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

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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, diaryl heterocycles, 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
drastically reduced inhibitory potency against both COX enzymes (19). 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 transcription dependent on 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 [3H]-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-
was chosen to eliminate potential physical mice for 4 d. Intraperitoneal administration of 5-hydroperoxyeicosatetraenoic acid (5-HPETE) to leukotriene C4 as judged by the 5-lipoxygenase product 5-HPETE.

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 pSVgαl. Cells were treated with 1 (solid bars) or indomethacin (open bars) for 12 h, and relative luciferase/gαl 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.

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 compatible, 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 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 (S) 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
fixed twice in the acetic acid–methanol 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 randomized 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 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 12 h, followed by serum-free medium containing the compounds for 12 h. The assays were performed in the absence of dithiothreitol.

Adipogenesis Assay. HCA7 cells (ATCC) were grown at 37 °C in 5% CO₂ in DMEM 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 a Leica DM IRB fluorescence 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 CO₂ 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-
nally, the cartridge was washed with 1 mL of hexane, dried for 2 min with air, and eluted with 1.6 mL of acetonitrile. The eluent was dried under N₂, reconstituted in 100 µL of acetonitrile plus 100 µL of water, and injected onto the Waters 2695 separations module. Peaks were separated isotropically in reverse-phase mode using a Phenomenex Synergi Max-RP column (7.5 µm × 0.2 mm) held at 40 °C. The mobile phase was 0.3 mL/min where A = H₂O with 0.1% acetic acid and B = acetonitrile with 0.1% acetic acid. Chromatograms were collected at a wavelength of 214 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, r² 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.

**REFERENCES**


