LETTER

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Desmethyl Derivatives of Indomethacin and Sulindac as Probes for Cyclooxygenase-Dependent Biology

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ABSTRACT Cyclooxygenases (COX) have been implicated in the etiology of a number of diseases, but defining the precise contribution of COXs to these diseases is challenging. Potent COX inhibitors exist, but they display off-target effects. 2′-Desmethyl derivatives of indomethacin and sulindac sulfide were synthesized that demonstrated reduced COX inhibitory activity but were inducers of peroxisome proliferator-activated receptor γ-dependent transcription, adipocyte differentiation, or apoptosis of colon cancer cell lines. 2′-Desmethylindomethacin demonstrated gastrointestinal toxicity lower than that of indomethacin in C57BL6 mice, highlighting the importance of COX activity in maintaining gastrointestinal homeostasis and establishing that COX inhibition contributes to gastrointestinal toxicity by nonsteroidal antiinflammatory drugs. These compounds serve as useful probes of COX-dependent biology and may represent leads for antidiabetic and anticancer drugs.

Cyclooxygenase (COX) enzymes play an important role in many complex physiological and pathophysiological responses, and COX inhibition is associated with beneficial pharmacological effects including the relief of inflammation, pain, and fever (*1, 2*). COX inhibitors comprise a variety of structural classes including arylacetic acids, arylpropionic acids, diarylheterocycles, anthranilates, and salicylates (*3*). These nonsteroidal antiinflammatory drugs (NSAIDs) are low molecular weight compounds (MW 200–350) with relatively simple functional groups. Consequently, they associate with many proteins in addition to COXs and exhibit a continuum of binding affinities. This can lead to a range of "off-target" effects that can be beneficial or deleterious. Evaluating the importance of COX inhibition in a given pharmacological response is extremely important for dissecting the components of complex signaling networks and for defining new strategies for treating diseases. In addition, definition of the role of COX-independent effects within a structural class of NSAIDs represents a strategy for new-drug development that builds on existing compounds with long histories of human use.

Indomethacin and sulindac sulfide are powerful, slow, tight-binding inhibitors of COX-1 and COX-2 (*4, 5*). Sulindac sulfide is the active metabolite of the pro-drug sulindac (Figure 1) (*6*). COX inhibition is a major factor in the antiinflammatory, analgesic, and antipyretic activities of both drugs (*2*). However, indomethacin and sulindac sulfide exert actions such as activation of peroxisome proliferator-activated receptor γ (PPARγ), inhibition of γ-secretase, induction of apoptosis, and induction of the tumor suppressor NAG-1, that may be unrelated to their ability to inhibit COX (*7–11*).

Our laboratory has had a long-standing interest in defining the molecular determinants of COX inhibition by different classes of NSAIDs (*12*). We recently described a critical interaction between indomethacin and COX enzymes that is a major determinant of its time-dependent inhibitory activity (*13*). The 2-methyl group of the indole ring inserts into a hydrophobic depression in the side of the COX active site, strengthening its association with the protein (Figure 1). Site-directed mutagenesis of residues bordering this hydrophobic depression alters the kinetics of indomethacin binding and modulates its inhibitory potency. Removal of the methyl group generates **1,** which exhibits

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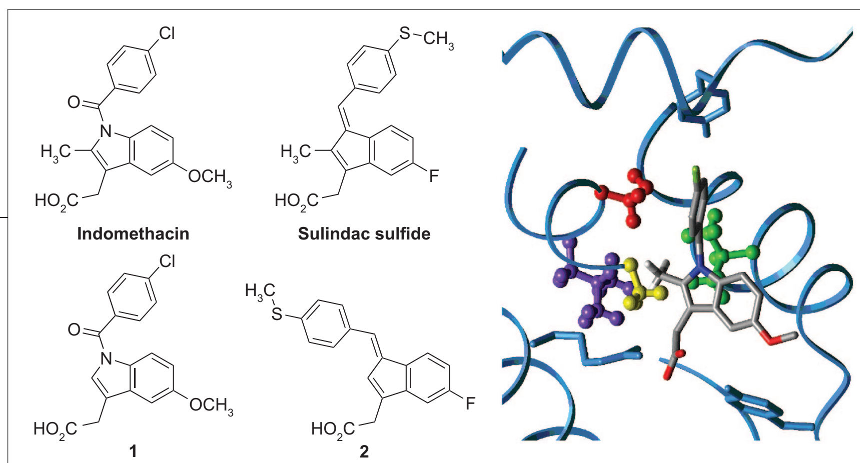


Figure 1. Structures of indomethacin, sulindac sulfide, 1, **and** 2. **The right panel shows the structure of the active site of COX-2 with indomethacin bound. The 2-methyl group on the indole ring inserts into a hydrophobic depression composed of Val349 (green), Ala527 (yellow), Ser530 (red), and Leu531 (violet).**

drastically reduced inhibitory potency against both COX enzymes (*13*). Since sulindac sulfide is an indomethacin analogue that contains a methyl group at the 2-position of the indene ring, we hypothesized that **1** and **2** would represent useful tools for defining the role of COX inhibition in complex biological responses.

Indomethacin and **1** were compared for their ability to activate the nuclear transcription factor PPARγ in the human colon cancer cell line HCA-7, which basally expresses this nuclear receptor (*14, 15*) Cells were transfected with an expression vector containing luciferase under the control of a PPRE. Addition of either compound triggered a concentration-dependent increase in luciferase activity (Figure 2, panel a). The concentration dependence of both compounds was comparable, although the magnitude of the response was slightly greater for indomethacin. Each compound induced the expression of a PPARγ-dependent gene, liver fatty acid binding protein (L-FABP) (*16*) (data not shown). Indomethacin and **1** also were compared for their toxicity to the human colon cancer cell line RKO. Viable cells were quantified by incubation with the dye WST-1, which is reduced by mitochondrial electron transport to a formazan that is quantified by absorbance at 405 nm (*17*). Figure 2, panel b demonstrates that both compounds exhibited similar concentration dependence for induction of cell death. These experiments indicate that the 2-methyl group of the indole ring of indomethacin, which is essential for COX inhibition, is not required for activation of PPARγ or for induction of tumor cell apoptosis.

Compound **2** was synthesized by a route that mimicked the synthesis of sulindac sulfide (Supplementary Scheme 1) (*18*). *p*-Fluorophenylpropionic acid was cyclized to the indanone then alkylated *via* a Reformatsky reaction. The tertiary alcohol was dehydrated, and the indene product was condensed with *p*-thiomethoxybenzaldehyde. The stereochemistry of the benzylidine double bond was established to be (*E*) by nuclear Overhauser effect spectroscopy. This contrasts with the (*Z*) stereochemistry of sulindac sulfide, indicating a key role for the 2-methyl group in controlling the stereochemistry of the elimination of water in the condensation reaction. As anticipated, **2** did not inhibit COX-1 or COX-2 at concentrations up to 250 μM.

Sulindac sulfide and **2** reduced the viability of RKO cells with similar dose–response curves (Supplementary Figure 1, panel a). The toxic response was greater at 48 h than at 24 h for both agents. This result not only indicates that **2** exhibits similar activity to sulindac sulfide but also establishes that the cytotoxic effect of sulindac sulfide toward colorectal carcinoma cells is unrelated to its ability to inhibit COX enzymes. The observed toxicity was due to apoptosis as judged by nuclear condensation, the induction of caspase-3 activity, and the cleavage of caspase-3 targets such as poly(ADP-ribose)polymerase (Supplementary Figure 1).

The effects of **2** also were assessed on PPARγ activation. Transfection of HCA-7 cells with a PPRE-luciferase construct followed by treatment with **2** led to robust induction of luciferase activity (Figure 3, panel a). The induction of luciferase reflected binding of **2** to PPARγ as demonstrated by a scintillation proximity assay in which **2** displaced [^{3}H]-troglitazone, a known PPARγ ligand (Figure 3, panel b) (*19, 20*) Treatment of HCA-7 cells with compound **2** led to the dose-dependent induction of L-FABP and another PPARγ target gene, aP2 (*21, 22*) (Figure 3, panel c). Induction of L-FABP and aP2 by **2** was suppressed by GW9662, a PPARγ antagonist (Figure 3, panel d) (*23*). We also evaluated the effects of troglitazone, indomethacin, **1**, sulindac sulfide, and **2** on adipogenesis in 3T3-L1 cells, a murine fibroblast cell line. As shown in Figure 3, panel e, staining with Oil Red O revealed formation of lipid droplets in cells treated with indomethacin, **1**, sulindac sulfide, and **2** in a manner similar to that of the known PPARγ activator troglitazone (*24*). Thus, **1** and **2** bind to PPARγ, activate transcription dependent on a PPRE, induce the expression of PPARγ target genes, and activate a complete program of PPARγ-dependent cellular differentiation. These experiments conclusively demonstrate that the ability of indomethacin and sulindac sulfide to activate PPARγ is not dependent on its COX inhibitory activity. Furthermore, these data establish that the ability of **1** and **2** (and by inference, indomethacin and sulindac sulfide) to activate PPARγ-dependent transcription is not indirect but depends entirely on their ability to bind to this nuclear transcription factor.

Sulindac sulfide and **2** were compared for their effects on leukotriene biosynthesis by resident peritoneal macrophages stimulated with zymosan (Supplementary Figure 2). Neither compound inhibited total metabolite production or 5-hydroxyeicosatetraenoic acid (5-HETE) synthesis. In fact, there was some stimulation of total metabolism at high concentrations of sulindac sul-

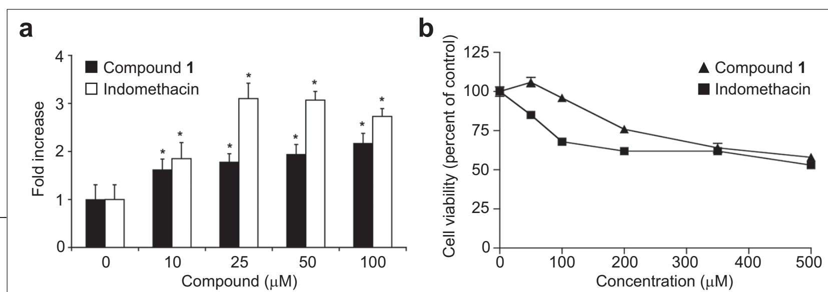


Figure 2. Compound 1 activates PPRE-dependent transcription and induces apoptosis. a) HCA7 cells were transfected with 1.8 μg of PPRE luciferase and 0.2 μg of pSVβgal. Cells were treated with 1 (solid bars) or indomethacin (open bars) for 12 h, and relative luciferase/βgal induction was quantified. Columns, means; bars, SD; $n = 6$; *, $p < 0.001$. The maximum fold induction observed by the PPARγ antagonist troglitazone was 3-fold in the same set of experiments. b) Dose–response for toxicity of indomethacin (solid squares) and 1 (solid triangles) in RKO cells.

fide or **2**. However, both compounds appeared to inhibit the transformation of 5-hydroperoxyeicosatetraenoic acid (5-HPETE) to leukotriene C_4 as judged by the decrease of the latter metabolite and the increase in 5-HETE synthesis. Thus, neither compound is a 5-lipoxygenase inhibitor but each exhibits an unprecedented ability to inhibit leukotriene production from the 5-lipoxygenase product 5-HPETE.

The fact that compounds **1** and **2** induce apoptosis and activate PPARγ-dependent transcription demonstrates that removal of the 2-methyl group on the indole or indene ring selectively eliminates COX inhibition from the biochemical properties of indomethacin and sulindac sulfide while leaving their collateral biochemical properties intact. Thus, both molecules should be broadly applicable probes of the role of COX inhibition in complex biological responses. To test this hypothesis, we compared the gastrointestinal toxicity of indomethacin and **1** in C57BL6 mice. COX inhibition is believed to be a component of this undesired side effect of NSAIDs, but other possible mechanisms, including injury due to direct physical contact with the lining of the stomach, have been suggested (*25*). Thus, controversy surrounds the role of COX inhibition in the gastrointestinal toxicity of NSAIDs in general and indomethacin in particular. C57BL6 mice are extremely sensitive to the toxic actions of indomethacin, so they represent a useful strain with which to test the utility of **1** as a probe for COX inhibition. Varying doses of indomethacin and **1** were administered once daily by intraperitoneal injection to male C57BL6 mice for 4 d. Intraperitoneal administration was chosen to eliminate potential physical toxicity to the stomach and intestines resulting from delivery by gavage. Sick or moribund animals were sacrificed, and their gastrointestinal tracts were removed for histological examination. Figure 4 compares stomach sections from an animal treated with 5 mg/kg indomethacin or compound **1**. The gastric mucosa in the indomethacin-treated mouse shows superficial hemorrhagic necrosis, with loss of glandular epithelium and little associated inflammation. The gastric antrum was more severely affected than the body of the stomach. In contrast, in the mouse treated with compound **1**, the gastric mucosa is histologically normal, with no ulcers or epithelial necrosis.

The apparent LD_{50}'s paralleled the dramatic histological differences displayed in Figure 4, 3.5 mg/kg for indomethacin and 70 mg/kg for compound **1**. To evaluate the possibility that reduced toxicity was due to differential metabolism, the plasma levels of indomethacin and **1** were quantified following a single intraperitoneal administration of 5 and 50 mg/kg, respectively. The plasma level of indomethacin at 5 mg/kg was 40 μM, whereas the plasma level of **1** at 50 mg/kg was 348 μM. The time courses of disappearance of both molecules were comparable, so it appears that the 20-fold difference in toxicity between indomethacin and **1** is not due to more rapid metabolic disposition of the latter. These results demonstrate that the toxicity of indomethacin is primarily attributable to COX inhibition.

These data indicate that a subtle structural modification, removal of the 2-methyl group from the indole or indene ring, selectively eliminates the COX inhibitory activity of indomethacin and sulindac sulfide while retaining activity at non-COX targets. This makes compounds **1** and **2** excellent tools for differentiating the contribution of COX enzymes to complex biological responses. Furthermore, the non-COX pharmacological effects exhibited by **1** and **2** may be directly translatable to the clinic. Both compounds are close structural analogues of compounds that have extensive human clinical histories, so they should exhibit bioavailability and pharmacokinetics similar to those of indomethacin and sulindac (*26, 27*). The subtle chemical modification that eliminated COX inhibitory activity should imbue them with reduced gastrointestinal and cardiovascular side effects relative to those of the parent drugs, thereby allowing higher doses to be administered. This eliminates the major hurdle that has prevented the use of indomethacin or sulindac as agents that act at non-COX targets. Potential indications for the desmethyl analogs include cancer prevention and therapy, treatment of diabetes, and treatment of Alzheimer's disease. Finally, the ability of **2** to bind to and activate PPARγ-dependent transcription indicates that the (*E*) geometry of the benzylidene ring is tolerated and may represent a structural element that can be exploited for further optimization of novel PPARγ activators.

METHODS

COX Inhibition Assay. Cyclooxygenase inhibition was determined as described previously (*13*).

Cell Viability Assay. RKO cells (ATCC) were cultured in 96-well plates in a final volume of 100 μL of culture medium with 10% FBS. Each well contained 2,000–4,000 cells per well and desired concentrations of chemicals. Cells were incubated in a humidified atmosphere for 24–48 h. To the cultures was added 10 μL of WST-1 reagent (Roche, Indianapolis, IN), and the mixtures were incubated for an additional 1–3 h. The absorbance of samples was determined using a microtiter plate reader at a wavelength of 450 nm against a background control; the reference wavelength was 690 nm.

Hoechst Staining and Apoptotic Cells Counting. Following treatment with drugs, cells in 6-well plates were centrifuged for 5 min at 300*g* to pellet apoptotic cells and then prefixed with 2 drops of glacial acetic acid and methanol (1:3, v/v) for 2 min. After the mixture was aspirated, cells were

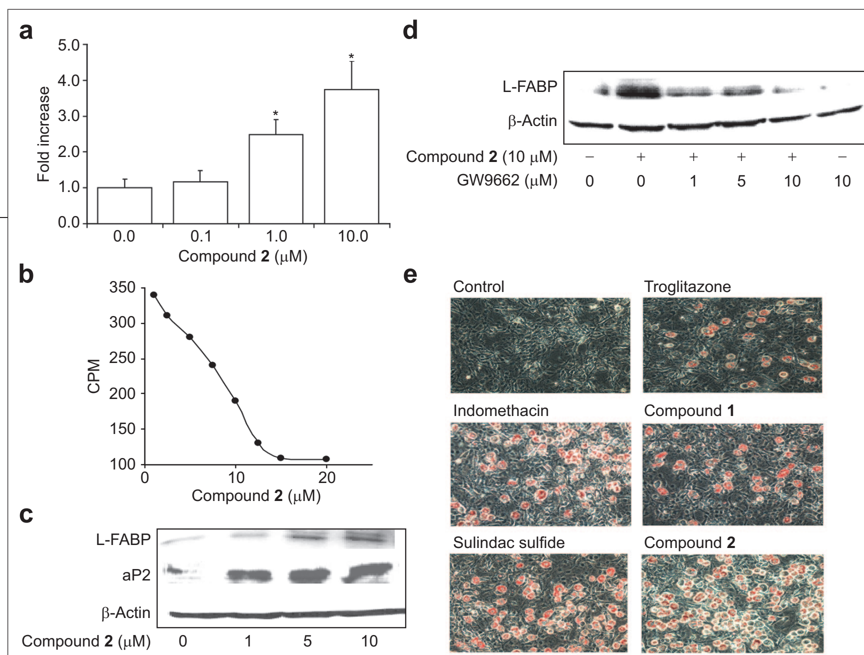


Figure 3. Compound 2 is a PPARγ agonist. a) HCA7 cells were transfected with 1.8 μg of PPRE luciferase and 0.2 μg of pSVβgal. Cells were treated under serum-free conditions with 0–10 μM 2 for 12 h. Columns, means; bars, SD; $n = 6$; *, $p < 0.001$. The maximum fold induction observed by the PPARγ antagonist troglitazone was 4-fold in the same set of experiments. b) Compound 2 binds to PPARγ. Competitive binding assays were performed by SPA for human PPARγ-ligand binding domain and 50 nM ^{3}H-troglitazone in the presence of increasing concentrations of nonradioactive 2 as a competitor. c) Compound 2 binds to PPARγ (*27*). HCA7 cells were treated in serum-free medium with 0–10 μM 2 for 24 h. Immunoblots were probed with antibodies to L-FABP, aP2, and β-actin. d) GW9662, a PPARγ antagonist, suppresses compound 2-mediated induction of L-FABP and aP2. Cells were treated as indicated with vehicle, 2 or GW9662 alone, or 2 and GW9662 for 24 h. e) Compound 2 induces adipogenesis. Differentiation of 3T3-L1 cells was induced by the indicated compounds under serum-free conditions. Adipogenesis was indicated by Oil Red O stained lipid droplets (200×).

fixed twice in the acetic acid–methanol solution for 5 min and stained with Hoechst solution (0.1 μg/mL in PBS) followed by several washes with deionized water to remove the excess Hoechst. Apoptotic cells (heavily stained cells with rounded and fragmented nuclei) were visualized under a fluorescence microscope. For quantitative determination, 3–5 fields of cells were randomly chosen and counted. The total counted cells (apoptotic and non-apoptotic) were at ≥300.

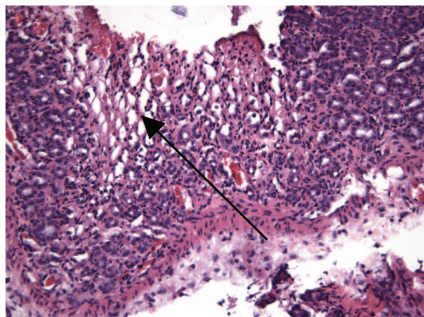
Transient Transfection Assay. The HCA7 human colon cancer cell line was established from a moderately differentiated adenocarcinoma of the colon (*14*). The cell line was maintained in DMEM supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% FBS. Cells were grown to 40% confluence in 6-well dishes. For each well, 2 μg of plasmid DNA (1.8 μg of PPRE-luciferase and 0.2 μg of pSVβgal) was introduced into cells using 6 μg of Lipofectamine 2000 as per the manufacturer's instructions. After 6 h of incubation, the medium was replaced with growth medium for 16 h, followed by serum-free medium containing the compounds for 12 h. The activities of luciferase and β-galactosidase were measured.

SPA. The assay was performed as described previously (*19*). The PPARγ ligand binding domain was isolated from *E. coli* as a polyhistidine-tagged fusion protein. Radiolabeled troglitazone was synthesized as described previously (*20*). The buffer for all assays was 50 mM HEPES (pH 7), 50 mM KCl, 5 mM CHAPS, 0.1 mg/mg BSA. The protein was biotinylated, and immobilized on streptavidin-modified SPA beads. Nonradioactive **2** was used to compete for binding to the PPARγ ligand binding domain using ^{3}H-troglitazone as the ligand. The assays were performed in the absence of dithiothreitol.

Adipogenesis Assay. 3T3-L1 cells (ATCC) were grown at 37 °C in 5% CO_2 in DMEM supplemented with 10% CBS to 60% confluence. The cells were then placed in growth medium supplemented with 0.1% 3-isobutyl-1-methylxanthine and 1.0% dexamethasone to initiate adipogenesis for 48 h. The cells were then treated in growth medium containing 10 μg/mL recombinant human insulin supplemented with the test compound for an additional 2 days. The medium was then replaced with fresh growth medium for an additional 48 h prior to staining with Oil Red O. Images were taken using Q-Imaging Retina EX camera and Olympus IX51 bright field microscope (200×).

***In Vivo* Evaluation.** C57BL6 male mice (20 g) were treated with 100 μL of vehicle (DMSO), indomethacin, or **1** by intraperitoneal injections for 4 consecutive days. The dosages of indomethacin were 1, 2.5, or 5 mg/kg, and the dosages of **1** were 1, 10, 25, 50, or 100 mg/kg. Animals were monitored closely and were sacrificed by CO_2 asphyxiation when they appeared moribund. There were 6 mice per group; the experiment was performed in duplicate. The gastrointestinal tracts were removed from representative animals, fixed in formalin, and subjected to histological evaluation. All procedures were approved by the Vanderbilt IACUC.

Determination of Plasma Levels. Indomethacin and **1** in mouse plasma were determined by HPLC–UV analysis after sample clean-up *via* solid-phase extraction. Plasma samples were stored at −20 °C, thawed, and aliquoted (100 μL) into 13 mm ×; 100 mm test tubes. Each sample was spiked with 10 nmol of internal standard (**1** served as the internal standard for indomethacin analyses and vice versa) then diluted with 900 μL of 0.5% aqueous acetic acid solution. The dilute sample was loaded onto a 1 cc OASIS HLB solid-phase extraction cartridge (Waters Corp., Milford, MA), which was preconditioned with 1 mL of methanol followed by 1 mL of 0.5% aqueous acetic acid. The cartridge was washed with 1 mL of 0.5% aqueous acetic acid followed by 1 mL of 0.5% aqueous acetic acid with 40% methanol. Air was drawn through the cartridge for 1 min. Fi-



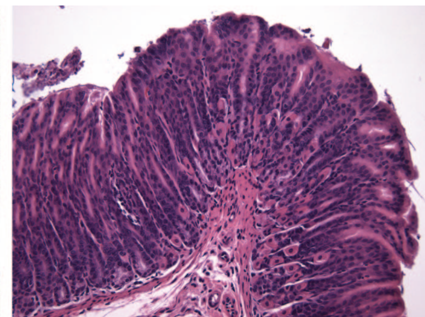


Figure 4. Comparison of gastrointestinal toxicity of indomethacin and 1. Indomethacin or 1 (5 mg/kg) was injected intraperitoneally in 100 μL of DMSO once daily for 4 d. Animals were monitored closely for hypothermia or lethargy and were sacrificed if they became moribund. All animals were sacrificed after 4 days, and their stomachs were removed for histopathological evaluation. The left frame represents hematoxylin- and eosin-stained slides of a section from an indomethacin-treated animal, and the right frame is from an animal treated with compound 1. The section from the latter is identical to a tissue section from a control animal and represents healthy tissue. The arrow in the left frame points to tissue erosion.

nally, the cartridge was washed with 1 mL of hexanes, dried for 2 min with air, and eluted with 1.6 mL of acetonitrile. The eluent was dried under N_2, reconstituted in 100 μL of acetonitrile plus 100 μL of water, and injected on the Waters 2695 separations module. Peaks were separated isocratically in reverse-phase mode using a Phenomenex Synergi Max-RP column (7.5 mm ×; 0.2 mm) held at 40 °C. The mobile phase was 1:1 A:B at a flow of 0.3 mL/min where A = H_2O with 0.1% acetic acid and B = acetonitrile with 0.1% acetic acid. Chromatograms were collected at a wavelength of 318 nm. Indomethacin and **1** were quantified against a standard curve. Standard samples were prepared by spiking blank mouse plasma (Pel-Freez, Carlsbad, CA) with indomethacin or **1** and then subjecting the samples to the clean-up procedure described above, alongside samples to be quantified. Analyte response (analyte peak area/internal standard peak area) was plotted against nanomoles/sample to generate a linear standard curve. For both indomethacin and **1** standard curves, r^2 value was >0.999.

Statistics. Comparisons between groups were made with Student's *t* test. A difference of $p < 0.05$ was considered significant.

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Supporting Information Available: This material is available free of charge *via* the Internet.

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