Letter

The 2′-Trifluoromethyl Analogue of Indomethacin Is a Potent and Selective COX-2 Inhibitor

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Ⓢ *Supporting Information*

ABSTRACT: Indomethacin is a potent, time-dependent, non-selective inhibitor of the cyclooxygenase enzymes (COX-1 and COX-2). Deletion of the 2′-methyl group of indomethacin produces a weak, reversible COX inhibitor, leading us to explore functionality at that position. Here, we report that substitution of the 2′-methyl group of indomethacin with trifluoromethyl produces CF_3−indomethacin, a tight-binding inhibitor with kinetic properties similar to those of indomethacin and unexpected COX-2 selectivity (IC_{50} mCOX-2 = 267 nM; IC_{50} oCOX-1 > 100 μM). Studies with site-directed mutants reveal that COX-2 selectivity results from insertion of the CF_3 group into a small hydrophobic pocket formed by Ala-527, Val-349, Ser-530, and Leu-531 and projection of the methoxy group toward a side pocket bordered by Val-523. CF_3−indomethacin inhibited COX-2 activity in human head and neck squamous cell carcinoma cells and exhibited in vivo anti-inflammatory activity in the carrageenan-induced rat paw edema model with similar potency to that of indomethacin.

Inhibits COX-1 > COX-2   COX-2-Selective

KEYWORDS: *Cyclooxygenase, inflammation, nonsteroidal anti-inflammatory drug, coxib, prostaglandin, arachidonic acid*
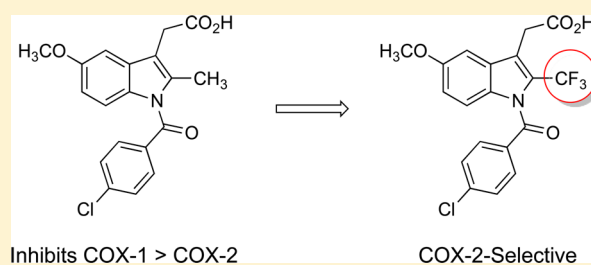
Cyclooxygenase (COX) enzymes, which catalyze the conversion of arachidonic acid (AA) to prostaglandin H₂, are the pharmacological targets of nonsteroidal anti-inflammatory drugs (NSAIDs).[1] The two COX isoforms, COX-1 and COX-2, have high sequence identity (60%), very similar three-dimensional structures, and nearly indistinguishable kinetic parameters with AA as substrate.[2] COX-1 is constitutively expressed in most tissues and is involved in the production of prostaglandins that mediate basic cellular housekeeping functions. In most contexts, COX-2 is an inducible enzyme, the expression of which is activated by cytokines, mitogens, endotoxin, and tumor promoters. The anti-inflammatory and analgesic properties of traditional NSAIDs are primarily due to inhibition of COX-2.[3]

Indomethacin is a potent NSAID that exhibits an approximately 15-fold higher selectivity for COX-1 relative to COX-2 (Figure 1).[4−7] It is a slow, tight-binding inhibitor that establishes a rapidly reversible equilibrium with the enzyme followed by a slow transition to a much more tightly bound COX-indomethacin complex. Formation of the tightly bound complex is responsible for indomethacin's strong COX inhibitory activity.[4] Indomethacin is a powerful anti-inflammatory agent and a strong tocolytic.[8] It also exhibits anticancer activity as suggested by a report demonstrating that indomethacin significantly increased the lifespan of a group of terminally ill patients suffering from a range of cancers, mainly gastrointestinal.[9]

The use of indomethacin is limited by its gastrointestinal toxicity and its inhibition of platelet function leading to increased bleeding times.[10,11] A significant component of both of these side effects results from the ability of indomethacin to inhibit COX-1. Thus, multiple attempts to decrease the COX-1 inhibitory activity of indomethacin have been reported. Since the COX-2 active site is approximately 25% larger than that of COX-1, indomethacin analogues have been synthesized that increase its size by, e.g., lengthening the carboxylic acid side chain or augmenting the steric bulk of the acyl group attached to the indole nitrogen.[12] In addition, many different amides and esters of indomethacin exhibit significant COX-2 selectivity, and this approach has been used to construct COX-2-targeted molecular imaging agents.[13,14]

Several years ago, our laboratory reported that deletion of the 2′-methyl group on the indole ring of indomethacin produces a *des*-methyl derivative that is a weak, reversible inhibitor of COX-2 and, to some extent, COX-1.[15] The explanation for the loss of COX inhibitory activity resulting from deletion of the 2′-methyl group is provided by the crystal structure of a complex of indomethacin with COX-2.[16] The 2′-methyl inserts into a hydrophobic pocket comprising Ala-527, Val-349, Ser-530, and Leu-531 (Figure 1C). Mutations of Val-349 to Ala or Leu alter
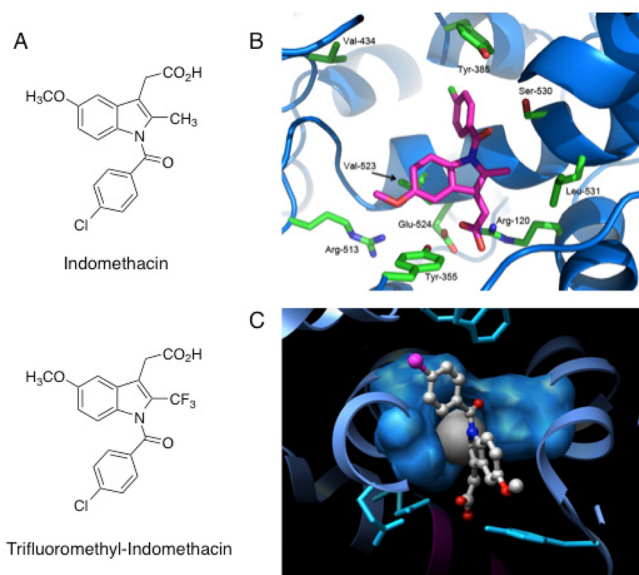
Figure 1. Structures of indomethacin and trifluoromethyl-indomethacin (A). Representation of indomethacin in the active site of mouse COX-2 (B) and a space-filling model of the methyl-binding pocket in mouse COX-2 for indomethacin comprising Ala-527, Val-349, Ser-530, and Leu-531 (C).

Figure 2. Synthesis of CF_3−indomethacin from CF_3−levulinic acid lactone.

the size of the pocket and lead to an increase or decrease, respectively, in the potency of indomethacin.[15]

The importance of the 2′-methyl group in mediating slow, tight-binding of indomethacin prompted us to explore other functionality at this position. These efforts led to the discovery of 2′-trifluoromethyl-indomethacin (CF_3−indomethacin), in which the 2′-methyl group was replaced by a CF_3 group (Figure 1). This modest chemical change results in a very significant and unexpected shift in the selectivity of COX inhibition, creating a molecule that is a highly potent, slow, tight-binding inhibitor of COX-2 lacking significant COX-1 inhibitory activity. Here, we describe the synthesis, inhibitory mechanism, and pharmacology of CF_3−indomethacin.

We initially attempted to synthesize CF_3−indomethacin through the condensation of 1-(4-methoxyphenyl)-1-(4-chlorobenzoyl)hydrazine hydrochloride with CF_3−levulinic acid using a classic Fisher indole synthesis approach. Although this route occasionally yielded the desired product, it proved to be unreliable, affording a phenylhydrazone derivative with no cyclization to the intended indole on most attempts. This led us to investigate a number of alternative approaches, including a Fisher indole synthesis starting with CF_3-levulinic acid and 2-methyl-1-(4-methoxyphenyl)-1-(4-chlorobenzoyl) hydrazine, synthesized from 1-(4-methoxyphenyl)-1-(4-chlorobenzoyl)-hydrazine hydrochloride by reaction with paraformaldehyde followed by reduction with $NaBH_4$. We also attempted condensation of CF_3-levulinic acid with 2-acetyl-1-(4-methoxyphenyl)-1-(4-chlorobenzoyl)hydrazine. No indole product was detected in either of these reactions.

We hypothesized that failure of indole formation was due to the electron-withdrawing effect of the CF_3 group, which disfavors tautomerization of the hydrazone to the enamine intermediate required for cyclization to the indole product. To address this problem, we designed a synthetic route utilizing the reaction of the hydrazine with CF_3-levulinic acid lactone in hopes that direct formation of the enamine could occur without the intermediacy of the hydrazone (Figure 2). Consistently, we
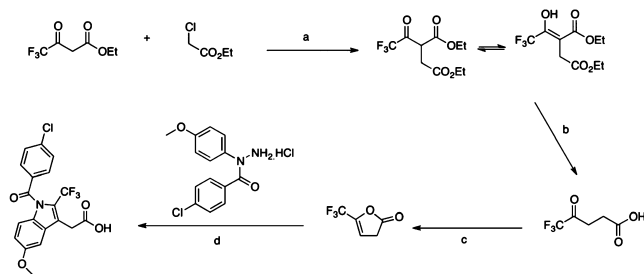
detected the desired CF_3−indomethacin by mass spectrometry of the reaction mixture of 1-(4-methoxyphenyl)-1-(4-chlorobenzoyl)hydrazine hydrochloride and the lactone. The success of this approach suggests that a significant lactone impurity in commercial CF_3-levulinic acid may have accounted for our occasional ability to synthesize CF_3−indomethacin using the more traditional synthetic route.

Mass spectral analysis (Figure S1, Supporting Information, shown for CF_3−indomethacin), one-dimensional proton NMR spectroscopy (Figures S2 and S3, Supporting Information, shown for CF_3−indomethacin), and HPLC analysis were performed on each of the reaction intermediates and final products to confirm both the structure and purity of the compounds. Each was shown by HPLC analysis with evaporative light scattering detection to elute as a single peak (>98% pure). Under optimal conditions, the yield of CF_3−indomethacin from the reaction of the hydrazine and the lactone was 20%; however, the synthesis was reproducible, and sufficient amounts of material could be generated for subsequent testing.

Following a 3 min equilibration of purified and hematin-reconstituted mouse or human COX-2 (mCOX-2 or hCOX-2) or ovine COX-1 (oCOX-1) at 37 °C, CF_3−indomethacin and $[1-^{14}C]$-AA were added simultaneously and incubated for 30 s. No significant enzyme inhibition was observed over a wide range of inhibitor and substrate concentrations, indicating that CF_3−indomethacin is not a pure competitive inhibitor of COX (data not shown). Next, CF_3−indomethacin was preincubated with purified COX enzymes using a protocol designed to evaluate time-dependent inhibition. Figure 3A shows the inhibition curve for indomethacin itself. As expected, indomethacin was a potent and nonselective inhibitor of mCOX-2, hCOX-2, and oCOX-1, with IC_{50} values of 127, 180, and 27 nM, respectively, consistent with previous reports.[15] In contrast, Figure 3B shows that CF_3−indomethacin potently and selectively inhibited both mCOX-2 and hCOX-2 (IC_{50} = 267 and 388 nM, respectively) with no appreciable effect on oCOX-1 up to very high inhibitor concentrations (100 μM).

Time-dependent inhibition of COX enzymes is most simply described by the two-stage equilibrium summarized in eq 1.[4] To evaluate the magnitude of the steps in this equilibrium, the dependence of COX inhibition by CF_3−indomethacin on preincubation time and inhibitor concentration was determined. The decrease in substrate conversion at different inhibitor concentrations was plotted against the preincubation times and fit to a single-exponential decay with a plateau to determine a value for k_{obs}. The dependence of k_{obs} on inhibitor concentration is represented by eq 2, where K_I corresponds to the inhibitor concentration that yields a k_{obs} value equal to half the limiting k_{obs}, and k_2 represents the limiting forward rate
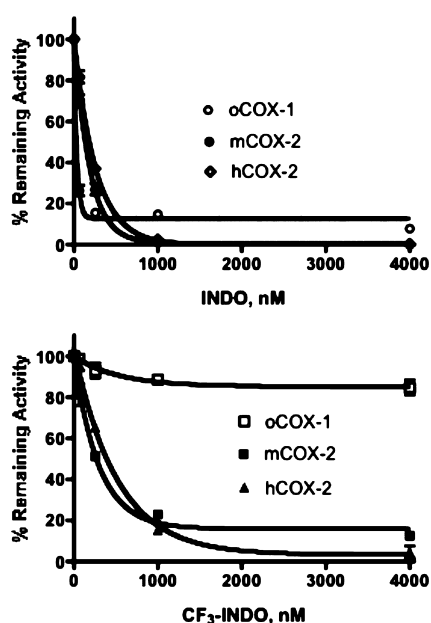
Figure 3. Inhibition of purified oCOX-1, mCOX-2 and hCOX-2 by indomethacin (INDO) and CF_3−indomethacin (CF_3−INDO). The IC_{50} values of indomethacin for oCOX-1, mCOX-2 and hCOX-2 were 27, 127, and 180 nM, respectively. The corresponding IC_{50} values of CF_3−indomethacin were >4 μM, 267 nM, and 388 nM, respectively.

Figure 4. Kinetics of the time-dependent inhibition of hCOX-2 by CF_3−indomethacin (CF_3−INDO). Individual k_{obs} values were obtained from a plot of the decrease in AA conversion versus preincubation time (A) and plotted against inhibition concentration (B) to calculate the kinetic constants, K_I, k_2, and k_{-2}.

constant for inhibition. The reverse rate constant, k_{-2}, is equal to the y-intercept and is zero for compounds that display extremely tight binding.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [EI] \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI^* \tag{1}$$

$$k_{obs} = ((k_2[I])/(K_I + [I])) + k_{-2} \tag{2}$$

where $K_I = k_{-1}/k_1$.

Figure 4A shows the kinetics for the time- and concentration-dependent inhibition of wild-type hCOX-2 by CF_3−indomethacin. Inhibition proceeded rapidly, and plateaus were reached at short time points for the higher inhibitor concentrations. The time-dependent inhibition curve for hCOX-2 approached 0% remaining activity at higher inhibitor concentrations, indicating tight binding to the enzyme by CF_3−indomethacin. Interestingly, CF_3−indomethacin interacted with hCOX-2 with a greater affinity than indomethacin, as indicated by a lower K_I (1.5 ± 0.26 μM versus 13 ± 2.3 μM, respectively). The forward rate constants (k_2) were similar between indomethacin (0.061 ± 0.004 s^{-1}) and CF_3−indomethacin (0.020 ± 0.001 s^{-1}). A reverse rate constant (k_{-2}) was not determinable for either compound. Similar results were demonstrated for wild-type mCOX-2 (data not shown).

The crystal structure of indomethacin complexed with COX-2 shows that the carboxyl group of the inhibitor hydrogen bonds and ion pairs with Tyr-355 and Arg-120 at the constriction site at the base of the active site (Figure 1B). In contrast, some carboxylic acid-containing inhibitors, including diclofenac and lumiracoxib, bind to COX-2 in an orientation that places their carboxylates at the top of the active site, allowing them to form hydrogen bonds with Ser-530 and Tyr-385.[17,18] Table 1 shows that a Ser-530 to Ala mutation has little effect on the IC_{50} value observed for CF_3−indomethacin, indicating an orientation similar to that of indomethacin.
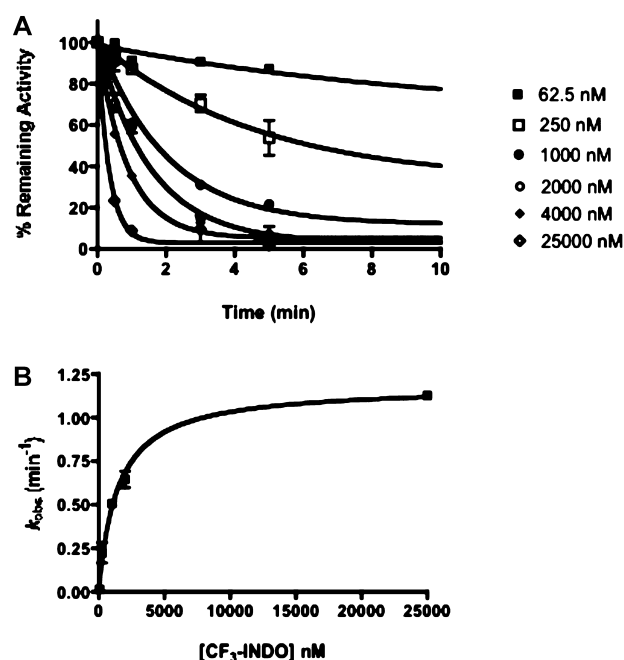
Table 1. Inhibition of COX Enzymes by Indomethacin and CF_3−Indomethacin

enzyme	INDO IC_{50}[a]	CF_3−INDO IC_{50}[a]
wt mCOX-2	127 nM	267 nM
wt oCOX-1	27 nM	
mV349A	71 nM (1.8↑)	120 nM (2.2↑)
mV349I	315 nM (2.5↓)	163 μM (6.1↓)
mV349L	1.34 μM (10.6↓)	
mS530A	220 nM (1.7↓)	435 nM (1.6↓)
mR120A		
mR120Q	326 nM (2.6↓)	1.56 μM (5.8↓)
mY355F		
mV523I	475 nM (3.7↓)	>4 μM (15↓)

[a]Indomethacin (INDO) and CF_3−indomethacin (CF_3−INDO) were screened against purified wild-type (wt) oCOX-1, mCOX-2, and the indicated mCOX-2 mutants as described in the Experimental Procedures in the Supporting Information. Values in parentheses indicate the fold increase or decrease in IC_{50} values for the mutants compared to wt mCOX-2. Dashed lines indicate that no inhibition was observed for inhibitor concentrations of up to 100 μM.

Kinetics studies of inhibition by esterified NSAIDs or of site-directed mutants of Arg-120 have revealed that carboxylic acid-containing, time-dependent NSAIDs (e.g., indomethacin and flurbiprofen) form an ion-pair and/or hydrogen bond with Arg-120 and that this interaction is critical for inhibition. For COX-2, this interaction plays a role but is less important for inhibitor binding and potency than it is for COX-1.[4,7,19,20] Table 1 shows that both indomethacin and CF_3−indomethacin are unable to inhibit an Arg-120 to Ala mutant or a Tyr355 to Phe mutant of COX-2, suggesting that the carboxylate of each inhibitor interacts with those residues at the constriction site. An R120Q mutant eliminates a potential ion-pair interaction with the inhibitors but retains the ability to hydrogen bond. Both

indomethacin and CF_3—indomethacin were able to inhibit the R120Q mutant, although the potency of inhibition was decreased by nearly 3-fold and 6-fold, respectively, indicating the importance of both hydrogen bonding and ion-pairing in the inhibition of COX by these compounds.

As noted above, the potent, time-dependent inhibition of COX by indomethacin is attributed to the insertion of the 2′-methyl group of the inhibitor into a small hydrophobic pocket comprising four residues (Ala-527, Val-349, Ser-530, and Leu-531). Mutation of Val-349 to Ala enlarges the pocket and increases the potency of indomethacin 3-fold, whereas mutation to Leu reduces the pocket size and decreases the potency of the inhibitor by 16-fold. A Val-349 to Ile mutant resembles wild-type enzyme.[15] A similar, though more severe, trend was observed for CF_3—indomethacin with regard to mutating Val-349 (Table 1), suggesting that the trifluoromethyl group of CF_3—indomethacin also inserts into or interacts with the residues in the hydrophobic pocket.

The first X-ray crystal structures solved for hCOX-2 showed that there is an overall difference in the size and shape of the COX-2 active site compared to that of COX-1.[16,21] The nearly 25% larger active site of COX-2 is accounted for by a side pocket resulting from a Val-523 substitution (Ile in COX-1) in the active site and by Arg-513 and Val-434 substitutions (His-513 and Ile-434, respectively, in COX-1) in the secondary shell. This side pocket is the primary determinant of inhibition of the diarylheterocycle class of COX-2-selective inhibitors (e.g., celecoxib and rofecoxib). The crystal structure of mCOX-2 with the celecoxib analogue SC-558 reveals that the sulfonamide group of the inhibitor binds in the side pocket adjacent to Val-523.[16] Mutagenesis of Val-523 to Ile in COX-2 abolishes the selectivity of diarylheterocycle inhibitors.[22,23] Thus, the current data suggest that the selective, time-dependent step in diarylheterocycle-mediated inhibition of COX-2 is most likely the insertion of the methylsulfonyl or sulfonamide group of the inhibitor into the pocket adjacent to Val-523 in COX-2. This is precluded in COX-1 by the extra steric bulk of Ile-523.

The cumulative results of the site-directed mutagenesis studies described above suggested that CF_3—indomethacin binds to COX-2 in essentially the same orientation as indomethacin and that the two inhibitors share the same molecular determinants of inhibition. However, this conclusion was contradicted by results from a V523I mutant that clearly differentiated CF_3—indomethacin and indomethacin. As noted above, V523I is a conserved COX-2 → COX-1 amino acid change that has a dramatic effect on the binding of diarylheterocycles such as celecoxib and rofecoxib to COX-2.[22,23] Although the V523I mutant COX-2 was sensitive to inhibition by indomethacin, the mutation resulted in a 3.7-fold reduction in inhibitor potency compared to wild-type enzyme. This reduction in potency was much more striking for CF_3—indomethacin (>15-fold) (Table 1). The 5-methoxy group of indomethacin is located near Val-523, but it does not insert into the side pocket.[16] Thus, the reduced inhibition of the V523I mutant by indomethacin may arise from steric interactions with the bulky Ile residue. The greater loss of activity observed with CF_3—indomethacin suggests that the larger trifluoromethyl group at the 2′ position displaces the indole ring toward the center of the active site and puts more pressure on the 5-methoxy-Ile-523 interaction in the mutant. It is noteworthy that the V523I mutant is less sensitive to inhibition by indomethacin even though it represents a COX-2 → COX-1 substitution.

This is contrary to expectations because indomethacin is a more potent inhibitor against COX-1 than COX-2.[7] The reduced sensitivity of V523I to indomethacin inhibition is a reminder that a drug's action on its target reflects the ensemble of multiple interactions in the binding site and is rarely dictated by the interaction with a single protein residue.

Having determined its key molecular determinants of COX-2 inhibition, we next evaluated CF_3—indomethacin's ability to inhibit COX-2 in intact cells. For these studies, we selected 1483 human head and neck squamous cell carcinoma cells that express high levels of the enzyme. Cells were pretreated with varying concentrations of CF_3—indomethacin for 30 min followed by incubation with $[1-^{14}C]AA$ for 20 min. In this assay, CF_3—indomethacin inhibited COX-2-dependent AA oxygenation with an IC_{50} value of 0.15 μM.

Finally, we assessed the in vivo anti-inflammatory activity of CF_3—indomethacin in the footpads of rats injected with carrageenan. CF_3—indomethacin or indomethacin was administered orally in corn oil at 1 h postcarrageenan injection, and footpad volume was measured 2 h later. The results showed that both CF_3—indomethacin and indomethacin exhibited anti-inflammatory activity with EC_{50} values of 1.7 mg/kg and 1.0 mg/kg, respectively (Figure 5). The highest concentration of
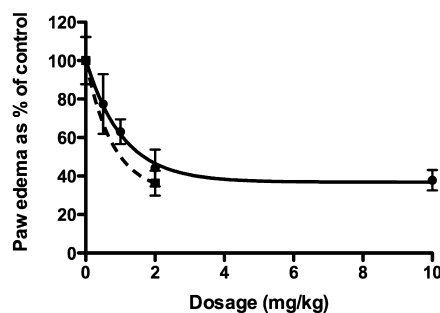

Figure 5. In vivo anti-inflammatory activity of indomethacin and CF_3—indomethacin in the rat paw edema model. Carageenan was injected into one paw, then 1 h later indomethacin or CF_3—indomethacin was administered by oral gavage. Paw volume was measured 2 h later. Indomethacin, ▲; CF_3—indomethacin, ●.

indomethacin tested was 2 mg/kg because previous studies in Sprague−Dawley rats had indicated substantial gastric erosion and bleeding at higher doses. CF_3—indomethacin was tested up to 10 mg/kg with no evidence of gastrointestinal bleeding. In fact, once testing was complete, the CF_3—indomethacin-treated animals were maintained overnight for further observation. The animals appeared healthy, and no passage of blood from the gastrointestinal tract was observed.

In conclusion, we report here the remarkable finding that replacement of the 2′-methyl group of indomethacin with trifluoromethyl completely eliminates COX-1 inhibitory activity while retaining potent, time-dependent inhibition of COX-2. The K_I for initial binding to COX-2 is approximately 9-fold lower for CF_3—indomethacin than for indomethacin, but the k_2 is 3-fold slower. Thus, the k_2/K_I for CF_3—indomethacin (0.013 s^{-1} μM) is approximately 3-fold greater than the k_2/K_I for indomethacin (0.0046 s^{-1} μM). CF_3—indomethacin inhibits COX-2 oxygenation of AA in cultured human head-and-neck (1483) cancer cells and is equipotent to indomethacin as an inhibitor of edema in the rat footpad model of inflammation. Given its comparable effects to indomethacin on COX-2 combined with its inability to inhibit COX-1, CF_3—

indomethacin may represent an attractive substitute for indomethacin for the relief of pain, prevention of tumor growth, or cessation of premature labor in individuals highly sensitive to the gastrointestinal and antiplatelet effects of indomethacin.

■ ASSOCIATED CONTENT

ⓈSupporting Information

Experimental procedures and spectral data for CF₃−indomethacin. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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■ ABBREVIATIONS

indomethacin, 2-(1-(4-chlorobenzoyl)-5-methoxy-2′-methyl-1H-indol-3-yl)ethanoic acid; CF₃−indomethacin, 2-(1-(4-chlorobenzoyl)-5-methoxy-2′-trifluoromethyl-1H-indol-3-yl)-ethanoic acid; COX, cyclooxygenase; AA, arachidonic acid; NSAID, nonsteroidal anti-inflammatory drug

■ REFERENCES

(1) Vane, J. R.; Botting, R. M. Mechanism of Action of Anti-Inflammatory Drugs. *Scand. J. Rheumatol.* **1996**, *25* (Suppl. 102), 9−21.

(2) Smith, W. L.; DeWitt, D. L.; Garavito, R. M. Cyclooxygenases: Structural, Cellular, and Molecular Biology. *Annu. Rev. Biochem.* **2000**, *69*, 145−182.

(3) Marnett, L. J. The Coxib Experience: A Look in the Rear-View Mirror. *Annu. Rev. Pharmacol. Toxicol.* **2009**, *49*, 265−290.

(4) Rome, L. H.; Lands, W. E. M. Structural Requirements for Time-Dependent Inhibition of Prostaglandin Biosynthesis by Anti-Inflammatory Drugs. *Proc. Natl. Acad. Sci.U.S.A.* **1975**, *72*, 4863−4865.

(5) Kulmacz, R. J.; Lands, W. E. M. Stoichiometry and Kinetics of the Interaction of Prostaglandin H Synthase with Anti Inflammatory Agents. *J. Biol. Chem.* **1985**, *260*, 12572−12578.

(6) Laneuville, O.; Breuer, D. K.; DeWitt, D. L.; Hla, T.; Funk, C. D.; Smith, W. L. Differential Inhibition of Human Prostaglandin Endoperoxide H Synthases-1 and -2 by Nonsteroidal Anti-Inflammatory Drugs. *J. Pharmacol. Exp. Ther.* **1994**, *271*, 927−934.

(7) Kalgutkar, A. S.; Crews, B. C.; Rowlinson, S. W.; Marnett, A. B.; Kozak, K. R.; Remmel, R. P.; Marnett, L. J. Biochemically Based Design of Cyclooxygenase-2 (Cox-2) Inhibitors: Facile Conversion of Nonsteroidal Antiiflammatory Drugs to Potent and Highly Selective Cox-2 Inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 925−930.

(8) Macones, G. A.; Marder, S. J.; Clothier, B.; Stamilio, D. M. The Controversy Surrounding Indomethacin for Tocolysis. *Am. J. Obstet. Gynecol.* **2001**, *184*, 264−722.

(9) Lundholm, K.; Gelin, J.; Hyltander, A.; Lonnroth, C.; Sandstrom, R.; Svaninger, G.; Korner, U.; Gulich, M.; Karrefors, I.; Norli, B.; Hafstrom, L. O.; Kewenter, J.; Olbe, L.; Lundell, L. Anti-Inflammatory Treatment May Prolong Survival in Undernourished Patients with Solid Tumors. *Cancer Res.* **1994**, *54*, 5602−5606.

(10) Simon, L. S. Nonsteroidal Anti-Inflammatory Drug Toxicity. *Curr. Opin. Rheumatol.* **1993**, *5*, 265−275.

(11) Buchanan, G. R.; Martin, V.; Levine, P. H.; Scoon, K.; Handin, R. I. The Effects of "Anti-Platelet" Drugs on Bleeding Time and Platelet Aggregation in Normal Human Subjects. *Am. J. Clin. Pathol.* **1977**, *68*, 355−359.

(12) Black, W. C.; Bayly, C.; Belley, M.; Chan, C.-C.; Charleson, S.; Denis, D.; Gauthier, J. Y.; Gordon, R.; Guay, D.; Kargman, S.; Lau, C. K.; Leblanc, Y.; Mancini, J.; Ouellet, M.; Percival, D.; Roy, P.; Skorey, K.; Tagari, P.; Vickers, P.; Wong, E.; Xu, L.; Prasit, P. From Indomethacin to a Selective Cox-2 Inhibitor: Development of Indolalkanoic Acids as Potent and Selective Cyclooxygenase-2 Inhibitors. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 725−730.

(13) Kalgutkar, A. S.; Marnett, A. B.; Crews, B. C.; Remmel, R. P.; Marnett, L. J. Ester and Amide Derivatives of the Nonsteroidal Antiinflammatory Drug, Indomethacin, As Selective Cyclooxygenase-2 Inhibitors. *J. Med. Chem.* **2000**, *43*, 2860−2870.

(14) Uddin, M. J.; Crews, B. C.; Blobaum, A. L.; Kingsley, P. J.; Gorden, D. L.; McIntyre, J. O.; Matrisian, L. M.; Subbaramaiah, K.; Dannenberg, A. J.; Piston, D. W.; Marnett, L. J. Selective Visualization of Cyclooxygenase-2 in Inflammation and Cancer by Targeted Fluorescent Imaging Agents. *Cancer Res.* **2010**, *70*, 3618−3627.

(15) Prusakiewicz, J. J.; Felts, A. S.; Mackenzie, B. S.; Marnett, L. J. Molecular Basis of the Time-Dependent Inhibition of Cyclooxygenases by Indomethacin. *Biochemistry* **2004**, *43*, 15439−15445.

(16) Kurumbail, R. G.; Stevens, A. M.; Gierse, J. K.; McDonald, J. J.; Stegeman, R. A.; Pak, J. Y.; Gildehaus, D.; Miyashiro, J. M.; Penning, T. D.; Seibert, K.; Isakson, P. C.; Stallings, W. C. Structural Basis for Selective Inhibition of Cyclooxygenase-2 by Anti-Inflammatory Agents. *Nature* **1996**, *384*, 644−648.

(17) Rowlinson, S. W.; Kiefer, J. R.; Prusakiewicz, J. J.; Pawlitz, J. L.; Kozak, K. R.; Kalgutkar, A. S.; Stallings, W. C.; Kurumbail, R. G.; Marnett, L. J. A Novel Mechanism of Cyclooxygenase-2 Inhibition Involving Interactions with Ser-530 and Tyr-385. *J. Biol. Chem.* **2003**, *278*, 45763−45769.

(18) Clark, K.; Kulathila, R.; Koehn, J.; Rieffel, S.; Strauss, A.; Hu, S.; Kalfoglou, M.; Szeto, D.; Lasala, D.; Sabio, M.; Wang, X.; Marshall, P. Crystal Structure of the Lumiracoxib:Cyclooxygenase-2 Complex. *American Chemical Society: Book of Abstracts* **2004**, 22−26.

(19) Bhattacharyya, D. K.; Lecomte, M.; Rieke, C. J.; Garavito, R. M.; Smith, W. L. Involvement of Arginine 120, Glutamate 524, and Tyrosine 355 in the Binding of Arachidonate and 2-Phenylpropionic Acid Inhibitors to the Cyclooxygenase Active Site of Ovine Prostaglandin Endoperoxide H Synthase-1. *J. Biol. Chem.* **1996**, *271*, 2179−2184.

(20) Greig, G. M.; Francis, D. A.; Falgueyret, J. P.; Ouellet, M.; Percival, M. D.; Roy, P.; Bayly, C.; Mancini, J. A.; O'Neill, G. P. The Interaction of Arginine 106 of Human Prostaglandin G/H Synthase-2 with Inhibitors Is Not a Universal Component of Inhibition Mediated by Nonsteroidal Anti-Inflammatory Drugs. *Mol. Pharmacol.* **1997**, *52*, 829−38.

(21) Luong, C.; Miller, A.; Barnett, J.; Chow, J.; Ramesha, C.; Browner, M. F. Flexibility of the Nsaid Binding Site in the Structure of Human Cyclooxygenase-2. *Nat. Struct. Biol.* **1996**, *3*, 927−933.

(22) Guo, Q.; Wang, L.-H.; Ruan, K.-H.; Kulmacz, R. J. Role of Val509 in Time-Dependent Inhibition of Human Prostaglandin H Synthase-2 Cyclooxygenase Activity by Isoform-Selective Agents. *J. Biol. Chem.* **1996**, *271*, 19134−19139.

(23) Gierse, J. K.; McDonald, J. J.; Hauser, S. D.; Rangwala, S. H.; Koboldt, C. M.; Seibert, K. A Single Amino Acid Difference between Cyclooxygenase-1 (Cox-1) and-2 (Cox-2) Reverses the Selectivity of Cox-2 Specific Inhibitors. *J. Biol. Chem.* **1996**, *271*, 15810−15814.