THE 2'- TRIFLUOROMETHYL ANALOG OF INDOMETHACIN IS A POTENT AND SELECTIVE COX-2 INHIBITOR*

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SUPPORTING INFORMATION

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Experimental Procedures

Reagents and Solvents

Indomethacin was purchased from Sigma (St. Louis, MO). The starting materials, ligands, and catalysts for all chemical reactions were commercially available and were purchased from Aldrich (Milwaukee, WI). HPLC grade solvents for chromatography were obtained from Fisher (Pittsburg, PA). 1-(4-methoxyphenyl)-1-(4-chlorobenzoyl)hydrazide hydrochloride was synthesized as previously described.[1] TLC was performed on silica plates (Silica Gel 60 F_{254} precoated) obtained from Analtech (Newark, DE). The plates were analyzed by UV fluorescence (254 nm) or by staining with phosphomolybdic acid followed by heating. Silica gel column chromatography was performed using Sorbent silica gel standard grade, porosity 60Å, particle size 32-63 μm (230 x 450 mesh), surface area 500 – 600 m^2/g, bulk density 0.4 g/mL, pH range 6.5 – 7.5, purchased from Sorbent Technologies (Atlanta, GA). Column chromatography was performed using a Biotage SP1 purification system (Uppsala, Sweden). For COX inhibition studies, [1-^{14}C]-arachidonic acid was purchased from PerkinElmer Life Sciences (Boston, MA).

Synthesis of CF_3-indomethacin

Diethyl trifluoroacetosuccinate. Ethyl chloroacetate (6.2 g, 0.05 mole) was added dropwise to a mixture of KOH (2.8 g, 0.05 mole), ethyl 4,4,4-trifluoroacetoacetate (9.2 g, 0.05 mole), and benzyltriethylammonium chloride (0.093 g, 0.0005 mole). The reaction mixture was stirred for 2 h at 45 °C, cooled to room temperature, acidified with dilute HCl, and extracted thrice with ether. The combined organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to afford the crude product, which was purified by distillation at 145-155°C (15 mmHg). This afforded the pure product as a colorless oil (10 g, 80%). ^{1}H NMR (DMSO-d_6) δ 1.17-1.22 (m, 6H), 4.05-4.17 (m, 4H), 4.35 (s, 2H), 5.68 (s, 1H, enol). Mass (ESI): $(M-H)^-$ 269.16.

5,5,5-Trifluorolevulinic acid. A 40% H_2SO_4 solution (5 mL) was added to diethyl trifluoroacetosuccinate (2.0 g). The reaction mixture was refluxed for 5 h and distilled under reduced pressure (140-145 °C, 15 mmHg) to afford 5,5,5-trifluorolevulinic acid (0.6 g, 46%). After cooling, the compound solidified to a pale yellow semi-solid. ^{1}H NMR ($CDCl_3$) δ 2.34-1.38 (m, 2H), 2.62-2.65 (m, 2H). Mass (ESI): $(M-H)^-$ 169.13.

5,5,5-Trifluorolevulinic acid lactone. Conc. H_2SO_4 (5 mL) was added to 5,5,5-trifluorolevulinic acid (0.5 g). The reaction mixture was refluxed for 2 h and distilled under reduced pressure (180-185°C, 15 mmHg) to afford the lactone (0.08 g, 10%) as a colorless heavy oil. ^{1}H NMR ($CDCl_3$) δ 3.16-3.21 (m, 2H), 5.11-5.18 (m, 1H). Mass (ESI): $(M+H)^+$ 153.07.

CF_3-indomethacin. 5,5,5-Trifluoromethyl levulinic acid lactone (0.05 g, 0.89 mmol) and 1-(4-methoxyphenyl)-1-(4-chlorobenzoyl)hydrazide hydrochloride (0.25 g, 0.80 mmol) were dissolved in glacial acetic acid (5 mL) and stirred for 1 h at 40 °C and 16 h at 90 °C. After cooling to room temperature, water (2 mL) and CH_2Cl_2 (2 mL) were added, and the organic phase was separated. The aqueous phase was extracted with an additional portion of CH_2Cl_2, and the combined layers were washed with water and

saturated $NaHCO_3$ (2 x 10 mL). The combined aqueous extracts were acidified with 15% HCl, and the resulting mixture was extracted with CH_2Cl_2 (3 x 10 mL). The combined layers were dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography using $CHCl_3$: MeOH : NH_4OH (35 : 7 : 1), which afforded CF_3-indomethacin as a white solid (0.09 mg, 20 %). ^{1}H NMR (DMSO-d_6) δ 3.76 (s, 3H), 3.77 (s, 2H), 6.52 (d, J = 9.2 Hz, 1H), 6.88 (dd, J = 2.3, 9.2 Hz, 1H), 7.30 (d, J = 2.3 Hz, 1H), 7.69 (d, J = 8.5 Hz, 2H), 7.80 (d, J = 8.5 Hz, 2H). Mass (ESI): $(M-H)^-$ 410.12.

Instrumental Analysis

^{1}H NMR spectra were obtained on a Bruker AV-I console operating at 400.13 MHz. ^{1}H COSY experiments were executed using a 9.4 T Oxford magnet equipped with a Bruker AV-I console operating at 400.13 MHz. Experimental conditions included 2048 x 512 data matrix, 13 ppm sweep width, recycle delay of 1.5 sec, and 4 scans per increment. The data were processed using a squared sine-bell window function, symmetrized, and displayed in magnitude mode. ^{13}C direct detection, HSQC, and HMBC NMR experiments were conducted using an 11.7 T Oxford magnet equipped with a Bruker DRX console operating at 500.13 MHz. Multiplicity-edited HSQC spectra were acquired using a 2048 x 256 data matrix, a J(C-H) value of 145 Hz resulting in a multiplicity selection delay of 34 msec, a recycle delay of 1.5 sec, and 16 scans per increment along with GARP decoupling on ^{13}C during the acquisition time (150 ms). The data were processed using a p/2 shifted squared sine window function and displayed with CH/CH_3 signals phased positive and CH_2 signals phased negative. J_1(C-H) filtered HMBC experiments were acquired using a 2048 x 256 data matrix, a J(C-H) value of 9 Hz for detection of long range couplings resulting in an evolution delay of 55ms, a J_1(C-H) filter delay of 145 Hz (34 ms) for the suppression of one-bond couplings, a recycle delay of 1.5 sec, and 128 scans per increment. The HMBC data were processed using a p/2 shifted squared sine window function and displayed in magnitude mode. Mass spectrometry was performed on a ThermoElectron Surveyor pump and autosampler operated in-line with a Quantum triple quadrupole instrument in ESI positive or negative ion mode.

Enzymes

The expression and purification of murine COX-2 (mCOX-2) and human COX-2 (hCOX-2) from insect cells and of ovine COX-1 (oCOX-1) from ram seminal vesicles were performed according to published methods.[2, 3] Site-directed mutagenesis on mCOX-2 to generate the various active site mutants (V349A, V349I, V349L, S530A, R120A, R120Q, Y355F, V523I, and L472M) was performed as described.[2] All activity or inhibition studies were performed in 100 mM Tris-HCl buffer containing 500 μM phenol. Inhibitors were dissolved in dimethyl sulfoxide. Reaction mixtures contained hematin-reconstituted proteins at final enzyme concentrations adjusted to give approximately 30-35% substrate consumption (hCOX-2 = 94 nM, mCOX-2 = 77 nM, oCOX-1 = 22.5 nM, V349A = 250 nM, V349I = 268 nM, V349L = 113 nM, S530A = 165 nM, R120A = 100 nM, R120Q = 106 nM, Y355F = 43 nM, V523I = 64 nM, and L472M = 85 nM).

COX Inhibition Screening Assay

Concentration-dependent inhibition reactions were performed by pre-incubating the inhibitor and enzyme for 17 min at 25 ^{o}C, followed by 3 min at 37 ^{o}C prior to the addition of 50 μM [1-^{14}C]-AA (~55 mCi/mmol, Perkin Elmer, Waltham, MA) for 30 sec at 37 ^{o}C. Reactions were terminated, and the resulting mixtures were analyzed for substrate consumption by thin-layer chromatography as previously described.[4] Inhibitor concentrations required for 50% inhibition of enzyme activity (IC_{50}) were determined graphically using Prism (GraphPad Software, La Jolla, CA) and were the average of at least two independent determinations.

Time-Dependent COX Inhibition Assays

Time-dependent inhibition assays were conducted by pre-incubating increasing concentrations of the inhibitor with m/hCOX-2, oCOX-1, or the active site mCOX-2 mutants for various time points (0, 0.125, 0.25, 0.5, 1, 3, 5, 15, 30 and 60 min) at 37 ^{o}C prior to the addition of 50 μM [1-^{14}C]-AA for 30 sec at 37 ^{o}C. Reactions were terminated and analyzed by thin layer chromatography as described above. The values of the kinetic parameters were the average of at least three independent determinations.

Inhibition of COX-2 Activity in Intact Cells

The inhibition of COX-2 activity in intact 1483 human head and neck squamous cell carcinoma (HNSCC) cells was determined as previously described.[5] Cells were pretreated with inhibitor dissolved in dimethyl sulfoxide (0 – 2.5 μM, final concentration) for 30 min at 37°C followed by the addition of [1-^{14}C]-arachidonic acid (10 μM) for 20 min at 37°C. The culture medium was extracted and analyzed by thin layer chromatography.

Carrageenan-Induced Inflammation in the Rat

The in vivo anti-inflammatory activity of CF_3-indomethacin was evaluated using the carrageenan-induced rat footpad edema model.[4] Sprague Dawley rats (125 – 150 g) received a single subplantar injection of 100 μL of a sterile 1% solution of carrageenan in saline followed, 1 h later, by oral administration of inhibitor or vehicle (corn oil). After 2 h, footpad volume was measured with a plethysmometer and compared with the preinjection volume of the same paw. Swelling (in mL) was then calculated, and percent inhibition was derived through comparison with the vehicle control group. These studies were carried out with the approval of the Vanderbilt Institutional Animal Care and Use Committee.

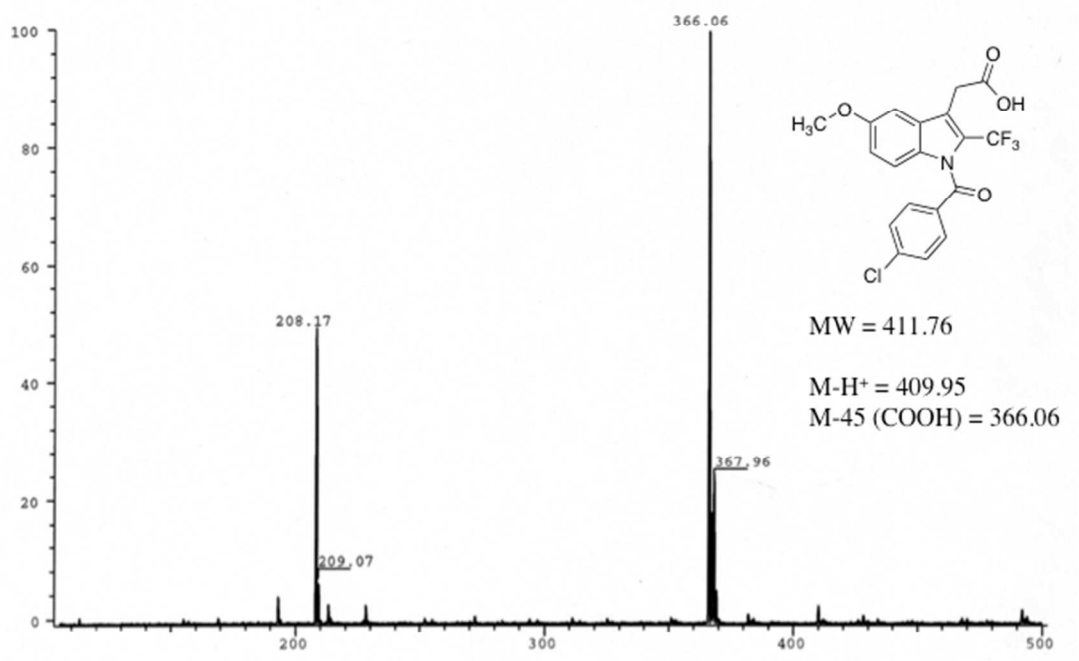

Figure S1 Mass spectral characterization of CF_3-indomethacin in negative ion mode. MW = 411.76, M-H^+ = 409.95, Decarboxylation (M-45) = 366.06.

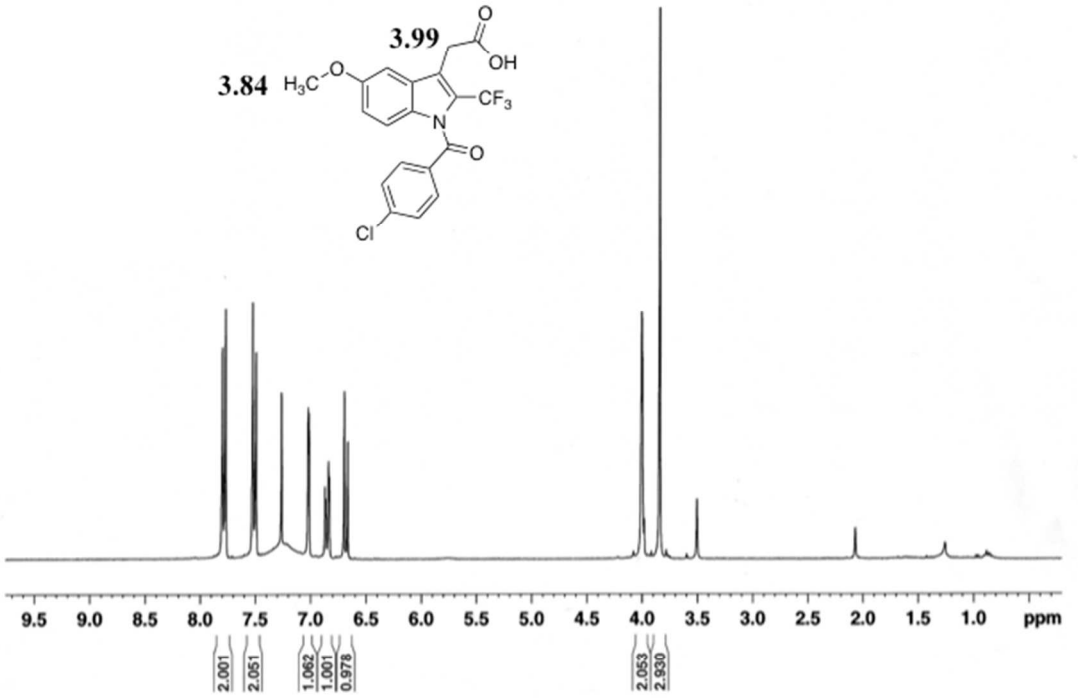

Figure S2 Proton NMR of CF_3-indomethacin in $CDCl_3$. δ 7.78 (d, *J* = 8.43 Hz, 2H), 7.51 (d, *J* = 8.43 Hz, 2H), 7.02, (d, *J* = 2.3 Hz, 1H), 6.85 (dd, *J* = 2.3, 9.2 Hz, 1H), 6.68 (d, *J* = 9.2 Hz, 1H), 3.99 (s, 2H), 3.84 (s, 3H); ESI 410 ($M\text{-}H^+$). Solvents: $CDCl_3$ (7.26 ppm), methanol (3.5ppm), ethylacetate (2.1).

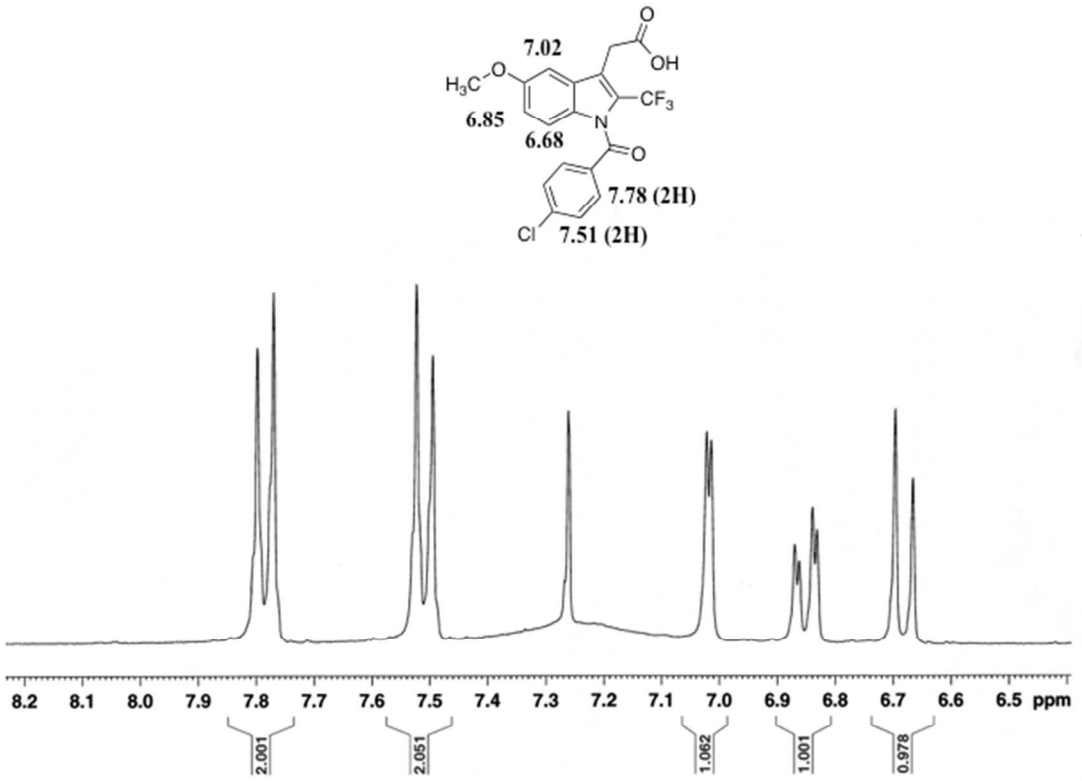

Figure S3 Proton NMR of CF_3-indomethacin - aromatic region. δ 7.78 (d, *J* = 8.43 Hz, 2H), 7.51 (d, *J* = 8.43 Hz, 2H), 7.02, (d, *J* = 2.3 Hz, 1H), 6.85 (dd, *J* = 2.3, 9.2 Hz, 1H), 6.68 (d, *J* = 9.2 Hz, 1H), 3.99 (s, 2H), 3.84 (s, 3H); ESI 410 ($M\text{-}H^+$). Solvents: $CDCl_3$ (7.26 ppm).

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