indomethacin

Table 1 – Kinetic parameters determined for the reduction of PQ and ketosteroids catalyzed by the AKR1C isoforms

Enzyme	Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
AKR1C1	PQ	0.73 ± 0.2	35 ± 6.1	48,000
AKR1C2	PQ	1.1 ± 0.4	110 ± 18	100,000
AKR1C3	PQ	1.5 ± 0.7	84 ± 18	55,000
AKR1C1	Progesterone	5.7 ± 0.4	0.93 ± 0.9	210
AKR1C2	5α-DHT	4.6 ± 1.4	3.8 ± 0.3	820
AKR1C3	Δ^4-Androstene-3,17-dione	6.6 ± 2.4	0.16 ± 0.02	24

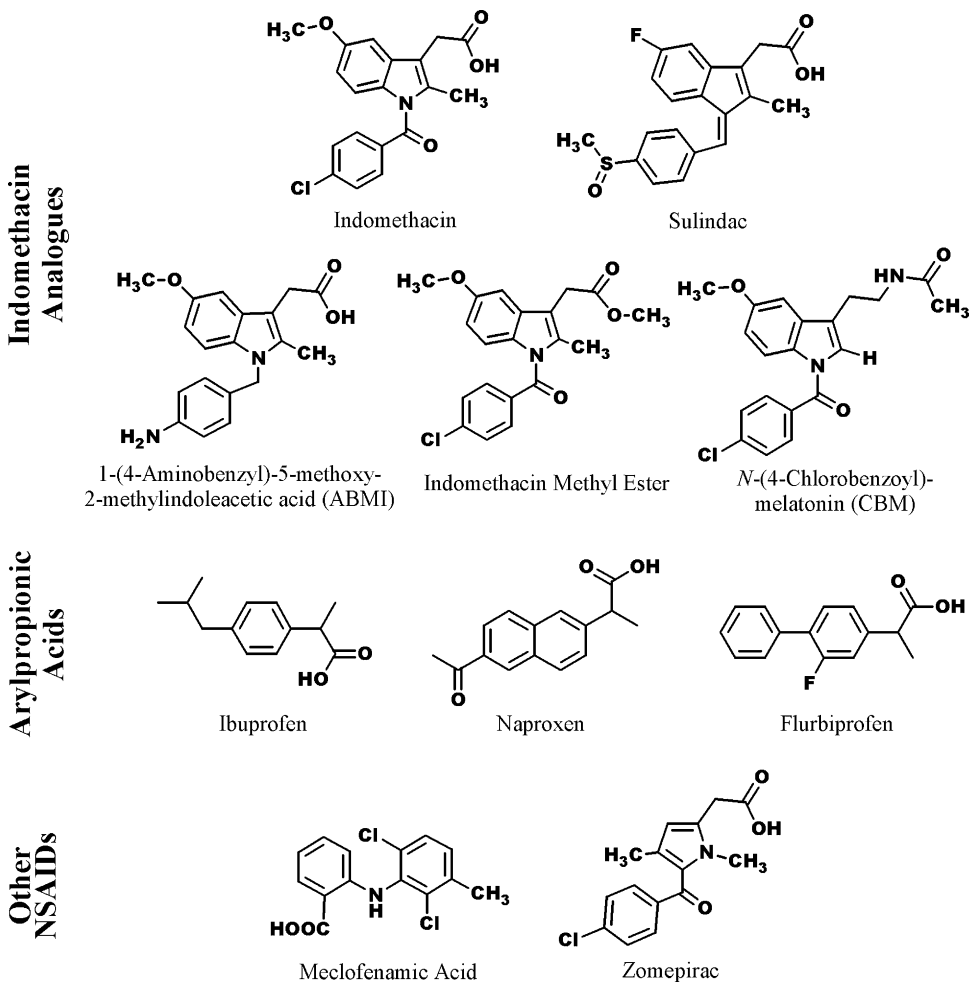


Fig. 1 – Structures of NSAIDs and NSAID analogues tested for inhibition of AKR1C enzymes.

methyl ester (Fig. 1) [22]. For these studies we elected to follow the NADPH-dependent reduction of PQ, which provided a rapid method for determining the effects of inhibitors on the reduction of a single substrate for each AKR1C isoform. Inhibition studies were performed with the PQ concentration set to the K_M of the respective enzyme.

All of the tested NSAIDs were effective inhibitors of the AKR1C enzymes, demonstrating inhibition that was comparable to their inhibition of the COX enzymes (Table 2) [24,25]. Sulindac, meclofenamic acid, and the arylpropionic acids were effective inhibitors of all three isoforms, but provided little isoform selectivity. Only indomethacin and indomethacin methyl ester demonstrated a strong preference for AKR1C3 over the other AKR1C isoforms. Indomethacin inhibited AKR1C3 at concentrations that were 20-fold lower than the concentrations required to inhibit the other AKR1Cs (Fig. 2A). Indomethacin methyl ester, which could only be tested up to 20 μM because its UV absorbance interfered with the assay at

Table 2 – IC_{50} values for the inhibition of the AKR1C catalyzed reduction of PQ by NSAIDs and indomethacin derivatives

Compound	IC_{50} (μM)			Preference for AKR1C3 versus AKR1C2
	AKR1C1	AKR1C2	AKR1C3	
Indomethacin	96	50	2.3	22
Sulindac	10	6.6	3.4	1.9
ABMI	28	62	52	1.2
Indomethacin Methyl ester	≫20	≫20	2.3	≫8.7
Meclofenamic Acid	2.9	1.7	0.7	2.4
Zomepirac	>50	23	40	0.58
Ibuprofen	29	1.9	9.9	0.19
Naproxen	280	1.2	1.4	0.86
Flurbiprofen	51	3.2	7.8	0.24

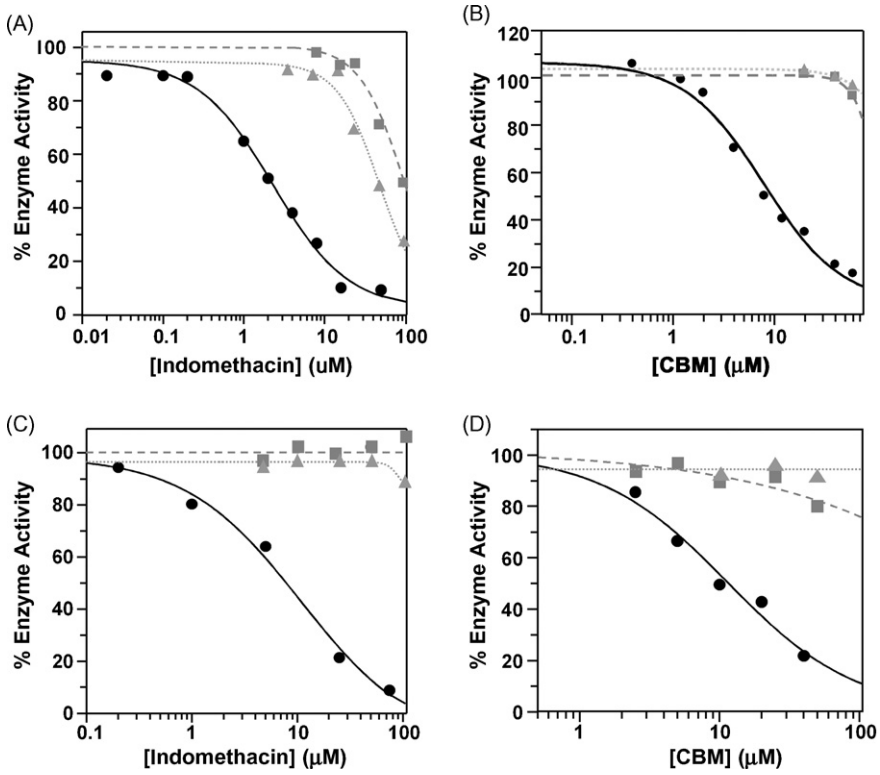
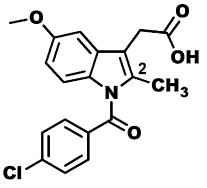


Fig. 2 – Indomethacin and its analogue CBM are selective inhibitors of reduction reactions catalyzed by AKR1C3. Inhibition of the reduction of PQ catalyzed by AKR1C1 (■), AKR1C2 (▲), or AKR1C3 (●) by increasing concentrations of (A) indomethacin or (B) CBM, with the concentration of PQ fixed at K_M for each enzyme. Effect of (C) indomethacin and (D) CBM on the reduction of progesterone (5 μM) catalyzed by AKR1C1 (■), the reduction of 5α-DHT (3.5 μM) catalyzed by AKR1C2 (▲), and the reduction of Δ^4-androstene-3,17-dione (5 μM) catalyzed by AKR1C3 (●).

higher concentrations, effectively inhibited only AKR1C3 and had no effect on PQ reduction by the other AKR1C enzymes at the concentrations tested. Previously, Matsuura et al. [10] had shown that indomethacin had high affinity for AKR1C3, while Hara et al. [26] showed that indomethacin was a relatively weak inhibitor of AKR1C1 and AKR1C2. However, these studies used widely varied concentrations of substrate relative to the K_M of the respective enzymes, making the results difficult to compare across isoform.

3.3. *Development of a specific inhibitor of AKR1C3*

Indomethacin represented a promising lead compound for the development of an AKR1C3-selective inhibitor, given its strong preference for the inhibition of AKR1C3 over the other AKR1C isoforms. The inhibition data provided insights into the aspects of indomethacin's structure that contribute to the preferential inhibition of AKR1C3 over the other AKR1C isoforms (Fig. 3). Zomepirac, which has similar functional



Modification	AKR1C1 & AKR1C2	AKR1C3	COX-1	COX-2
Removal of Bridge Carbonyl	Improved Inhibition	No Change	No Change [30]	No Change [30]
Modification of Carboxylic Acid	Reduced Inhibition	No Change	Reduction or Loss of Inhibition [21;28;29]	Variable [21;28;29]
Removal or Modification of 2-Methyl Group	No Change	No Change	Loss of Time-Dependent Inhibition [27]	Loss of Time-Dependent Inhibition [27]
Polar Substitution on the Benzoyl ring	Reduced Inhibition	No Change	Loss of Inhibition [31]	Loss of Inhibition [32]

Fig. 3 – Predicted effects of structural modifications to indomethacin on the inhibition of the AKR1C and COX enzymes based on the crystal structures of the targets and the reported inhibition of COX-1 and COX-2 by indomethacin analogues.

groups to indomethacin, but lacks the complete indole ring, is a weak and nonspecific inhibitor of AKR1C3, indicating the importance of the indole structure. Sulindac and ABMI lack the bridge carbonyl group and have little or no preference for AKR1C3 over AKR1C2. Indomethacin methyl ester retained its selectivity for AKR1C3, suggesting that modifications to the acetic acid moiety could be tolerated.

Before embarking on syntheses of indomethacin analogues we analyzed the crystal structure of the AKR1C3·$NADP^+$·indomethacin complex (Fig. 4, protein data bank accession number 1S2A) [20]. This structure demonstrates coordination between indomethacin's bridge carbonyl group and the catalytic Tyr55. This interaction may position indomethacin in the AKR1C active site in a way that favors the binding to AKR1C3, but which results in steric hindrance in the slightly smaller binding pockets of AKR1C1 and AKR1C2. The crystal structure also indicates modifications to indomethacin that would be accommodated by the active site of AKR1C3, but not by the other AKR1C isoforms or the COX isozymes (Fig. 3) [20,21,27–32]. In particular, the AKR1C3 amino acids that surround the carboxylic acid and *p*-chlorobenzoyl groups of indomethacin are less bulky than the corresponding amino acids in the AKR1C2·$NADP^+$·ursodeoxycholate structure (protein data bank accession number 1IHI) [33]. In addition, the lack of amino acid residues in proximity to the 2-methyl group implies that modification at this position would not affect the inhibition of the AKR1C enzymes. This methyl group is required for the time-dependent inhibition of the COX isozymes [27].

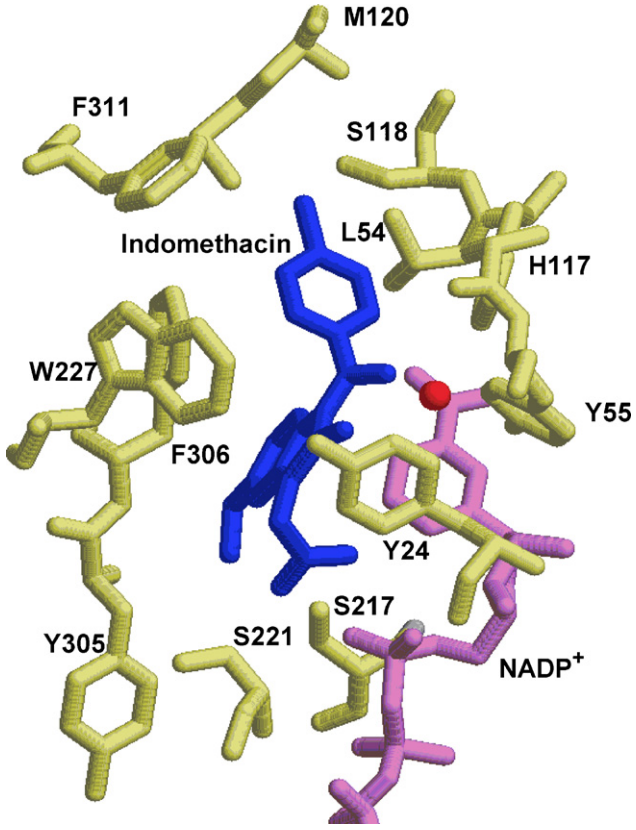


Fig. 4 – Position of indomethacin within the active site of AKR1C3 [20].

N-(4-Chlorobenzoyl)-melatonin (CBM) was one compound predicted to fit easily into the active site of AKR1C3, but not the other AKR1C isoforms or the COX enzymes. In the structure of CBM, the acetic acid group of indomethacin is replaced by a reverse amide, which will eliminate COX-1 inhibition and reduce the inhibition of COX-2 [21]. In addition, the loss of the 2-methyl group prevents the time-dependent inactivation of both COX isoforms. Because CBM retains the elements of indomethacin that confer selectivity for AKR1C3 over the other AKR1C isoforms and has a bulkier reverse amide in place of the acetic acid group, it was predicted to be at least as selective for AKR1C3 over the other isoforms as indomethacin.

CBM was synthesized and screened for COX inhibition by Kalgutkar et al. [21] and did not significantly inhibit either of the COX enzymes ($IC_{50} > 66$ μM). We repeated the synthesis of the compound and screened CBM for inhibition of the AKR1C isoforms (Fig. 2B). It potently inhibited AKR1C3-mediated reduction of PQ, with an IC_{50} of 7.8 μM, but only inhibited the reduction of PQ catalyzed by AKR1C1 and AKR1C2 by less than 10% at the highest concentration tested (60 μM).

3.4. *Inhibition of AKR1C-mediated steroid hormone metabolism*

Next we examined whether the selective inhibition of AKR1C3 over the other AKR1C isoforms by indomethacin and CBM was retained with physiologically-relevant steroid hormone substrates. To this end, we examined the inhibition of AKR1C3-mediated reduction of Δ^4-androstene-3,17-dione (5 μM), AKR1C2-mediated reduction of DHT (3.5 μM), and the AKR1C1-mediated reduction of progesterone (5 μM). Indomethacin and CBM inhibited the reduction of Δ^4-androstene-3,17-dione by AKR1C3, with IC_{50} values of 8.5 and 11.4 μM, respectively, but did not affect the reduction of DHT catalyzed by AKR1C2 and exhibited only weak inhibition of progesterone reduction catalyzed by AKR1C1 (Fig. 2C and D).

3.5. *Patterns of enzyme inhibition*

The patterns of inhibition by indomethacin and CBM for the reduction of PQ and Δ^4-androstene-3,17-dione catalyzed by AKR1C3 were examined. Inhibition of PQ reduction by indomethacin and CBM was best fit by an uncompetitive model, yielding K_I values of 0.7 and 3.4 μM, respectively (Fig. 5A and B). The inhibition of PQ reduction catalyzed by AKR1C3 by indomethacin was reversible, as no time-dependent loss of enzyme activity was observed when indomethacin was preincubated with enzyme (data not shown). These results indicated the formation of a nonproductive ternary or quaternary complex subsequent to substrate binding. We then examined the inhibition patterns regarding the AKR1C3 catalyzed reduction of Δ^4-androstene-3,17-dione to testosterone. Indomethacin and CBM competitively inhibited this reaction yielding K_I values of 8.2 and 6.0 μM, respectively (Fig. 5C and D). These results indicated the formation of nonproductive ternary complexes that preceded substrate binding. To provide further insight into the mechanism of AKR1C3 inhibition, we also examined the inhibition of the AKR1C3-mediated, $NADP^+$ dependent, oxidation of testosterone by indomethacin. In the oxidation direction, indomethacin also

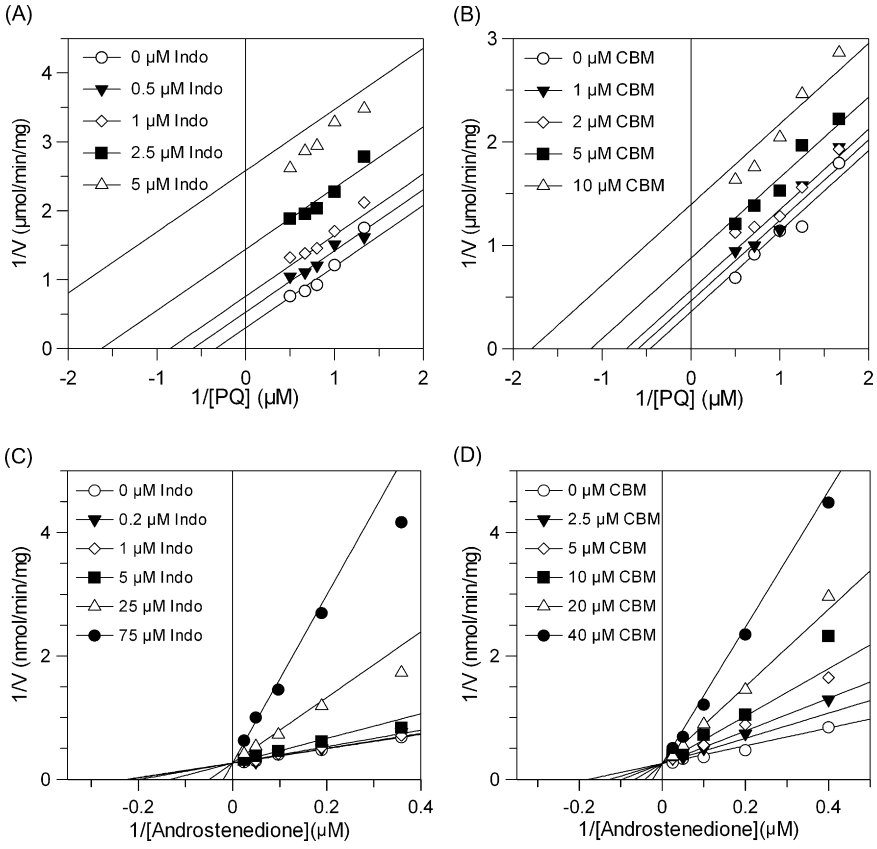


Fig. 5 – Indomethacin and CBM exhibit uncompetitive inhibition patterns for the reduction of PQ catalyzed by AKR1C3 but competitive inhibition patterns for the reduction of Δ^4-androstene-3,17-dione catalyzed by AKR1C3. Lineweaver–Burke plots demonstrating the pattern of inhibition for the reduction of PQ catalyzed by AKR1C3 by (A) indomethacin or (B) CBM and the inhibition of the reduction of Δ^4-androstene-3,17-dione catalyzed by AKR1C3 by (C) indomethacin and (D) CBM.

exhibited a competitive pattern of inhibition yielding a K_I of 5.0 μM (data not shown).

4. Discussion

We previously described N-phenylanthranilic acid analogues that inhibit the AKR1C subfamily of enzymes, but do not inhibit COX activities [19]. However, the close sequence identity (>86%) between the human AKR1C isoforms presented a challenge for the development of isoform selective inhibitors. Recently, estradiol derivatives bearing a lactone ring on the D-ring were found to be potent inhibitors of AKR1C3 [34]. However, since AKR1C1, AKR1C2, and AKR1C3 are all capable of 17-ketosteroid reduction, we anticipate that these compounds will not be isoform selective. In addition, steroidal inhibitors of AKR1Cs have the potential to bind nuclear receptors. The current study explored the inhibition of the peripherally expressed human AKR1C isoforms by NSAIDs. The observation that indomethacin preferentially inhibited AKR1C3 over the other isoforms was used to develop an isoform-specific inhibitor of AKR1C3 that does not affect cyclooxygenase activity. Structure–activity relationships derived from the differences between indomethacin analogues in AKR1C inhibition patterns and the crystal structure of the AKR1C3·$NADP^+$·indomethacin complex [20] led to the discovery of a compound, CBM, that potently inhibits AKR1C3 but does not inhibit the other AKR1C isoforms or the COX enzymes. CBM provides a tool for the determination of the roles of AKR1C3 in normal and pathological physiology.

By acting on both steroid hormone and prostaglandin mediated signaling pathways, AKR1C3 may play an integral part in the autocrine and paracrine regulation of hormone-dependent and hormone-independent cancer growth. Increased testosterone and estrogen production through the 17-ketosteroid reductase activity of AKR1C3 will result in activation of the androgen and estrogen receptors and stimulate proliferation of hormone-dependent cancers, such as prostate and breast. In addition, the prostaglandin F synthase activity of AKR1C3 will alter signaling pathways and stimulate the proliferation of both hormone-dependent and hormone-independent cancers. The PGF_2 isomers will stimulate the Gq-coupled F prostanoid receptor and activate signal cascades associated with proliferation and the inhibition of differentiation [11–13]. Reduction of PGD_2 levels by AKR1C3 will prevent its dehydration and rearrangement to form the J-series prostaglandins. The PGJ_2 products inhibit cell proliferation and stimulate apoptosis through several mechanisms, including covalent modification and activation

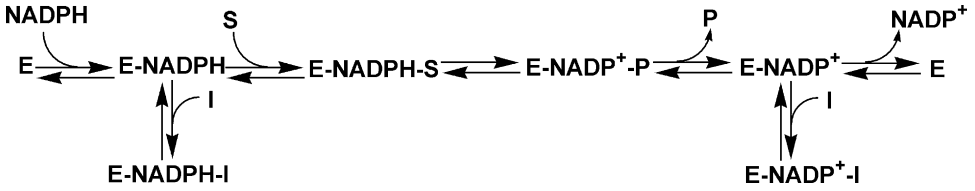


Fig. 6 – Proposed formation of inhibitory complexes by indomethacin and indomethacin analogues during the ordered bi bi reaction catalyzed by AKR1C3. E = enzyme, I = inhibitor, S = substrate, and P = product.

of PPARγ and covalent modification and inactivation of NF-κB and its upstream kinase [16–18,35,36].

Accumulating evidence suggests that AKR1C3 contributes to the development of human cancer. AKR1C3 is over-expressed in leukemia and cancers of the prostate, breast, endometrium, and head and neck [8,37–41]. In addition, a variant allele of the *AKR1C3* gene decreases the risk of lung and prostate cancer [42,43]. In HL-60 leukemia cells, over-expression of AKR1C3 protected cells from differentiation and apoptosis, while indomethacin treatment potentiated these effects [15]. Given these data and its potential to stimulate proliferative steroid hormone and prostaglandin signaling, AKR1C3 represents a potential target for the prevention or treatment of cancer.

AKR1C enzymes catalyze an ordered bi bi mechanism in which cofactor binds first followed by substrate [44–46]. Two mechanisms contribute to the inhibition of AKR1C3 by indomethacin and its analogues. With the reduction of PQ, which displays high catalytic efficiency, indomethacin and its analogues exhibit an uncompetitive pattern of AKR1C3 inhibition, while with the less efficient substrate Δ^4-androstene-3,17-dione, a competitive pattern of inhibition was observed. Koda et al. [47] found that prostaglandin H_2 reduction catalyzed by AKR1C3 was competitively inhibited by a prostamide H_2 analogue, but was noncompetitively inhibited by the prostamide $F_{2\alpha}$ analogue bimatoprost. Crystal structures indicate that both indomethacin and bimatoprost bind the active site of AKR1C3, suggesting that a classical noncompetitive mechanism of inhibition is unlikely (Fig. 4) [20,48]. Instead, inhibition patterns can be explained if AKR1C3 forms two different inhibitory complexes in the kinetic sequence. Inhibitors can bind either to E·NADPH or E·$NADP^+$ (Fig. 6). The relative contribution of these two complexes to the overall inhibition pattern will depend on both the inhibitor and the substrate. Inhibitors based on substrates likely favor formation of the E·NADPH·I complexes, while those based on products would be more likely to form E·$NADP^+$·I complexes. With substrates that display high catalytic efficiency, such as PQ [9,10], the predominate rate-limiting step for reduction is likely $NADP^+$ release [49]. This rate determining step will be unaffected by the formation of the E·NADPH·I complex but slowed by the formation of the abortive E·$NADP^+$·I complex, yielding uncompetitive inhibition. A similar uncompetitive mechanism of inhibition has been observed with the type 2 5α-reductase inhibitor epristeride [50]. With a less efficient substrate, such as Δ^4-androstene-3,17-dione, the binding of the second substrate and the reduction chemistry are important determinants of the catalytic rate. These steps will be inhibited by the E·NADPH·I complex, but unaffected by the formation of the E·$NADP^+$·I complex, yielding competitive inhibition. Indomethacin binds with a higher affinity to the E-$NADP^+$ complex than to the E-NADPH complex, possibly because of its negative charge, which the crystal structure indicates is in close proximity to the nicotinamide ring of $NADP^+$ [20]. This effect is reflected by the lower K_I for indomethacin observed for both the uncompetitive inhibition of PQ reduction and the competitive inhibition of testosterone oxidation as compared to the higher K_I value observed for the competitive inhibition of Δ^4-androstene-3,17-dione reduction. Inhibition of the AKR1C3-mediated formation of the PGF_2 isomers by indomethacin and its analogues may occur through either one or both of these complexes as these reactions have catalytic efficiencies that are intermediate between the reduction of PQ and the reduction of Δ^4-androstene-3,17-dione [10].

CBM will provide a tool for the examination of the role of AKR1C3 in cell proliferation and cancer. Because it does not affect the COX-mediated production of prostaglandins, CBM will allow us to determine those aspects of proliferative prostaglandin signaling that are a direct result of AKR1C3 activity. Because it has a strong preference for AKR1C3 over the other human AKR1C isoforms, CBM will also allow the determination of whether AKR1C3 plays a significant role in the regulation of proliferative steroid hormone signaling. Previous efforts at determining the roles of AKR1C3 in cellular proliferative signaling, both by us and others [15], have focused on the effects of over-expression of AKR1C3 or inhibition with non-specific inhibitors. However, ectopic expression does not allow for the determination of AKR1C3 effects at physiologically relevant expression levels, and may have unpredicted effects on the expression of other proteins. By using CBM to reduce the activity of endogenous AKR1C3, we can determine what impact these relevant levels have on the proliferative signaling of the cell.

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