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Synthesis and Biological Evaluation of Orally Active Prodrugs of Indomethacin

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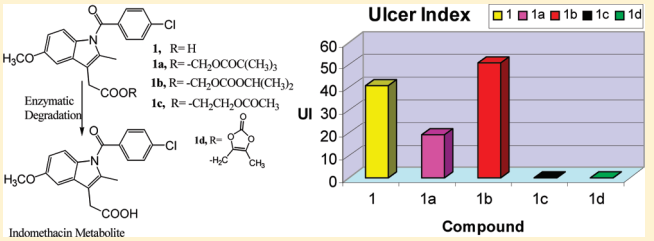
Supporting Information

ABSTRACT: Synthesis and biological evaluation of orally active prodrugs (**1a**−**d**) of indomethacin are described. Prodrugs **1a**−**c** showed a similar degree of anti-inflammatory activity, and prodrug **1d** was found to be less potent than the parent drug indomethacin (**1**). Ulcer index (UI) data indicated that **1a** (UI = 19), **1c** (UI = 0), and **1d** (UI = 0) were substantially less ulcerogenic and **1b** (UI = 62) was more ulcerogenic than parent drug **1** (UI = 47). These prodrugs demonstrated good stability at acidic and basic pH and found to be more lipophilic than parent drug compound **1**, indicated by partition coefficients measured in octanol−buffer system at pH 7.4 and 3.0. On the basis of in vivo studies, **1a** and **1c** compounds were selected for metabolic stability in rat liver microsome (RLM) and rat plasma (RP), and both were found to be enzymatically labile. Prodrugs **1a** and **1c** emerged as potent anti-inflammatory agents with a lesser potential for ulcer than the parent drug indomethacin.



INTRODUCTION

Indomethacin (**1**, Figure 1) is a well-known nonsteroidal anti-inflammatory drug (NSAID) and is effective against severe rheumatoid arthritis, ankylosing spondylitis, osteoarthritis of large joints, and other inflammations.[1−5] The beneficial effect is associated with inhibition of cyclooxygenases (COX) that convert arachidonic acid into prostaglandins in inflammatory processes.[6] The major limitation of long-term therapeutic use of NSAIDs (COX-1 inhibitors) is their gastrotoxicity. These side effects produced by NSAIDs are believed to involve two different mechanisms: inhibition of prostaglandin synthesis in the stomach responsible for inducing mucus production and a local action exerted by direct contact of the drugs with gastric mucosa due to the acidic nature of the NSAIDs.[7] The most common effects associated with NSAID therapy are upper GI irritation, ulceration, dyspepsia, bleeding, and in some cases death.[8] To overcome the GI ulceration side effect of drug, more COX-2 selective inhibitor NSAID is preferred, which does not significantly inhibit cyclooxygenase in the stomach and appears to be less likely to cause GI ulceration.[9] Unfortunately very effective COX-2 selective inhibitor drugs, i.e., rofecoxib and celecoxib, were withdrawn from the market because of the increased risk of heart attack and stroke associated with long-term, high-dosage use. Hence considerable attention has been focused on the development of bioreversible derivatives, such as prodrugs, to temporarily mask the acidic group of NSAIDs as a promising means of reducing or abolishing the GI toxicity due to the local action mechanism.

The prodrug approach afforded compounds with better anti-inflammatory activity, differentiated pharmacokinetic profile, and reduced gastric ulcerogenic activity.[10−14] Prodrugs are pharmacologically inactive derivatives of active agents, which undergo chemical and/or enzymatic biotransformation, resulting in the release of active drug after administration. The metabolic product (i.e., parent drug) subsequently elicits the desired pharmacological response.[15,16] Most prodrugs of NSAIDs have been prepared by derivatization of the carboxylic group. The esters have dominated prodrug research because they have the ideal characteristic of exhibiting reasonable in vitro chemical stability which allows them to be formulated with adequate shelf lives. In addition, by virtue of their ability to function as esterase substrates, esters are suitably labile in vivo.[17,18] By use of the prodrug approach, one strategy that could be useful is to temporarily mask the carboxylic acid function of the NSAIDs so that the prodrug hydrolyzes in vivo to release the active parent NSAID.[19−21]

The present work was initiated with the aim to develop prodrugs of indomethacin (**1**), possessing a high enzymatic bioconversion rate, favorable physicochemical properties, and fewer ulcerogenic properties. Thus, indomethacin ester prodrugs

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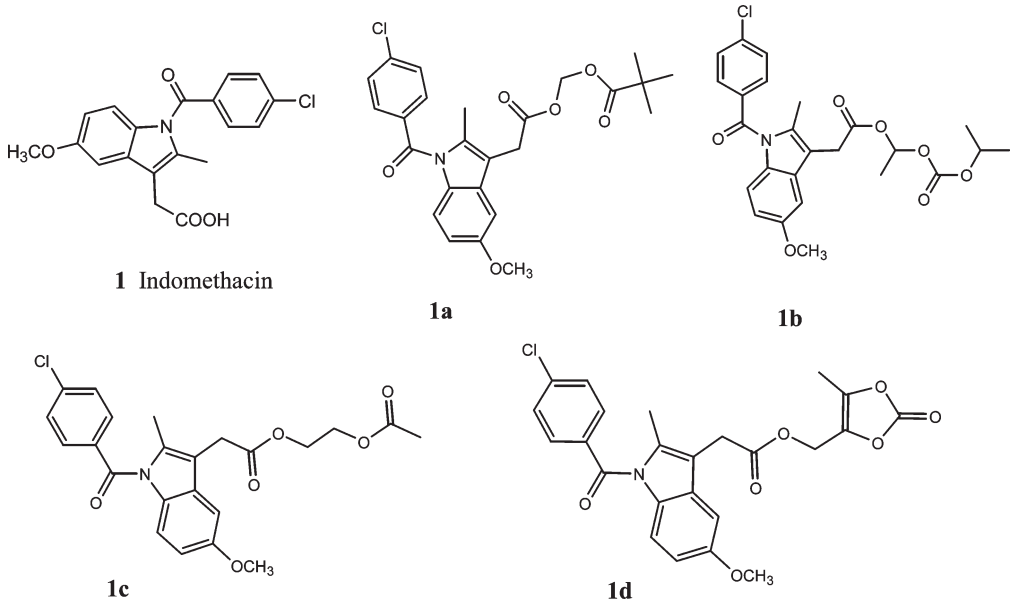

Figure 1. Chemical structures of indomethacin (**1**) and prodrugs **1a**−**d**.

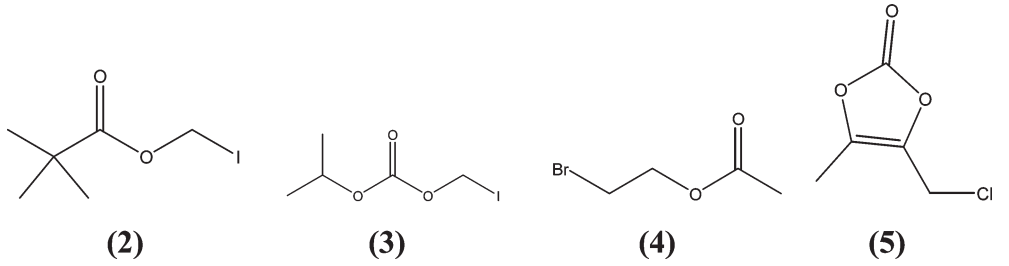

Figure 2. Chemical structures of 1-iodomethyl pivalate (**2**), 1-iodomethylisopropyl carbonate (**3**), 2-bromoethyl acetate (**4**), and 4-chloromethyl-5-methyl-1,3-dioxol-2-one (**5**).

(**1a**−**d**) were prepared and evaluated for their potential use as prodrug for oral delivery. The physicochemical properties of a drug play a major role in the development of formulation and bioavaibility. Thus, in addition to characterization of the proposed structures, physicochemical parameters like the partition coefficient (log *P*), aqueous solubility, aqueous stability, in vitro metabolic stability in rat liver microsomes and rat plasma, and ulcerogenicity (GI toxicity) were studied.

CHEMISTRY

A series of novel ester prodrugs (**1a**−**d**) were prepared as depicted in Scheme 2 in good to excellent yields (95.5%, 96.6%, 78%, and 81.24%) by condensation of **1** with the appropriate promoiety in the presence of organic base like TMG or sodium carbonate in DMAc. The structures of all prodrug compounds were established by ^{1}H NMR, ^{13}C NMR, and mass spectrometry. All compounds were analyzed by HPLC, and their purity was confirmed to be in excess of 98.0%.

Promoieties **2** and **3** (Figure 2) were prepared from their corresponding chloro compounds for fast reaction rate and better yield by treatment with sodium iodide in acetonitrile as shown in Scheme 1. The synthesis of 2-bromethyl acetate (**4**) was carried out according to the procedure reported in U.S. patent number 5,155,256, issued October 13, 1992. Thus, the reaction of ethylene glycol with acetic acid in the presence of hydrogen bromide afforded **4**. Commercially available 4-chloromethyl-5-methyl-1,3-dioxol-2-one (**5**) was used in the preparation of prodrug **1d** (Scheme 1). The structures of all promoieties were established by IR and ^{1}H NMR. All promoieties were analyzed by GC, and their purity was confirmed to be in excess of 96.5%.

RESULTS AND DISCUSSION

Prolonged administration of NSAIDs exhibits several undesired side effects. The most important side effects are gastrointestinal irritation and ulceration which still represent an unsolved therapeutic problem. With the aim of minimizing the ulcerogenicity, a series of ester prodrugs **1a**−**d** of **1** were synthesized and evaluated. The potential ulcerogenic effects of **1a**−**d** were determined after single dose administration in rats (100 mg/kg dose) and compared to those produced by the parent compound indomethacin (**1**) with identical conditions. Lesser degree of ulcers was observed in animals treated with prodrugs **1a**−**d**, compared to **1** (Table 1). Animals in each group (n = 6) treated with **1** developed an average of 41 ulcerogenic lesions with single dose administration. Around 4.9% of the lesions were considered to be large (higher than 3 mm diameter). This allowed the classification of lesions, which were scored depending upon the severity of mucosal damage. Prodrug **1a** on single dose administration led to the development of only 19 small lesions (<1 mm diameter), and **1b** on single dose administration

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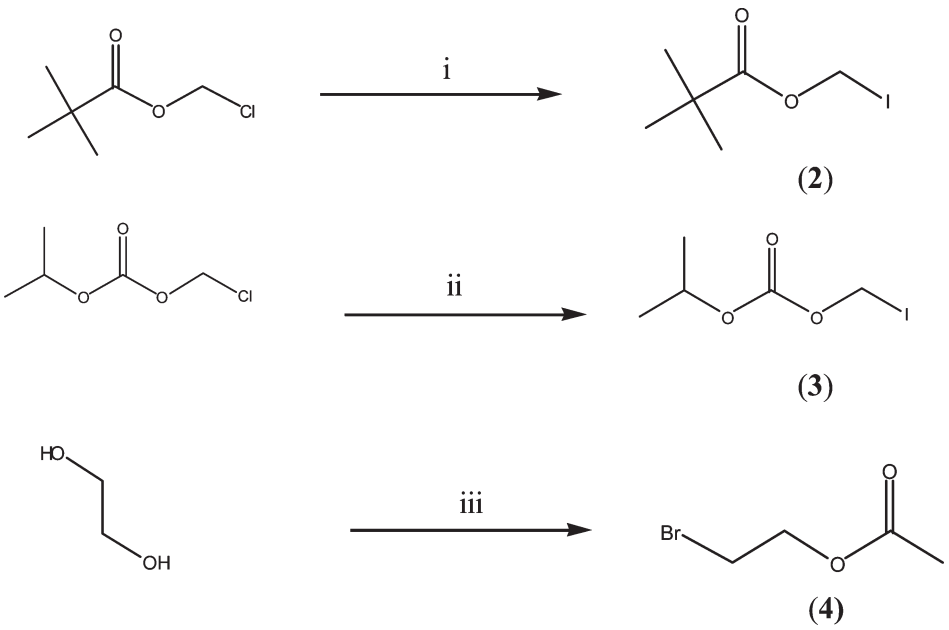
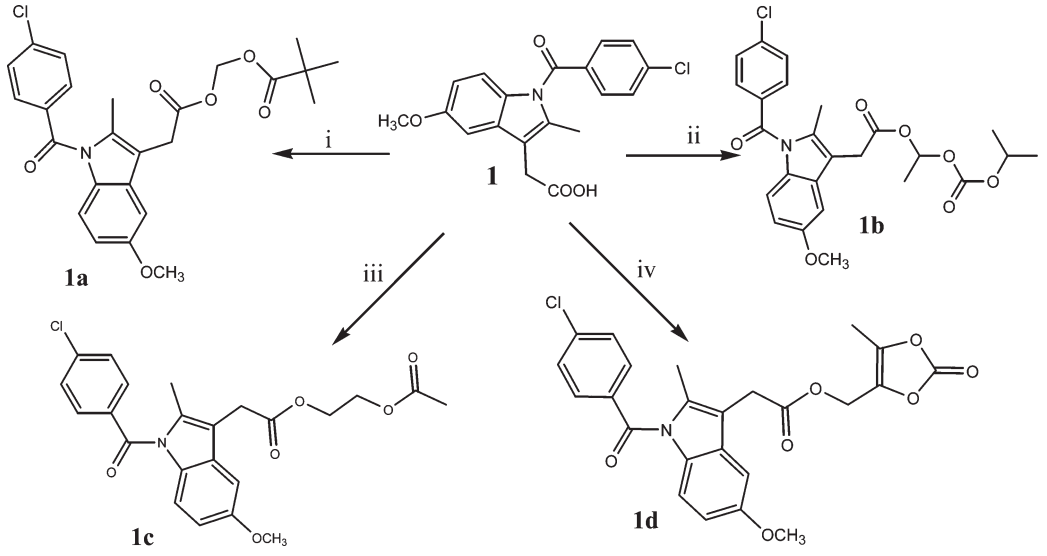
Scheme 1. Synthesis of Promoieties 2, 3, 4[a]



[a] Reagents and conditions: (i) NaI, ACN, 30 °C, 5 h; (ii) NaI, toluene, 18-crown-6, 105 °C, 2 h; (iii) CH_3COOH, 48% aqueous HBr, acetic anhydride, toluene, 110 °C, 90 min.

Scheme 2. Synthesis of Ester Prodrugs of Indomethacin (1a−d)[a]



[a] Reagents and conditions: (i) $ICH_2OCOC(CH_3)_3$, TMG, DMAc, −15 °C, 30 min; (ii) $ICH_2OCOOCH(CH_3)_2$, TMG, DMAc, −15 °C, 20 min; (iii) $BrCH_2CH_2OCOCH_3$, Na_2CO_3, DMAc, 55 °C, 10 h; (iv) Na_2CO_3, **5**, DMAc, 35 °C, 6 h.

led to the development of around 51 lesions of level I (86.3%), level II (5.9%), and level III (7.8%). However, **1c** and **1d** developed no ulcerogenic lesions on identical dose. These findings proved that masking of carboxylic function of indomethacin in prodrugs **1a**, **1c**, and **1d** successfully decreased the gastroulcerogenicity. All the prodrugs (except **1b**) showed an improved safety profile compared with the parent reference compound (Table 2). The prodrug **1b** (UI = 62) produced more detectable lesions on the gastric mucosa in the group of animals examined. **1a** (UI = 19) produced fewer detectable lesions, and **1c** and **1d** showed no visible lesions. This indicated that the prodrugs **1a**, **1c**, and **1d** were significantly less irritating to gastric mucosa than **1** (UI = 47).

The anti-inflammatory activity of prodrugs was evaluated by using the in vivo rat carrageenan induced paw edema method when administered orally to rats. Prodrugs **1a**, **1b**, and **1c** were found to be potent anti-inflammatory compounds and produced a significant anti-inflammatory effect (Table 3).

Partition Coefficient. The partition coefficient (log *P*) of parent compound indomethacin (**1**) and prodrugs **1a−d** were determined at room temperature in *n*-octanol−phosphate buffer at pH 7.4 and in 1-octanol−citric acid buffer at pH 3.0. The

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partition coefficient of all prodrugs at pH 7.4 was higher than **1**, indicating that prodrugs are more lipophilic than the parent drug. Partition coefficients of indomethacin and prodrugs are summarized in Table 4, and results are in line with the literature values.[24]

Aqueous Stability. The orally administered drug should be stable at various pH environments encountered in the gastrointestinal tract to deliver the intact prodrug to the systemic circulation. Four prodrugs candidates (**1a**−**d**) were evaluated for solubility and stability in a series of buffer solutions ranging from pH 1 to 9. All the prodrug compounds were found to be stable and practically insoluble in buffer solutions (below detection limit). Aqueous solubility of **1a**−**d** was lower than that of parent drug indomethacin (**1**), which is related to the increased lipophilicity of prodrugs. Limit of detection (LOD) of prodrugs **1a**−**d** was estimated by HPLC (LOD: **1a**, 0.084 μg/mL; **1b**, 0.119 μg/mL; **1c**, 0.111 μg/mL; **1d**, 0.0638 μg/mL). The aqueous solubility of **1** at 40 °C was 0.03, 24, 1500, and 1600 μg/mL at pH 1.0, 3.0, 5.2, 7.4, 9.0, as against that reported in the literature[25] (3.882 and 767.5 μg/mL at pH 1.2 and pH 7.2). Experimental solubility value of **1** was slightly different from the one reported earlier[25] and could be because of the difference in solubility study parameters. Solubility of indomethacin was pH dependent and increased with increasing pH. Insignificant hydrolysis of prodrug compounds to parent drug was observed in acidic to neutral pH buffer solutions (Table 5) at 40 °C, indicating that prodrugs **1a**−**d** are resistant toward hydrolysis at the pH of the stomach acidic environment, pH of the small intestinal mucosa, and at physiological pH.

Metabolic Stability. Indomethacin ester prodrugs **1a**−**d** were synthesized with the aim of obtaining enzymatic lability. Metabolic stability is an important property of drug candidates, since it affects parameters such as clearance, half-life, and bioavailability. A successful prodrug candidate is expected to undergo rapid, complete conversion to parent compound in the plasma or microsomes within 1−3 h. On the basis of in vivo studies, prodrugs **1a** and **1c** were selected for metabolic stability study in rat liver microsomes and rat plasma. Prodrugs **1a** and **1c** and reference parent drug **1** were subjected to metabolic stability in the presence of rat liver microsomes and rat plasma. The parent drug **1** was stable up to 60 min in both rat liver microsomes and rat plasma; however, prodrugs were highly metabolized (Table 6).

Formation of Parent Compound Indomethacin (1) from Prodrugs (1a, 1c) in Rat Liver Microsomes (RLM) and Rat Plasma (RP). The experimental findings depicted in Tables 7 and 8 proved that prodrugs **1a** and **1c** were enzymatically labile and converted rapidly to parent compound **1**. Indomethacin was the only metabolite observed after biotransformation in both RLM and RP. Release of parent drug from these ester prodrugs upon hydrolysis was confirmed by HPLC. Prodrug peaks in HPLC (retention time, **1a**, ∼14.1 min; **1c**, ∼11.1 min) disappeared in 30−60 min, and the peak area of the parent compound **1** (retention time, ∼9.15 min) was increased (Tables 7 and 8).

Solid State Morphology. The solid state morphology of active pharmaceutical ingredient (API) is a key parameter for their further utilization when several forms can coexist as crystalline or amorphous and/or as different polymorphs (allotropes).

Table 1. Ulcerogenic Effect of Indomethacin (1) and Prodrugs (1a−d) in Rats[a]

compd	no. of ulcers[§]	level I (<1 mm)	level II (1−3 mm)	level III (>3 mm)
1	41	37 (90.2%)	2 (4.9%)	2 (4.9%)
1a	19	19 (100.0%)	0	0
1b	51	44 (86.3%)	3 (5.9%)	4 (7.8%)
1c	0	0	0	0
1d	0	0	0	0

[a] The results were obtained with an average of six animals analyzed per group (n = 6).

Table 2. Ulcer Index for Compounds 1a−d and Indomethacin (1)

group	dose (mg/kg)	ulcer index (UI)[a]
normal		nil
1	100	47
1a	100	19
1b	100	62
1c	100	0
1d	100	0

[a] Ulcer index (UI) is calculated based on the lesions developed in the stomach on single dose administration in rats in each group (n = 6). Data are presented as the mean ± SEM at 6 h after oral administration of the test compound.

Table 4. Partition Coefficients of Indomethacin (1) and Prodrugs 1a−d in Buffer Solution

	partition coefficient (log P)[a]	
compd	pH 7.4	pH 3.0
1	2.1	4.2
1a	4.7	4.6
1b	5.0	5.1
1c	3.5	4.2
1d	2.9	4.5

[a] Results are reported as average of three experiments (n = 3).

Table 3. Anti-Inflammatory Activities of Prodrugs 1a−d and Parent Drug (1)

group	dose (mg/kg)	paw volume (mL)	inhibition at 180 min (%)
normal		1.88 ± 0.04	
carrageenan (1%), 0.1 mL		3.12 ± 0.04[a]	
1	100	2.52 ± 0.06[b]	48.39
1a	100	2.52 ± 0.08[c]	48.39
1b	100	2.56 ± 0.18[c]	45.16
1c	100	2.58 ± 0.09[c]	44.35
1d	100	2.96 ± 0.08	12.10

[a] P < 0.001, compared to normal control . [b] P < 0.001, compared to carrageenan control. [c] P < 0.01, compared to carrageenan control.

This is particularly important for API, as their morphology can have a significant impact on their bioavailability and stability. Hence, it was necessary to know the solid state morphology of prodrugs (**1a**−**d**). Prodrugs **1a**−**d** were crystalline when screened in a Bruker AXS D8 advance diffractometer. Powder XRD profiles of prodrugs are shown in Figures 3, 4, 5, and 6.

CONCLUSION

Indomethacin ester prodrugs were evaluated for their anti-inflammatory and ulcer potential by known experimental techniques. These prodrugs emerged as potent anti-inflammatory agents with lesser potential for ulcer than parent drug indomethacin. Low ulcer index (UI) was observed with three prodrug compounds (except **1b**). On the basis of in vivo evaluation, prodrugs **1a** and **1c** were selected for metabolic stability. Both these prodrugs were rapidly transformed enzymatically to the parent drug indomethacin in both rat liver chromosomes and rat plasma. Thus, on the basis of in vitro and in vivo evaluation, prodrugs **1a** and **1c** emerged as potent inflammatory drugs for prevention of gastrointestinal disorders and as substitutes for parental drug.

Table 5. Aqueous Solubility and Stability of Indomethacin (1) and Prodrugs 1a−d in Buffer Solutions at 40°C for 4 h[a]

	hydrolysis to parent drug (μg/mL)				solubility (μg/mL)			
buffer pH	**1a**	**1b**	**1c**	**1d**	**1a**	**1b**	**1c**	**1d**
1.0	0.0210	0.010	0.0103	0.0191	BDL	BDL	BDL	BDL
3.0	0.0075	0.00539	0.0092	0.0134	BDL	BDL	BDL	BDL
5.2	0.1525	0.01379	0.0197	0.0244	BDL	BDL	BDL	BDL
7.4	0.2940	0.01332	0.0093	0.0101	BDL	BDL	BDL	BDL
9.0	2.187	0.24614	0.369	0.5106	BDL	BDL	BDL	BDL

[a] BDL, below detection limit. Limit of detection (LOD) is estimated by the HPLC method: **1a** (0.084 μg/mL), **1b** (0.119 μg/mL), **1c** (0.111 μg/mL), **1d** (0.0638 μg/mL).

Table 6. Metabolic Stability of 1 and Prodrugs (1a and 1c) in RLM and RP

metabolic stability in RLM				metabolic stability in RP		
	% remaining in RLM[a]				% remaining in RP[a]	
compd	0 min	30 min	60 min	Time→	0 min	60 min
1	100	90.3	80.2	**1**	100	97.1
1a	100	0	0	**1a**	100	0
1c	100	1.14	0	**1c**	100	0

[a] The percentage of prodrug remaining after metabolism at respective time points was calculated by ratio of peak area at the respective time (min) to peak area found at 0 min multiplied by 100: (% remaining) = [(peak area at respective time (min))/(peak area at 0 min)] × 100.

EXPERIMENTAL SECTION

Materials and Methods. Melting points were determined on an MR VIS (Lab India) melting point apparatus and are uncorrected. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker Advance spectrophotometer using $CDCl_3$ and DMSO solvent. The chemical shifts are reported in ppm downfield from zero, and coupling constants are reported in hertz (Hz). IR spectra were acquired by using a FTIR Perkin-Elmer model RXI or FTIR spectrophotometer Nicolet 380 (Thermo Nicolet); software was Omnic. Mass spectra were recorded on a PE-SCIEX API-3000 LCMS/MS (Applied Biosystem) spectrophotometer. Commercially available solvents and reagents for reactions (ethyl acetate, DMAc, TMG, sodium carbonate) were procured from S.D.Fine-Chem Ltd., India. HPLC analysis was performed by using Shimadzu LC 2010 CHT with UV detector and Waters Alliance 2695, with PDA 2996 detector. All prodrug compounds were analyzed by HPLC, and their purity was confirmed to be in excess of 98.0%. HPLC grade solvents (acetonitrile, methanol, acetic acid, and toluene) were used in the analysis and were procured from a local Indian supplier. GC analysis was performed on Perkin-Elmer model Clarus 500 instrument. Promoieties were analyzed by GC for chromatographic purity, which was confirmed to be in excess of 96.5%. UV spectra were recorded by using a Shimadzu UV-2401 PC spectrophotometer. In vitro metabolic stability study was conducted in rat liver microsomes and rat plasma by the HPLC method, which was developed in-house in the laboratory. Powder XRD of prodrugs was performed on a Bruker AXS D8 Advance diffractometer. The in vivo anti-inflammatory and ulcer index assays were carried out by using protocol approved by the Institutional Animal Ethics Committee (IAEC) and compiled with National Institutes of Health (NIH) guidelines on handling of experimental animals. Male Wistar rats weighing 150−200 g were used for the study. Carrageenan was purchased from Sigma (St. Louis, MO, U.S.),

Table 7. Formation of Parent Compound 1 from Prodrugs 1a and 1c in RLM and RP

formation of indomethacin parent compound from prodrugs in RLM				formation of indomethacin parent compound from prodrugs in RP		
	indomethacin peak area in HPLC analysis				indomethacin peak area in HPLC analysis	
compd	0 min	30 min	60 min	compd	0 min	60 min
1a	0	1435186	1801169	**1a**	0	101220
1c	0	739541	738838	**1c**	0	855753

Table 8. Disappearance of Prodrugs by Metabolic Transformation in Presence of RLM and RP

prodrug peak area in RLM				prodrug peak area in RP		
	prodrug peak area in HPLC analysis				prodrug peak area in HPLC analysis	
compd	0 min	30 min	60 min	compd	0 min	60 min
1a	1157146	0	0	**1a**	491862	0
1c	794700	9082	0	**1c**	748633	0

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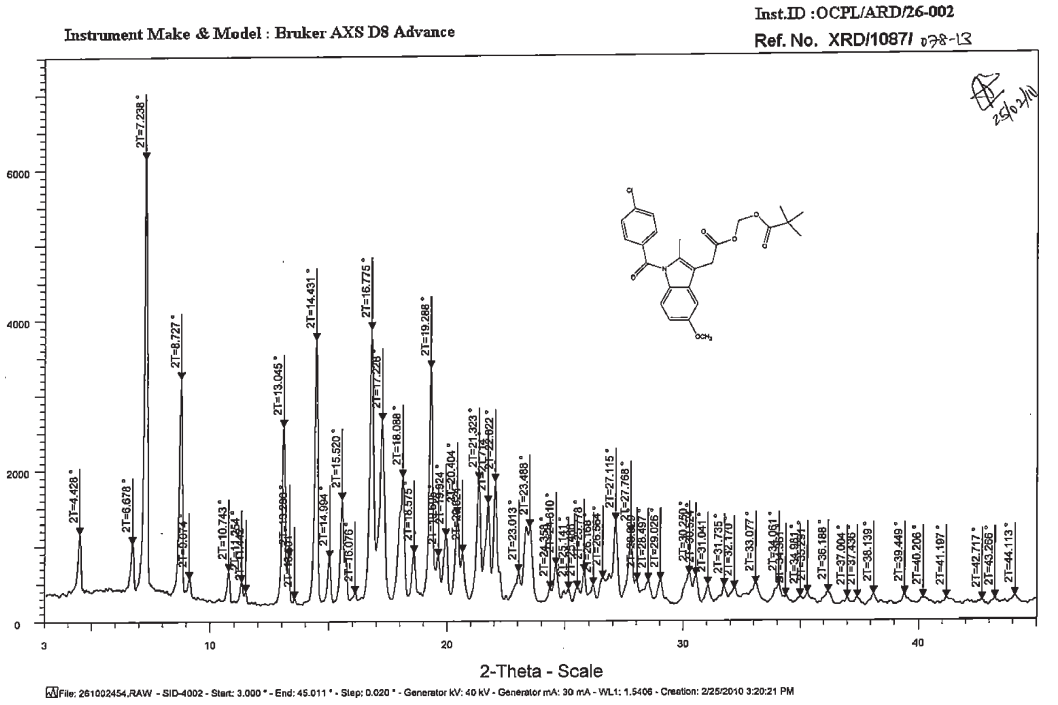

Figure 3. XRD diffractogram for prodrug **1a**.

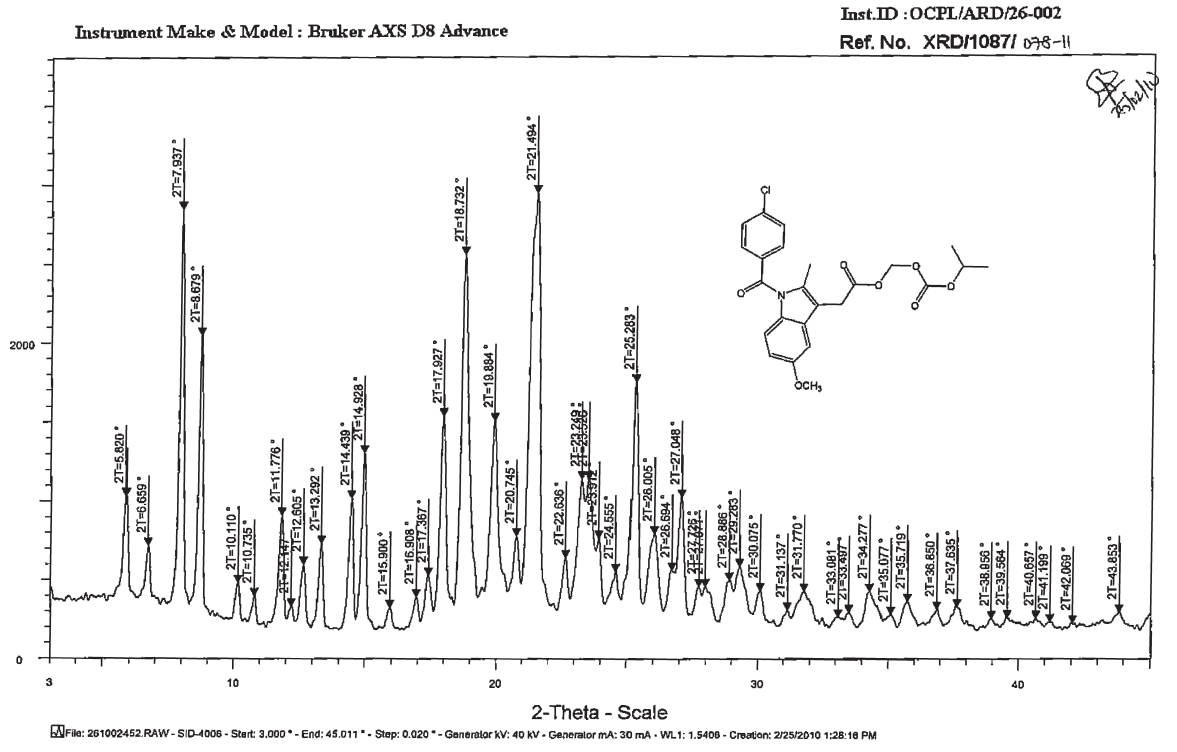

Figure 4. XRD diffractogram for prodrug **1b**.

Indomethacin was procured from a local bulk API manufacturer. 4-Chloromethyl-5-methyl-1,3-dioxol-2-one and chloromethylisopropyl carbonate reagents were gifted by M/s Matrix Laboratories, India, and M/s Huaren Chemical Co. Ltd., India, respectively. NADPH was purchased from Sigma (lot no. N-7785).

Iodomethyl Pivalate (2). A solution of chloromethyl pivalate (100 g, 0.664 mol) in acetonitrile (200 mL) was allowed to react with sodium iodide (180 g, 1.20 mol) at 30 °C for 5 h under N_2 atmosphere. Reaction progress was monitored by GC. After completion of reaction, the reaction mixture was transferred to a mixture of dichloromethane (1000 mL) and water (1000 mL), stirred for 10 min, and separated into two phases. The aqueous phase was discarded, and the organic phase was washed with 2% sodium thiosulfate ($Na_2S_2O_3$, 500 mL) and concentrated under vacuum to give **2** as a yellowish oil (138.8 g, 86.6%). Purity

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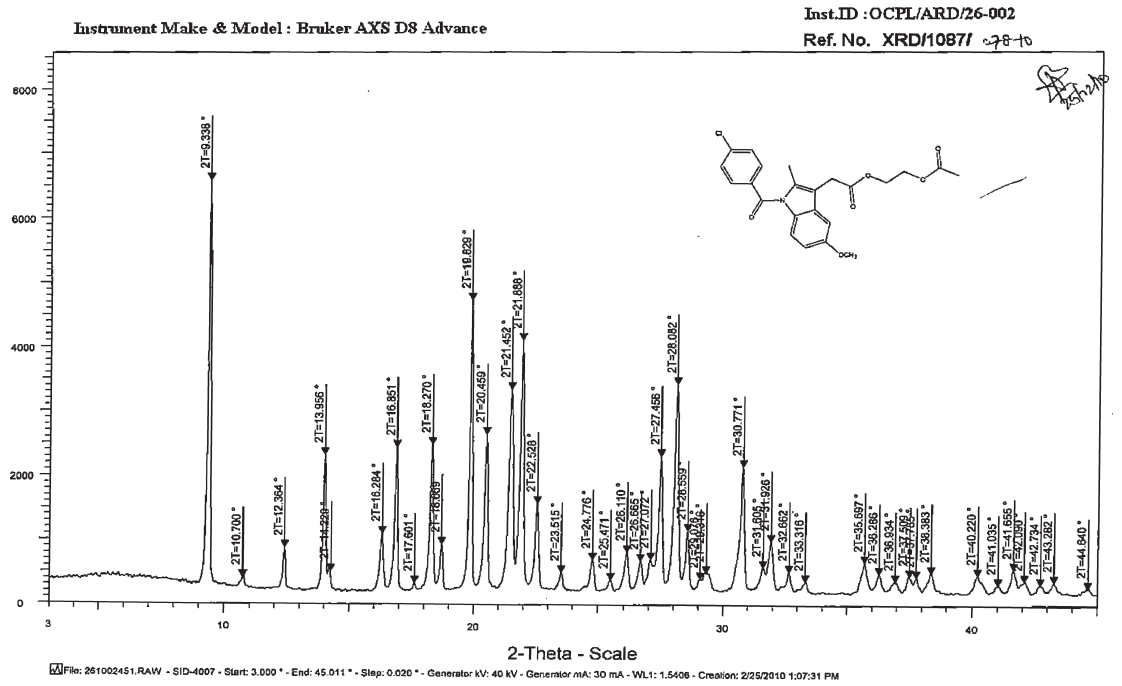

Figure 5. XRD diffractogram for prodrug **1c**.

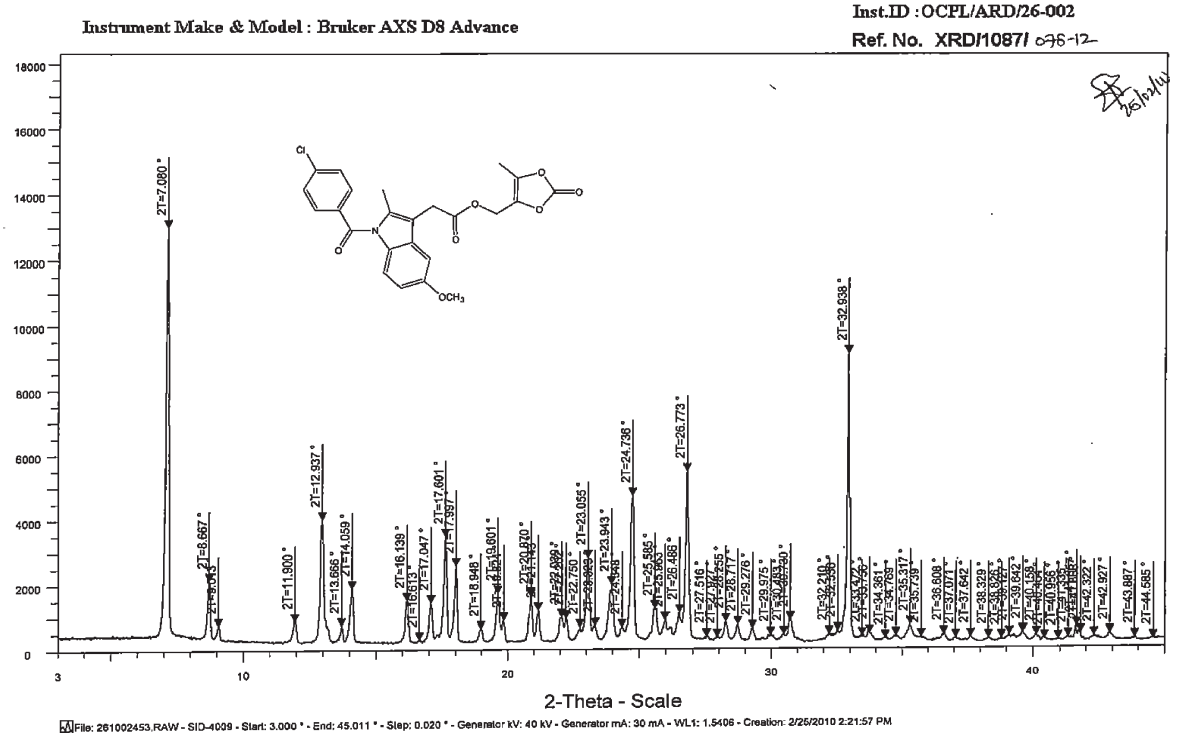

Figure 6. XRD diffractogram for prodrug **1d**.

by GC 99.01%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.93 (s, 2H, $-CH_2$)), 1.24 (s, 9H, $-C(CH_3)_3$); IR (Nujol) cm^{-1} 1759 ($C{=}O$), 1097 ($C-O$ str).

1-Iodomethylisopropyl Carbonate (3). Sodium iodide (112 g, 0.747 mol) and 18-crown-6 (2.5 g, 0.009 45 mol) were added to toluene (600 mL). The reaction mixture was stirred vigorously and heated to 110−112 °C under dry nitrogen atmosphere to remove total moisture. The reaction mixture was cooled to 105 °C, and chloromethyl isopropyl carbonate (50 g, 0.3276 mol) was added. The reaction mixture was stirred at 105 °C for 2 h. The reaction progress was monitored by GC. After completion of reaction, the reaction mixture was cooled to 5 °C and washed with 2% sodium thiosulfate (160 mL) followed by brine (670 mL). The organic layer was evaporated under vacuum to afford **3** as a pale yellow oil (69.5 g, 87%). Purity by GC, 99.61%.

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^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.93 (s, 2H, $-OCH_2I$)), 4.95 (m, 1H, J = 6.32 Hz, $-CH(CH_3)_2$), 1.35 (s, 6H, $-CH(CH_3)_2$); IR (KBr) cm^{-1} 1759 (C═O), 1077 ($C-O$).

2-Bromoethyl Acetate (4). A solution of ethylene glycol (51.6 g, 0.8322 mol), glacial acetic acid (75 g, 1.248 mol), toluene (20 mL), and 48% hydrogen bromide solution (140.3 g, 0.832 mol) was refluxed for 90 min to distill out azeotropically 130 mL of water under nitrogen atmosphere. The solution was cooled to 25 °C, and acetic anhydride (23.7 g, 0.232 mol) was added dropwise by controlling the temperature below 35 °C. Reaction was monitored by GC. After completion of reaction, sodium metabisulfite (0.25 g) and sodium carbonate (0.3 g) were added under stirring, and the reaction mass was maintained overnight without agitation. Reaction mixture was subjected to fractional distillation under vacuum, and the main fraction was collected at 40−47 °C. The main fraction was washed with chilled brine solution (100 mL) to remove acetic acid to afford **4** as a colorless liquid (55 g, 39.6%). Purity by GC, 96.72%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.1 (s, 3H, $-CH_3CO$), 3.50 (t, 2H, J = 3.96 Hz, $-CH_2Br$), 4.4 (t, 2H, J = 6.16 Hz, $-CH_2O$); IR (Nujol) cm^{-1} 1753 (C═O).

Pivaloyloxymethyl 1-(*p* -Chlorobenzoyl)-5-methoxy-2-methyl-3-indolylacetate (1a). Compound **1** (10 g, 27.9 mmol) was dissolved in DMAc (45 mL) at 20 °C and cooled to −15 °C under nitrogen atmosphere. TMG (3.37 g, 29.1 mmol) was added once, and the mixture was stirred for 20 min at −10 ± 2 °C. The reaction mixture was then cooled to −20 °C. To it was added iodomethyl pivalate (6.76 g, 27.9 mmol), and the mixture was stirred for 30 min at −15 °C. Reaction progress was monitored by HPLC. After completion of reaction, the reaction mixture was transferred into a mixture of ethyl acetate (120 mL), water (400 mL), and sodium thiosulfate (1 g) under vigorous stirring. The pH of the reaction mixture was adjusted to 10.5 by addition of sodium carbonate, and the organic layer was separated. The organic layer was washed with brine (125 mL), decolorized with activated charcoal (1 g), and filtered. After evaporation of solvent in vacuo, the desired product **1a** was obtained as an oil which solidified upon cooling to an off-white solid (12.6 g, 95.5%), mp 68 °C. Chromatographic purity (HPLC) 98.13%; MS (+ESI) m/z = 489.2 $(M + NH_4)^+$. UV max (ethanol): 272, 301, 320 nm (23 mM^{-1} cm^{-1}). IR (KBr) cm^{-1} 1757 (C═O), 1738 (C═O) 1673 (N−C═O); ^{1}H NMR (DMSO, 400 MHz) δ 1.04 (s, 9 H, $-(CH_3)_3$), 2.21 (s. 3H, $-CH_3$), 3.76 (s, 2H, $-OCH_3$), 3.85 (s, 2H, $-CH_2CO$), 5.72 (s, 2H, $-OCH_2O$), 6.71 (dd, 1H, J = 8.9 Hz and J = 2.2 Hz), 6.9 (d, 1H, J = 8.9 Hz), 7.01 (s, 1H, J = 2.0 Hz), 7.63−7.65 (m, 4H, Ar−H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.48, 26.85 (3C), 30.29, 38.81, 55.79, 79.73, 101.22, 111.84, 112.02, 115.1, 129.29 (2C), 130.49, 130.89, 131.25 (2C), 133.96, 136.25, 139.46, 156.24, 168.4, 169.61, 177.15.

[(1-Methyl)ethoxycarbonyloxy]methyl 1-(*p*-Chlorobenzoyl)-5-methoxy-2- methyl-3-indolylacetate (1b). Compound **1** (2.5 g, 6.98 mmol) was dissolved in DMAc (12.5 mL) at 10 °C. The reaction mixture was cooled to −15 °C under nitrogen atmosphere. TMG (0.87 g, 7.51 mmol) was added, and the mixture was stirred for 20 min at −15 °C. Then 1-iodomethylisopropyl carbonate (1.7 g, 6.98 mmol) was added at −15 °C. The reaction mixture was stirred for 20 min at −15 °C. Reaction completion was monitored by HPLC. The reaction mixture was transferred into a mixture of ethyl acetate (30 mL), water (100 mL), and sodium thiosulfate (1 g) under vigorous stirring. The pH of the reaction mixture was adjusted 10.6 by addition of sodium carbonate. The organic phase was separated, washed with brine (125 mL), decolorized with activated carbon (0.5 g), and filtered. After evaporation of solvent in vacuo, the desired product **1b** was obtained as an oil which solidified upon cooling to an off-white solid (3.2 g, 96.6%), mp 84.2 °C. Chromatographic purity (HPLC) 99.39%; MS (+ESI) m/z = 491.1$(M + NH_4)^+$; UV max (ethanol) 320, 272, 301.3 nm (23 mM^{-1} cm^{-1}), IR (KBr) cm^{-1} 1753 (C═O), 1686 ($N-C$═O); ^{1}H NMR (DMSO, 400 MHz) δ 1.20 (d, 6H, J = 6.2 Hz, $-2CH_3$), 2.22 (s, 3H, $-C-CH_3$), 3.76 (s, 3H, $-OCH_3$), 3.88 (s, 2H, $-CH_2CO$), 4.76 (h, 1H, J = 6.24 Hz, $-OCH(CH_3)_3$], 5.71 (s, 2H, $-OCH_2O$), 6.71 (dd, 1H, J = 9.0 Hz and J = 2.5 Hz, indole Ar−H), 6.90 (d, 1H, J = 9.0 Hz, indole Ar−H), 7.01 (d, 1H, J = 2.5 Hz, Ar−H), 7,63 − 7.68 (m, 4H, Ar−H); ^{13}C NMR ($CDCl_3$,100 MHz) δ 13.53, 21.75 (2C), 30.20, 55.81, 73.32, 82.19, 101.13, 111.72, 112.07, 115.13, 129.29 (2C), 130.51, 130.89, 131.37 (2C), 133.96, 136.32, 139.45, 153.43, 156.23, 168.42, 169.47.

Acetyloxyethyl 1-(*p*-Chlorobenzoyl)-5-methoxy-2-methyl-3-indolylacetate (1c). Compound **1** (5 g, 13.97 mmol) was dissolved in DMAc (20 mL) at 30 °C. Sodium carbonate (0.963 g, 9.08 mmol) was added, and the mixture was stirred for 30 min at 30 °C under nitrogen atmosphere followed by addition of 2-bromoethyl acetate (2.917 g, 17.46 mmol). The reaction mass was heated to 55 °C and stirred for 10 h, and completion of reaction was monitored by HPLC. Then the reaction mixture was transferred to a mixture of ethyl acetate (60 mL), water (200 mL), and sodium thiosulfate (2 g) under vigorous stirring. The pH of the reaction mixture was adjusted to 10.6 by addition of sodium carbonate, and the organic phase was separated. The organic phase was then washed with brine (240 mL) and filtered. After evaporation of the solvent in vacuo, the desired product **1c** was obtained as an oil which solidified upon cooling to off-white solid (4.8 g, 77.4%), mp 106.6 °C. Chromatographic purity (HPLC), 99.31%; MS (+ESI) m/z = 444.2 $(M + H)^+$; UV max (ethanol) 319.85, 271.1, 301.94 nm (21 mM^{-1} cm^{-1}); IR (KBr) cm^{-1} 1740 (C═O), 1685 ($N-C$═O); ^{1}H NMR (DMSO, 400 MHz) δ 1.92 (s, 3H, $-COCH_3$), 2.22 (s, 3H, $-C-CH_3$), 3.76 (s, 3H, $-OCH_3$), 3.79 (s, 2H, $-CH_2CO$), 4.20 (dd, 2H, J = 5.8 Hz and J = 2.5 Hz, $-OCH_2$), 4.26 (dd, 2H, J = 6.8 Hz and J = 3.2 Hz, $-OCH_2$), 6.71 (dd, 1H, J = 9.0 Hz and J = 2.5 Hz, indole Ar−H), 6.92 (d, 1H, J = 2.5 Hz, Ar−H), 7,63 − 7,69 (m, 4H, Ar−H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.19, 20.44, 29.26, 55.39, 61.89, 62.39, 101.65, 111.43, 112.61, 114.62, 129.1(2C), 130.22, 130.52, 131.20 (2C), 134.12, 135.51, 137.7, 155.6, 167.91, 170.25, 170.47.

(5-Methyl-2-oxo-1,3-dioxolene-4-yl)methyl 1-(*p*-Chlorobenzoyl)-5-methoxy-2-methyl-3-indolylacetate (1d). Compound **1** (3 g, 8.3 mmol) was dissolved in DMAc (15 mL) at 30 °C. Sodium carbonate (0.67 g, 6.2 mmol) and **5** (1.43 g, 8.5 mmol) were added. The reaction mass was stirred for 6 h at 35 °C under nitrogen atmosphere. Reaction progress was monitored by HPLC. After completion of reaction, the reaction mixture was transferred into a mixture of ethyl acetate (36 mL), water (120 mL), and sodium thiosulfate (1 g) under vigorous stirring. The pH of the reaction mixture was adjusted to 10.6 by addition of sodium carbonate, and the organic phase was separated. The organic phase was washed with brine (240 mL), treated with activated carbon (1 g), and filtered. After evaporation of solvent in vacuo, the desired product **1d** was obtained which on recrystallization in methanol gave a pale yellow solid (3.2 g, 81.24%), mp 118.2 °C. Chromatographic purity (HPLC) 99.54%; MS (+ESI) m/z = 487.2 $(M + NH_4)^+$; UV max (ethanol) 319.61, 268.9, 303.82 nm (29 mM^{-1} cm^{-1}); IR (KBr) 1811 (OCOO), 1739 (C═O), 1678 ($N-C$═O); ^{1}H NMR (DMSO, 400 MHz) δ 2.14 (s, 3H, $-CH_3-C$(OC=O)), 2.22 (s, 3H, $-C-CH_3$), 3.76 (s, 3H, $-OCH_3$), 3.85 (s, 2H, $-CH_2CO$), 5.01 (s, 2H, $-OCH_2C$═CCH_3), 6.71 (dd, 1H, J = 9.0 Hz and J = 2.5 Hz, indole Ar−H), 6.93 (d, 1H, J = 9.0 Hz, indole Ar−H), 7.03 (d, 1H, J = 2.5 Hz, indole Ar−H), 7.64−7.69 (m, 4H, Ar−H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 9.47, 14.33, 30.12, 54.36, 55.82, 101.36, 111.71, 111.8, 115.13, 129.28(2C), 130.49, 130.89, 131.30 (2C), 133.43, 133.88, 136.27, 139.46, 140.31, 152.13, 156.19, 168.38, 170.44.

GC Analysis. *Analysis Method for Promoiety* ***2***. The following equipment and parameters were used: instrument, Perkin-Elmer, model Clarus 500; column, DB-624, 30 mm × 0.53 mm, 3.0 μm; oven temperature, 40 °C; ramp rate, 10 °C/min up to 220 °C; final oven temperature, 220 °C; injection temperature, 200 °C; detector temperature, 250 °C; flow (carrier), 5.0 mL/min; injection volume, 0.2 μL; split, 20:1

Analysis Method for Promoiety ***3***. The following equipment and parameters were used: instrument, Perkin-Elmer, model Clarus 500; column, DB-1, 30 mm × 0.53 mm, 1.5 μm; oven temperature, 75 °C; ramp rate, 10 °C/min up to 200 °C; final oven temperature, 200 °C for 10 min; injection temperature, 150 °C; detector temperature, 250 °C; flow (carrier), 5.0 mL/min; injection volume, 0.2 μL; split, 20:1

Analytical Method for Promoiety ***4***. The following equipment and parameters were used: instrument, Perkin-Elmer, model Clarus 500; column, DB-5, 30 mm × 0.53 mm, 1.5 μm; detector, FID; carrier gas flow (N_2), 3.5 mL/min; initial oven temperature, 50 °C; initial time, 6.0 min; rate "1", 15 °C/min; final oven temperature "1", 110 °C; final time "1", 6 min; rate "2", 15 °C/min; final oven temperature "1", 250 °C; final time "1", 10 min; injection temperature, 125 °C; detector temperature, 270 °C; flow (carrier), 5.0 mL/min; injection volume, 0.2 μL; split, 1:10; run time, 30 min.

HPLC Analysis. *Testing Procedure for In-Process Analysis, Aqueous Solubility, Chromatographic Purity, Partition Coefficient Analysis.* A reverse phase HPLC method was used for analysis of metabolic stability, in-process analysis, and chromatographic purity of prodrugs. In-process analysis, chromatographic purity, partition coefficient, aqueous solubility were performed on an HPLC Shimadzu LC 2010 CHT with UV detector. Mobile phase A consisted of a mix of 2.0 mL of glacial acetic acid in 1000 mL of Milli Q water. Filtration was through a 0.45 μm filter, and the samples were degassed (pH of this solution was around 3.0). Mobile phase B involved filtered and degassed acetonitrile (HPLC grade). Mobile phase ratio was (mobile phase A (30%))/(mobile phase B (70%)). Chromatographic parameters were as follows: column, YMC-Pack C 8 (100 mm × 4.6 mm, 3 μm); flow rate, 0.8 mL/min; wavelength, 254 nm; injection volume, 20 μL; run time, 20 min.

Testing Procedure for Metabolic Stability. The procedure involved the following: HPLC system, Waters Alliance 2695 separation module with 2996 PDA detector; software, Empower; column C_{18}, ODS 3V, 250 mm × 4.6 mm, 5 μm; mobile phase A of 10 mM potassium dihydrogen phosphate; mobile phase B of acetonitrile; wavelength, 265 nm. The gradient elution program is depicted in Table 9.

Aqueous Solubility. The solubility of prodrugs **1a**−**d** was determined at 40 °C in 0.1 M boric acid buffer at pH 9, 0.2 M phosphate buffer at pH 7.4, 0.1 M phosphate buffer at pH 5.2, 0.1 M citric acid buffer at pH 3.0, and 0.2 M hydrochloric acid buffer at pH 1.0. Test compound (5 mg each) in buffer solution (10 mL) was incubated at 40 °C for 4 h at 400 rpm on a mechanical shaker. The solution was filtered through 0.20 μm membrane filter, and the filtrate was analyzed quantitatively by HPLC at a wavelength of 254 nm for its solubility and hydrolysis.

Determination of Partition Coefficients. The partition coefficients of indomethacin ester prodrugs **1a**−**d** were determined in octanol−buffer system by HPLC method at 25 °C. The aqueous phase was 0.2 M phosphate buffer of pH 7.4 and 0.1 M citric acid buffer of pH 3.0. Before use, the 1-octanol and buffer solution were mutually saturated for 24 h at 400 rpm on a mechanical shaker at 25 °C. A known concentration of compounds in 1-octanol (5 mL) and buffer solution (5 mL) was shaken on a mechanical shaker for 60 min at 400 rpm at 25 °C and centrifuged at 3500 rpm for 5 min. The concentration of the compound (solute) in both phases were analyzed quantitatively by HPLC at 254 nm. Each experiment was repeated in triplicate. The partition coefficient was calculated by following equation.

$$\log P_{\mathrm{oct/wat}} = \log\left(\frac{[\mathrm{solute}]_{\mathrm{octanol}}}{[\mathrm{solute}]^{\mathrm{un\text{-}ionized}}_{\mathrm{water}}}\right)$$

Anti-Inflammatory Assay. The test compounds **1a**−**d** and the reference drug indomethacin were evaluated by using the in vivo rat carrageenan-induced foot paw edema model reported previously.[22]

Table 9. Gradient Elution Program

time (min)	mobile phase A	mobile phase B
0−2	90	10
2−8	30	70
8−10	10	90
10−12	30	70
12−15	90	10
15−20	90	10

Chemicals. Carrageenan was purchased from Sigma (St. Louis, MO, U.S.). Indomethacin (EP-grade) is gifted by R & D Division of Orchid Chemicals and Pharmaceutical Ltd., and prodrugs **1a**−**d** were synthesized in the laboratory.

Animals. Male Wistar rats weighing 150−200 g were used for the study. All the experiments were approved by the Institutional Animal Ethics Committee (IAEC) and complied with the NIH guidelines on handling of experimental animals. The animals were housed in a group of three rats per cage under well-controlled conditions of temperature (22 ± 2 °C), humidity (55 ± 5%), and 12 h/12 h light−dark cycle. Animals had free access to diet purchased from vet care and water ad libitum.

Paw edema was induced with carrageenan 1% (0.1 mL) administered as a single subplantar injection under light ether anesthesia in the left paw. Animals were divided into groups, namely, normal control, carrageenan, and carrageenan treated with indomethacin and prodrug (100 mg/kg, po) 1 h prior to carrageenan administration. Paw volume was measured using the plethysmograph 3 h after the carrageenan administration. Percentage of inhibition was calculated by the following equation.

$$\%\ \text{inhibition} = \frac{\text{carrageenan} - \text{test compound}}{\text{carrageenan} - \text{normal}} \times 100$$

Acute Ulcerogenic Assay. The test compounds **1a**−**d** and the reference drug indomethacin were evaluated for ulcerogenic side effect by using distension ulcer model reported previously.[23]

Animals (n = 6/group) were fasted for 18 h with free access to water before administration of test compounds. Ulcerogenic activity was evaluated after 100 mg/kg single dose oral administration of indomethacin and prodrugs **1a**−**d**. Animals were sacrificed under ether anesthesia after a 6 h dosing of drug compounds. Stomach was removed, opened along the greater curvature, washed, mounted on a thermostat sheet, and examined for ulcers. Ulcerative lesions were scored as follows. (1) Length of all lesions was measured using a Vernier caliper. (2) The ulcers were classified as level I (ulcer area if <1 mm diameter), level II (ulcer area of 1−3 mm diameter), level III (ulcer area of >3 mm diameter). (3) Ulcerative lesion index (UI) was calculated as as 1(number of ulcer level I) + 2(number of ulcer level II) + 3(number of ulcer level III). Mean value of six animal readings was reported as ulcer index (UI).

Rat Plasma. Rat plasma was harvested from in-house rats. Fresh blood was collected from the male rat using the retro-orbital bleeding method in the tube containing heparin (100 IU/mL blood). After the collection of blood, plasma was separated from the blood by centrifugation at 9000 rpm for 5 min. The supernatant plasma was separated and utilized for further experiments.

In Vitro Physiological Stability of Prodrugs 1a−d and Indomethacin (1) in Rat Plasma. The test compound solution (5 μL of 5 mM) was dissolved in rat plasma (495 μL). Immediately after addition (0 min), aliquots (100 μL) were removed and added to ice-cold acetonitrile (100 μL) and mixed well by vortexing for 2 min. The mixture was centrifuged at 14 000 rpm for 10 min. The supernatant was diluted with acetonitrile and analyzed by HPLC. After 0 min, the remaining

sample was incubated at 37 °C for 60 min. After 30 and 60 min, the sample (100 μL) was treated with ice cold acetonitrile (100 μL) and centrifuged at 14 000 rpm for 10 min. The supernatant was diluted with acetonitrile and injected into the HPLC instrument. The percentage of prodrug remaining was calculated according to the following equation.

$$\%\ \text{remaining} = \frac{\text{peak area at respective time (min)}}{\text{peak area at 0 min}} \times 100$$

Rat Liver Microsomes. Rat liver microsomes were prepared in-house by a previously published method and used immediately in the experiments. Protein concentrations were determined by the Biorad protein assay (Bio-Rad, Hercules).

Microsomal Stability. *Materials.* Materials used were as follows: NADPH (Sigma, lot no. N 6674); acetonitrile (HPLC grade, Merck, India); Tris-HCl (S.D.Fine-Chem, India); rat liver chromosomes (in-house); prodrugs; indomethacin reference compound.

Requirement. Required items were as follows: rat liver microsomes (10 mg/mL protein concentration), NADPH (10 mM solution), Tris-HCl buffer (pH 7.4), Eppendorf tubes, test compound solutions (5 mM).

Study Conditions. Study conditions were as follows: incubation period, 60 min; incubation conditions, 37 °C with 60 rpm shaking; protein precipitation solvent, acetonitrile.

Assay Procedure. Tris-HCl buffer (395 μL), 10 mM NADPH solution (50 μL), and 50 μL of rat liver microsomes were mixed and vortexed for 10 s. To this mixture, 5 mM drug solution (5 μL) was injected and vortexed well. The sample (75 μL) was immediately taken out (0 min) and transferred to the centrifuge tube containing ice cold acetonitrile (75 μL), vortexed, and centrifuged at 14 000 rpm for 10 min. Aliquots of the supernatant were separated and used for analysis by HPLC. Then the assay mixture was incubated in a water bath at 37 °C for 60 min. At specific time points (30 min, 60 min), the assay mixture (75 μL) was taken out and added to the centrifuge tube containing an equal volume of cold acetonitrile. Then the tubes were vortexed and centrifuged at 14 000 rpm for 10 min. Aliquots of the supernatant were separated and used for analysis by HPLC.

The percentage of prodrug remaining was calculated according to the following equation.

$$\%\ \text{remaining} = \frac{\text{peak area at respective time (min)}}{\text{peak area at 0 min}} \times 100$$

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. ^{1}H NMR, IR, and chromatographic purity (GC) data for promoieties **2**, **3**, **4**; ^{1}H NMR, ^{13}C NMR, IR, chromatographic purity (HPLC), UV, and mass data for prodrugs. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

NSAID, nonsteroidal anti-inflammatory drug; Tris-HCl, tris-(hydroxymethyl)aminomethane hydrochloride; NADPH, nicotinamide adenine dinucleotide phosphate; HPLC, high performance liquid chromatography; GC, gas chromatography; RLM, rat liver microsomes; RP, rat plasma; API, active pharmaceutical ingredient; UI, ulcer index; GI, gastrointestinal; DMAc, dimethylacetamide; PXRD, powder X-ray diffraction; RT, room temperature; TMG, 1,1,3,3-tetramethylguanidine; 18-crown-6, 1,4,7,10,13,16-hexaoxacyclooctadecane; EP, European Pharmacopoeia; Ar, aromatic; BDL, below detection limit

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