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Synthesis, Characterization, and Biological Evaluation of Novel Diclofenac Prodrugs

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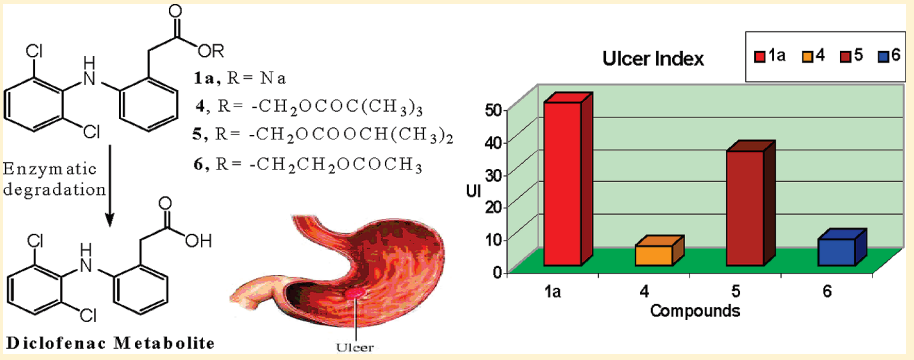
Supporting Information

ABSTRACT: Diclofenac ester prodrugs (**4**, **5**, **6**) were synthesized and evaluated in vitro and in vivo for their potential use for oral delivery, with the aim of obtaining enzymatically labile and less ulceration drugs than the parent drug diclofenac sodium (**1a**). Prodrugs **4**, **5**, **6** were found to be potent anti-inflammatory drugs with less ulcerogenic potential than the parent diclofenac sodium. Prodrugs **4**, **5**, **6** rapidly underwent enzymatic hydrolysis to release the parent drug diclofenac in 30−60 min in rat liver microsomes (RLM) and rat plasma (RP). Prodrugs were found to be more lipophilic when the partition coefficient was measured in 1-octanol and buffer system at pH 7.4 and 3.0. Diclofenac prodrugs **4**, **5**, **6** were found to be crystalline in nature (analyzed by PXRD). Prodrug **4** was found to be a superior candidate for the treatment of chronic inflammatory diseases.



INTRODUCTION

Diclofenac sodium (**1a**, Figure 1) is a nonsteroidal anti-inflammatory drug (NSAID) widely used for the treatment of pain and stiffness associated with a variety of inflammatory diseases including active inflammatory arthritis.[1−5] NSAIDs exert their therapeutic activity by inhibiting cyclooxygenase-derived prostaglandin synthesis, but this mechanism of action is inherently responsible for the gastrointestinal (GI),[6−10] renal,[11−13] and hepatic[14] side effects observed in patients undergoing a long-term treatment. The most common effects associated with NSAID therapy are upper GI irritation, ulceration, dyspepsia, bleeding, and in some cases death.[15] Prodrug formation through masking of the carboxylate moiety of these drugs has been considered as an approach to minimize such side effects and to improve their delivery characteristics.[16−18]

Among the many possible prodrugs, bioreversible esters have received considerable attention because of the presence of enzymes in the living system capable of hydrolyzing them. In both drug discovery and development, prodrugs have become an established tool for improving physicochemical, biopharmaceutical, or pharmacokinetic properties of pharmacologically potent compounds, thereby increasing developability and usefulness of a potential drug.[19,20] 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.[21−23]

In the present study, we have prepared diclofenac prodrugs **4**, **5**, **6** using promoities **1**, **2**, **3** (Figure 2) and evaluated them for oral drug application. The aqueous solubility, metabolic stability, and formation of drug from prodrug in rat liver microsomes and rat plasma, partition coefficient, ulceration index (UI), anti-inflammatory inhibition have been studied in comparison with the parent drug diclofenac sodium (**1a**).

CHEMISTRY

The novel diclofenac ester prodrugs **4**, **5**, **6** were synthesized in a straightforward manner starting from **1a** in good yields (83.8%, 87%, 85%, respectively) by treatment of **1a** with iodomethyl pivolate, iodomethylisopropyl carbonate, and 2-acetoxyethyl bromide, respectively, in the presence of sodium carbonate in DMAc, as illustrated in Scheme 2. The structures of all prodrug

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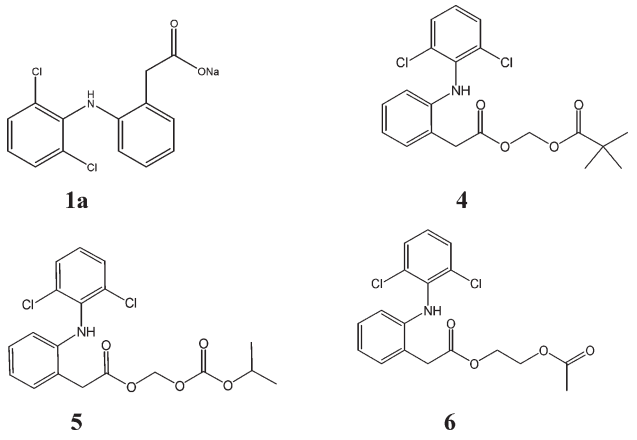


Figure 1. Chemical structures of diclofenac sodium (**1a**) and diclofenac prodrugs **4**, **5**, **6**.

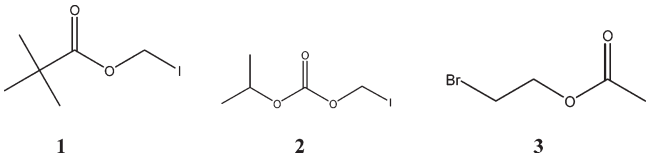
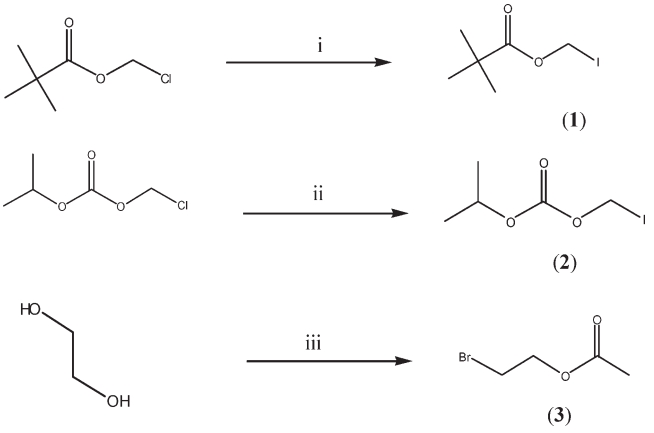


Figure 2. Chemical structures of iodomethyl pivalate (**1**), 1-iodomethyl-isopropyl carbonates (**2**), and 2-acetoxyethyl bromide (**3**).

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



[a] Reagents and conditions: (i) NaI, ACN, 30 °C, 5 h; (ii) NaI, ACN, 30 °C, 22 h; (iii) CH_3COOH, 48% aqueous HBr, acetic anhydride, toluene, 110 °C, 90 min.

compounds were established by ^{1}H NMR, ^{13}C NMR, and mass spectrometry, and their purity in excess of 98.0% was confirmed by HPLC analysis.

Promoieties used in the synthesis of prodrugs are synthesized either in iodo or bromo form for fast and better conversion. Thus, iodomethyl pivolate (**1**) was synthesized in 86.6% yield by reaction of chloromethyl pivolate with sodium iodide in acetonitrile at 30 °C for 5 h. Iodomethylisopropyl carbonate (**2**) was synthesized in 91.5% yield by reaction of chloromethylisopropyl carbonate with sodium iodide by using acetonitrile solvent at 30 °C for 22 h. Synthesis of 2-acetoxyethyl bromide (**3**) was carried out as per the procedure given in U.S. Patent 515556. Thus, the reaction of ethylene glycol with acetic acid in the presence of hydrogen bromide afforded **3** in 39.6% yield (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

Diclofenac ester prodrugs **4**, **5**, **6** were synthesized with the aim of obtaining enzymatically labile drugs and with less ulceration than the parent drug diclofenac sodium (**1a**). The prodrugs were evaluated in vitro and in vivo for their potential use as prodrugs for oral delivery.

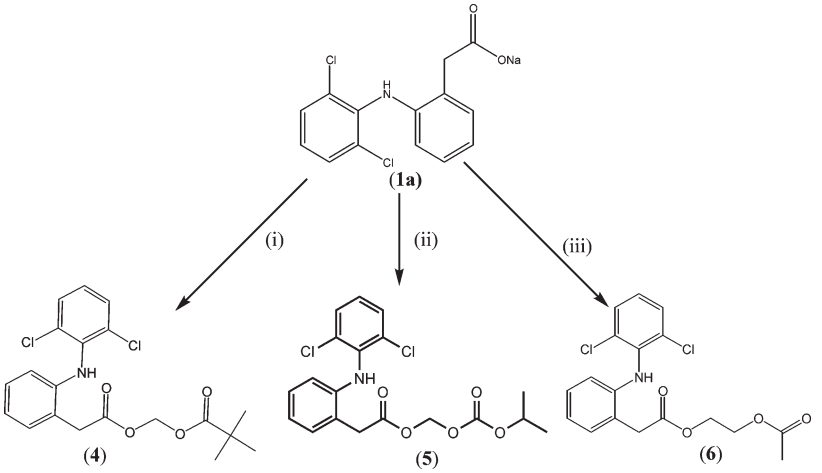
Metabolic Stability. Metabolic stability is an important property of drug candidate, since it affects parameters such as clearance, half-life, and bioavailability. A successful prodrug candidate is expected to undergo rapid, complete conversion to the parent compound in the plasma or microsomes within 1−3 h. Prodrugs **4**, **5**, and **6** and parent diclofenac sodium (**1a**) were subjected to metabolic stability in the presence of rat liver microsomes and rat plasma. Diclofenac sodium (**1a**) was fairly stable in rat liver microsomes and rat plasma; however, prodrugs **4**, **5**, and **6** were highly metabolized and converted to desirable parent compound **1a** in 30−60 min (Table 1). Chemical degradation was not observed during metabolic stability study. The corresponding loss of prodrug compounds and formation of parent drug **1** was determined by HPLC.

Formation of Parent Compound from Prodrugs in Rat Liver Microsomes (RLM) and Rat Plasma (RP). The experimental findings proved that all prodrugs were enzymatically labile and converted to parent compound diclofenac as metabolite by enzymatic hydrolysis of the ester group. The peak areas of the prodrugs in the HPLC analysis (retention time: **4**, 18.7 min; **5**, 11.97 min; **6**, 7.91 min) observed below detection level (BDL) after 60 min and correspondingly the parent drug diclofenac peak area (retention time, 4.4 min) were increased (Tables 2 and 3).

Aqueous Solubility. Aqueous solubility of diclofenac sodium (**1a**) and prodrugs **4**, **5**, **6** was determined in buffer solutions at pH 1−9. The solubility of **1a** depended on the pH of the dissolution medium at 25 °C and increased with increasing pH. Experimental values are in line with literature values.[27] The limit of detection for **1a** (0.008 μg/mL) was determined by HPLC at a wavelength 254 nm. The aqueous solubility of all prodrugs was independent of pH and was practically insoluble in acidic, neutral, and basic aqueous solution (Table 4). The limit of detection (LOD) of prodrugs **4**, **5**, **6** was determined by HPLC (**4**, 0.0837 μg/mL; **5**, 0.166 μg/mL; **6**, 0.0636 μg/mL) at a wavelength of 254 nm. Aqueous solubility of ester prodrugs (**4**, **5**, and **6**) was lower than that of parent diclofenac sodium, which is related to the increased lipophilicity of esters. Increased molecular size by introducing a bulky group through ester linkage, as well as masking the hydrophilic carboxylic group of diclofenac can lead to poor solubility of prodrugs.

Partition Coefficient (log *P*). The partition coefficients (log *P*) of diclofenac sodium (**1a**) and prodrugs **4**, **5**, **6** were determined by the HPLC method in octanol−buffer system. Partition coefficient values for all prodrugs were found to be higher than diclofenac sodium at pH 7.4. Results of log *P* at pH 3.0 and pH 7.4 are summarized in Table 5. Higher log *P* values of all prodrugs at pH 7.4 indicated that prodrugs are more lipophilic than the parent drug. Partition coefficient results of diclofenac sodium are in line with the value reported in the literature.[28−31]

Scheme 2. Synthesis of Diclofenac Prodrugs 4, 5, 6[a]



[a] Reagents and conditions: (i) $ICH_2OCOC(CH_3)_3$, Na_2CO_3, DMAc, −15 °C, 60 min; (ii) $ICH_2OCOOCH(CH_3)_2$, Na_2CO_3, DMAc, −10 °C, 45 min; (iii) $BrCH_2CH_2OCOCH_3$, Na_2CO_3, DMAc, 55 °C, 330 min.

Table 1. Metabolic Stability of Diclofenac Sodium and Prodrugs in Rat Liver Microsomes (RLM) and Rat Plasma (RP)

metabolic stability in rat liver microsomes				metabolic stability in rat plasma			
	% remaining in RLM[a]				% remaining in RP[a]		
compd	0 min	30 min	60 min	compd	0 min	60 min	120 min
1a	100	84.4	88.8	**1a**	100	86.2	69.4
4	100	2.5	0.0	**4**	100	0.0	0.0
5	100	2.2	1.2	**5**	100	0.0	0.0
6	100	0.0	0.0	**6**	100	0.0	0.0

[a] Percentage of prodrug remaining after metabolism was calculated by the ratio of the peak area at 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.

Table 2. Formation of Diclofenac Parent Compound (1a) from Prodrugs 4, 5, 6

formation of parent compound from prodrugs in rat liver microsomes				formation of parent compound from prodrugs in rat plasma			
	diclofenac peak area in HPLC analysis				diclofenac peak area in HPLC analysis		
compd	0 min	30 min	60 min	compd	0 min	30 min	60 min
4	0	250711	280652	**4**	0	100565	101220
5	0	396209	452071	**5**	0	46076	36528
6	0	256817	295451	**6**	0	105658	109889

Table 3. Disappearance of Prodrugs 4, 5, 6 by Enzymatic Biotransformation in RLM and RP

peak area of prodrugs in presence of rat liver microsomes				peak area of prodrugs in presence of rat plasma			
	prodrug peak area in HPLC analysis				prodrug peak area in HPLC analysis		
compd	0 min	30 min	60 min	compd	0 min	30 min	60 min
4	311622	7636	0	**4**	101079	0	0
5	569902	12271	0	**5**	46076	0	0
6	310206	0	0	**6**	117720	0	0

Anti-Inflammatory Assay. The anti-inflammatory activity of prodrugs was evaluated by using the in vivo rat carrageenan induced paw edema method.[24] All diclofenac produgs **4**, **5**, **6** produced a significant anti-inflammatory effect when administered

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orally in rats. Prodrug **4** emerges as a better anti-inflammatory drug than the prodrugs **5** and **6**. Inhibition of paw volume in carrageenan-induced paw edema is shown in (Table 6).

Acute Ulcer Assay. The most common side effects associated with the long-term administration of NSAIDs are gastric erosions, ulcer formation, and sometimes severe bleeding. Therefore, the potential ulcerogenic side effects of prodrugs were determined in rats and compared to those produced by the parent compounds after single dose oral administration.

The ulcerogenicity assay was performed with single dose diclofenac sodium (50 mg/kg) and prodrug (100 mg/kg) in rats. Lesser degree of ulcers was observed in rats that were treated with prodrugs **4**, **5**, **6**, compared to animals treated with diclofenac sodium **1a** (Table 7).

Animals treated with diclofenac sodium developed an average of 50 ulcerogenic lesions with only a single dose administration, and around 6.0% of the lesions were considered to be large (higher than 3 mm diameter). This allowed for the classification of lesions, which was scored depending upon the severity of mucosal damage. Prodrug **4** on 100 mg/kg single dose administration led to the development of only six small lesions (<1 mm diameter). Prodrug **5** on 100 mg/kg single dose administration led to the development of around 35 lesions of level I (91.42%), level II (2.86%), and level III (5.71%), and prodrug **6** developed only eight ulcerogenic lesions of level I (<1 mm diameter) with similar oral dose concentration. The results were obtained with an average of six animals analyzed per group (n = 6). These findings suggest that masking of carboxylic function of diclofenac successfully decreased gastroulcerogenicity.

Ulcer index (UI) is calculated based on the lesions developed on single dose administration in rats. All three prodrugs showed an improved safety profile compared with the parent reference compound even at higher dose (Table 8).

Table 4. Solubility of Diclofenac Sodium (1a) and Prodrugs 4, 5, 6 in Buffer Solution

		solubility, μg/mL				
pH	medium[a]	**1a**[c]	**1a**[b]	**4**[b]	**5**[b]	**6**[b]
1.0	0.2 M HCl buffer		1.27	BDL	BDL	BDL
1.2	0.1 M HCl	1.20				
3.0	0.1 M citric acid buffer		1.785	BDL	BDL	BDL
5.2	0.1 M phosphate buffer		43.7			
5.5	acetate buffer	36.0		BDL	BDL	BDL
7.4	0.2 M phosphate buffer	5150	5280	BDL	BDL	BDL
9.0	0.1 M alkaline borate buffer	15180	13960	BDL	BDL	BDL

[a] Buffer solutions were prepared as per USP 32. [b] Experimentally determined. Results are reported as the mean ± SD (n = 2). BDL: below detection limit. [c] Solubility is reported in the literature.[27]

Table 5. Partition Coefficient of Diclofenac Sodium (1a) and Prodrugs 4, 5, 6

	partition coefficient (log P)[a]	
compd	phosphate buffer, pH 7.4 (0.2 M)	citric acid buffer, pH 3.0 (0.1 M)
1a[b]	0.853 and 1.1	
1a	0.91	3.99
4	3.72	4.08
5	2.46	3.90
6	4.57	4.33

[a] Results are expressed as the mean of six tests (n = 6). [b] Reported in the literature.[28−31]

Table 7. Ulcerogenic Effect of Diclofenac Sodium (1a) and Prodrugs 4, 5, 6 in Rats

compd	no. of ulcers	level I (<1 mm)	level II (1−3 mm)	level III (>3 mm)
1a	50	40 (80.0%)	7 (14%)	3 (6.0%)
4	6	6 (100.0%)	0	0
5	35	32 (91.4%)	1 (2.86%)	2 (5.71%)
6	8	8 (100.0%)	0	0

Table 8. Ulcer Index for Prodrugs 4, 5, 6 and Diclofenac Sodium (1a)

	dose		
group	(mg/kg)	(μmol/kg)	ulcer index[a] (UI)
normal			0
1a	50	157	63
4	100	244	6
5	100	273	40
6	100	261	8

[a] Results are expressed as the mean ± SEM (n = 6).

Table 6. Anti-Inflammatory Activity of Prodrugs 4, 5, 6 and Parent Compound 1a in Carrageenan Paw Edema in Rats (Data Represented as the Mean ± SEM, n = 6)

	dose			
groups	(mg/kg)	(μmol/kg)	paw volume (mL)	% inhibition[d] at 180 min
normal			1.95 ± 0.05	
carrageenan (1%) 0.1 mL			###3.77 ± 0.21[a]	
1a	50	157	***2.88 ± 0.12[b]	49.17
4	100	244	***2.74 ± 0.07[b]	56.33
5	100	273	**3.03 ± 0.14[c]	40.88
6	100	261	***2.90 ± 0.07[b]	47.51

[a] P < 0.001, compared to normal control. [b] P < 0.001, compared to carrageenan control. [c] P < 0.01, compared to carrageenan control. [d] Inhibitory activity in a carrageenan-induced raw paw edema assay in the group of animals (n = 6) was calculated by using following equation: % inhibition = (carragennan − test compound)/(carragennan − normal) × 100.

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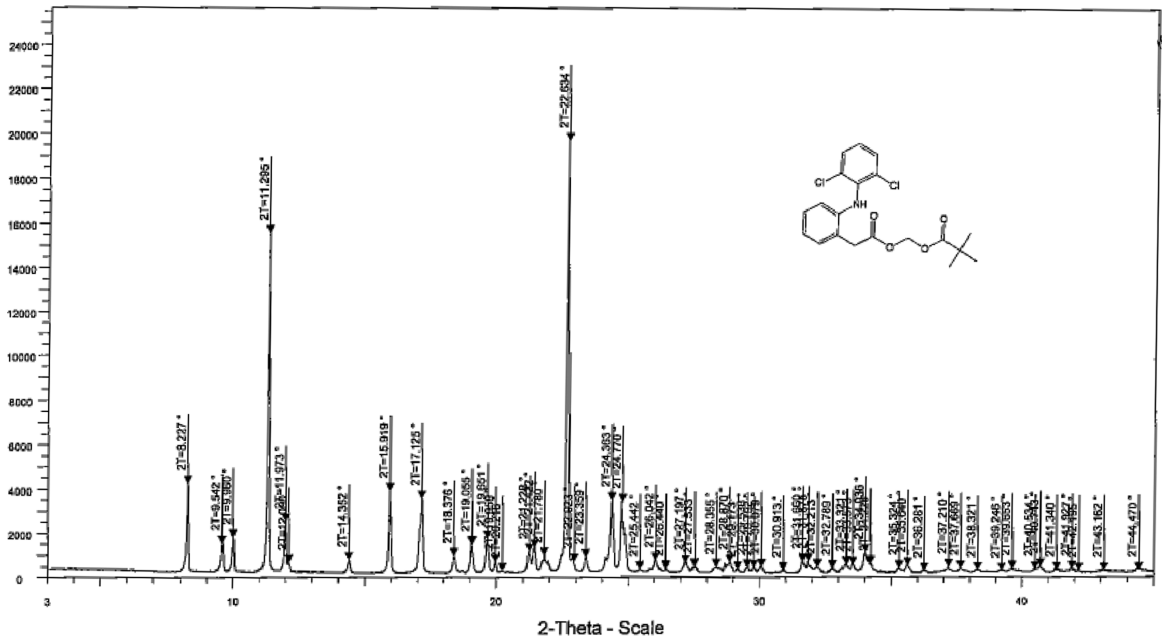

Figure 3. XRD diffractogram for prodrug **4**.

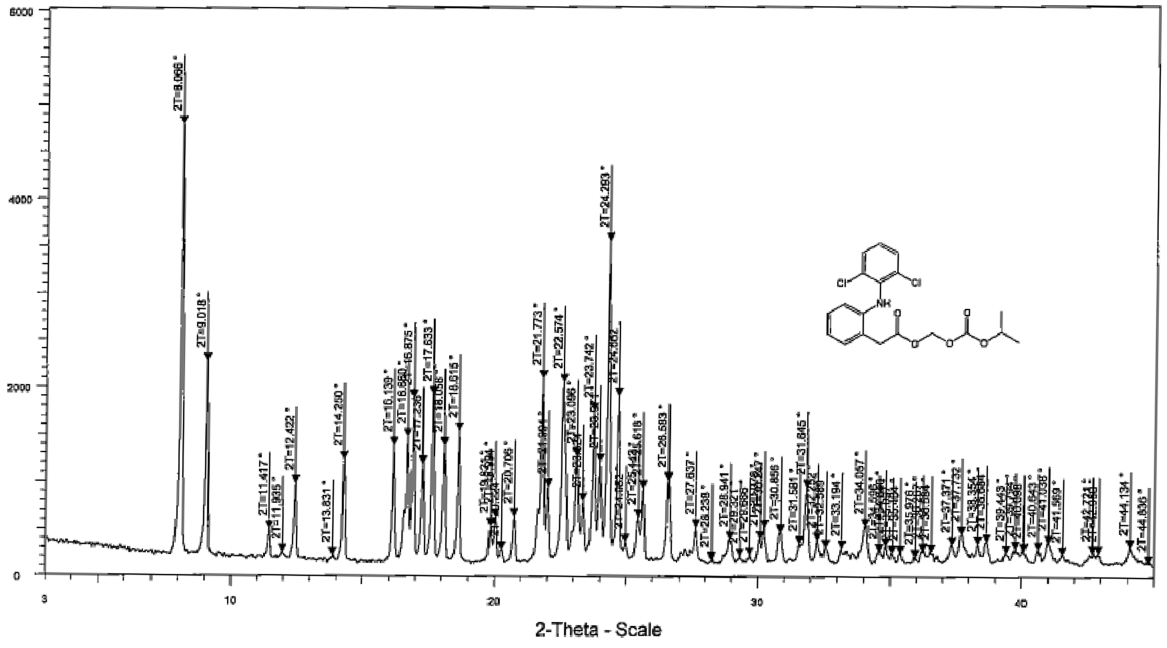

Figure 4. XRD diffractogram for prodrug **5**.

Unlike parent compound **1a** (ulcer index (UI) = 63), prodrug **4** (UI = 6) and prodrug **6** (UI = 8) produced very few detectable lesions on the gastric mucosa in the group of animals examined and prodrug **5** (UI 40) produced comparatively more detectable lesions. This represents a remarkable improvement considering that diclofenac sodium (UI = 63) was the most irritant compound on a molar basis.

Powder X-ray Diffraction of Prodrugs (PXRD). The solid state morphology of active pharmaceutical ingredient (API) is a key parameter for their further utilization when several forms can coexist as crystalline and amorphous and/or as different polymorphs (allotropes). Solid state morphology is particularly important for API, as their morphology can have a significant impact on their bioavailability and stability. Hence, it is necessary to know the solid state morphology of prodrugs **4**, **5**, **6** in which biological studies were performed. The diffraction spectrum of prodrugs **4**, **5**, **6** showed that the drugs are a highly crystalline powder when screened in a Bruker AXS D8 Advance diffractometer. The prodrug **4** possesses sharp peaks at 2θ equal to 8.27°, 9.54°, 9.96°, 11.29°, 15.91°, 17.15°, 22.63°. The prodrug **5** possesses sharp peaks at 2θ equal to 8.06°, 9.01°, 11.41°, 12.4°, 14.25°, 16.13°, 16.66°, 16.87°, 17.2°, 17.63°, 18.03°, 18.6°, 19.8°, 20.2°, 20.7°, 21.99°, 22.57°, 23.09°, 23.4°, 23.74°, 23.9°, 24.29°, 24.66°, 25.61°, 26.58°. The prodrug **6** possesses sharp peaks at

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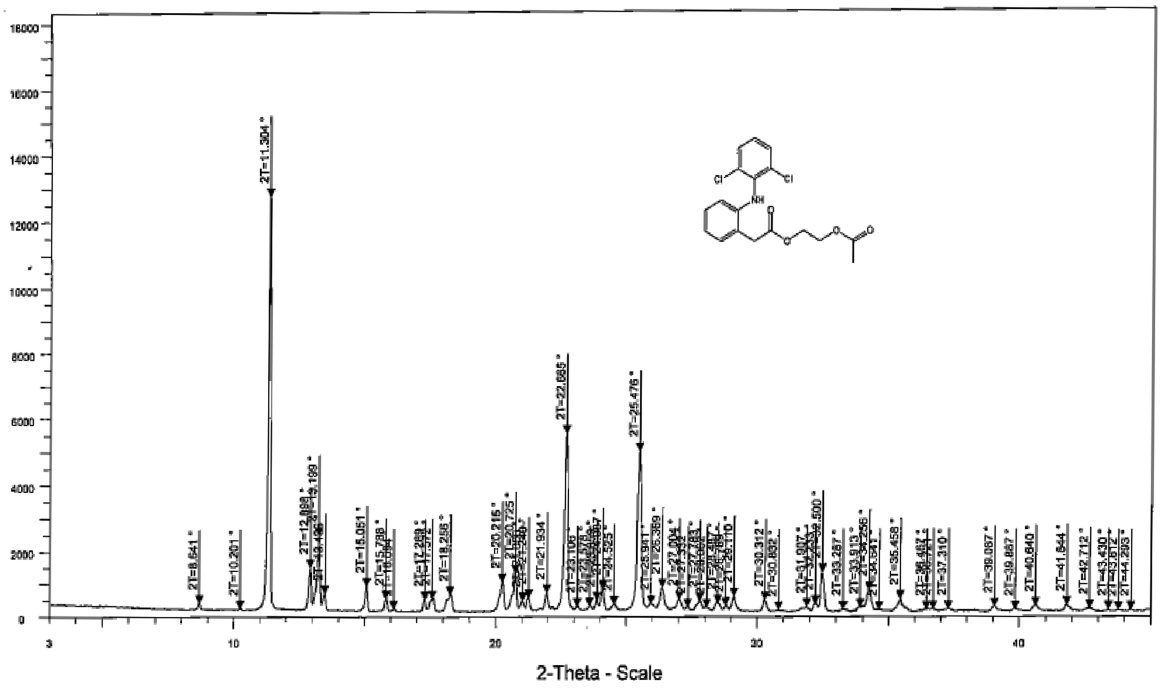

Figure 5. XRD diffractogram for prodrug **6**.

2θ equal to 11.3°, 12.89°, 13.19°, 15.05°, 22.66°, 25.47°. XRD patterns for prodrugs **4**, **5**, **6** are displayed in Figures 3, 4, and 5.

CONCLUSIONS

Diclofenac prodrugs were evaluated for their anti-inflammatory and ulcer potential by known experimental techniques. These prodrugs emerged as anti-inflammatory agents with lesser potential for ulcer than the parent drug diclofenac sodium. Ulcer index (UI) studies showed that the diclofenac prodrug **4** (UI = 6), prodrug **6** (UI = 8), and prodrug **5** (UI = 40) were substantially less ulcerogenic than the parent diclofenac sodium **1a** (UI = 63). Prodrugs **4**, **5**, and **6** were rapidly transformed enzymatically to the parent drug diclofenac in both rat liver chromosome and rat plasma. On the basis of in vitro and in vivo studies, prodrug **4** emerged as a potent and an alternative drug to diclofenac sodium for prevention of gastrointestinal disorders.

EXPERIMENTAL SECTION

General. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded using a Bruker Advance spectrophotometer. IR spectra were acquired by using a FTIR Perkin-Elmer model RXI spectrometer and THERMO IR spectrometer Model Nicolet-380. Mass of compounds was performed in PE-SCIEX API-3000 LCMS/MS, (Applied Biosystem). HPLC analysis was performed by using a Waters instrument, pump model 2695, and UV detector model 2487. All prodrug compounds were analyzed by HPLC, and their purity was confirmed to be in excess of 98.0%. Melting points were recorded in Lab India, using model MR VIS. GC analysis was performed in a Perkin-Elmer model Clarus 500 and a UV spectrophotometer (UV-2401 PC, Shimadzu). In vitro metabolic stability analysis was conducted by HPLC method. Promoieties were analyzed by GC for chromatographic purity, which was confirmed to be in excess of 96.5%. Powder XRD of prodrugs was performed on a Bruker AXS D8 Advance diffractometer. The anti-inflammatory activity of prodrugs was evaluated using the carrageenan induced rat paw edema method.[24] In vivo ulcer index (UI) study was conducted on Wister rats.

Iodomethyl Pivalate (1). Chloromethyl pivalate (100 g, 0.664 mol) was treated with sodium iodide (180 g, 1.20 mol) in acetonitrile (200 mL) 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 under stirring into a mixture of dichloromethane (1000 mL) and water (1000 mL), stirred for 10 min, and allowed to separate into phases for 10 min. The lower aqueous layer was separated and discarded. The organic layer was washed with 2% sodium thiosulfate ($Na_2S_2O_3$) and concentrated under vacuum to give **1** as a yellowish oil (138.8 g, 86.6%). Purity by GC, 99.01%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.24 (s, 9H, $-C(CH_3)_3$), 5.93 (s, 2H, $-CH_2$); IR (Nujol) cm^{-1}1759 ($C{=}O$), 1097 ($C{-}O$ streching).

1-Iodomethylisopropyl Carbonate (2). Chloromethylisopropyl carbonate (25 g, 163.8 mmol) was treated with sodium iodide (44.4 g, 296 mmol) in acetonitrile (50 mL) at 30 °C for 22 h under N_2 atmosphere. Reaction progress was monitored by GC. The reaction mixture was transferred into a mixture of dichloromethane (250 mL) and water (250 mL). The mixture was stirred vigorously for 10 min and allowed to separate into two phases for 15 min at 20 °C. The aqueous layer was discarded. The organic layer was washed with 2% sodium thiosulfate ($Na_2S_2O_3$) and the organic layer was evaporated under vacuum, affording **2** as brownish viscous oil (36.6 g, 91.5%). Purity by GC, 99.01%. Viscous oil was used in the next stage without further purification. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.93 (s, 2H, $-OCH_2I$)), 4.95 (h, 1H, J = 6.32 Hz, $-CH(CH_3)_3$), 1.35 (s, 6H, $-CH(CH_3)_3$); IR (KBr) cm^{-1} 1759 (C=O), 1077 ($C{-}O$).

2-Acetoxyethyl Bromide (3). 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 until 130 mL of water was obtained by azeotropic distillation 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. 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. The reaction mixture was subjected to fractional distillation in vacuo, and the main fraction was collected at 40−47 °C. The main

fraction was washed with a chilled brine solution (100 mL) to afford **3** 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, $-COCH_3$), 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).

Pivoloxymethyl 2-[(2,6-Dichlorophenyl)Amino]Benzene Acetate (4). A solution of **1a** (10 g, 31.4 mmol) in DMAc (45 mL) was treated with sodium carbonate (0.33 g, 3.1 mmol) at −5 °C for 10 min under a N_2 atmosphere. The mixture was cooled to −15 °C, followed by addition of iodomethyl pivalate **1** (7.6 g, 31.4 mmol). The mixture was stirred vigorously at −15 to −13 °C for 60 min under N_2 atmosphere. The reaction progress was monitored by HPLC. The reaction mixture was added under vigorous stirring to a mixture of ethyl acetate (120 mL), water (400 mL), and sodium thiosulfate (3.2 g). The organic layer was separated after 10 min of stirring at 25 °C. The organic layer was washed with brine solution (2 × 200 mL), filtered, and the solvent was removed in vacuum. Isopropyl ether (50 mL) was added. The solid product was filtered, washed with isopropyl ether (50 mL), and dried under vacuum at 45 °C for 8 h to afford **4** as a white crystalline powder (9.8 g, 83.8%), mp 95.2 °C. Chromatographic purity (HPLC): 99.50%. MS (ESI+) m/z = 410 $(M + H)^+$. UV max (methanol): 275 nm (26 mM^{-1} cm^{-1}). IR (KBr) cm^{-1} 3358 (N−H), 1750 (C=O); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.15 [s, 9H, $-C(CH_3)_3$], 3.86 (s, 2H, $-CO-CH_2-$Ar), 5.80 (s, 2H, $-O-CH2-O-$), 6.55 (d, 1H, J = 8.0 Hz, 7Ar−H), 6.71 (bs, 1H, −NH), 6.94−7.01 (m, 2H, Ar−H), 7.11 (td, 1H, J = 8.0 Hz and J = 1.4 Hz, Ar−H), 7.22 (dd, 1H, J = 7.5 Hz and J = 1.2 Hz, Ar−H), 7.33 (s, 1H, Ar−H), 7.35 (s, 1H, Ar−H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 26.95 [(3C, $(CH_3)_3$)], 38.38−38.89 (2C, CH_2), 79.84 (1C, OCH_2O), 118.7−142.8 (12C, ArC), 171.16 (1C, C=O), 177.23 (1C, C=O).

1-[(1-Methylethoxy)carbonyloxy]methyl 2-[(2,6-Dichlorophenyl)amino]phenyl Acetate (5). A solution of **1a** (5 g, 15.7 mmol) in DMAc (22.5 mL) was treated with micronized sodium carbonate (0.25 g, 2.35 mmol) at 30 °C for 10 min under N_2 atmosphere. The mixture was cooled to −10 °C, and iodomethylisopropyl carbonate **2** (3.76 g, 15.4 mmol) was added. The reaction mixture was stirred at −10 °C for 45 min under N_2 atmosphere. The reaction progress was monitored by HPLC. Reaction mixture was added under stirring to mixture of ethyl acetate (60 mL), water (200 mL), and sodium thiosulfate (2 g). The mass was stirred vigorously for 10 min at 25 °C. The organic layer was separated and washed with brine solution (2 × 125 mL). The organic layer was treated with activated carbon (1 g) for 10 min and filtered, and the solvent was evaporated completely under high vacuum followed by cooling of the sample at −15 to −10 °C to afford a yellowish solid product **5** (5.6 g, 86.4%). Chromatographic purity (HPLC), 99.58%. Mp 52.5 °C. MS (ESI+) m/z = 412 $(M + H)^+$. UV max (methanol) 274.2 nm (45 mM^{-1} cm^{-1}). IR (KBr) cm^{-1} 3306 (N−H), 1756 (C=O), 1735 (C=O); ^{1}H NMR (DMSO, 400 MHz) δ 1.22 (d, 6H, J= 6.3 Hz, $-CH(CH_3)_2$), 3.90 (s, 2H, $-Ph-CH_2-CO-$), 4.77 (sep, 1H, J = 6.3 Hz, $-O-CH(CH_3)_2$), 5.73 (s, 2H, $-O-CH_2-O-$), 6.21 (d, 1H, J = 8.0 Hz, Ar−H), 6.82 (t, 1H, J = 7.4 Hz, Ar−H), 7.02 (bs, 1H, −NH), 7.06 (t, 1H, J = 7.7 Hz, Ar−H), 7.18−7.24 (m, 2H, Ar−H), 7.53 (s, 1H, Ar−H), 7.55 (s, 1H, Ar−H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.77 (2C, $(CH_3)_2$), 38.28 (C, CH_2Ar), 73.34 (C, $-CH(CH3)_2$), 82.33 (C, $-OCH_2O-$), 118.73−142.83 (10C, Ar), 153.41 (C, −OCOO−), 170.96 (C, $-OCOCH_2-$).

2-Acetoxyethyl 2-[(2,6-Dichlorophenyl)amino]phenyl Acetate (6). A solution of **1a** (5 g, 15.7 mmol) was treated with sodium carbonate (0.33 g, 3.14 mmol) in DMAc (20 mL) at 30 °C for 10 min under N_2 atmosphere. 2-Acetoxyethyl bromide **3** (3.28 g, 19.64 mmol) was added at 35 °C, and the mixture was stirred at 40 °C for 60 min, 55 °C for 4 h under N_2 atmosphere. The reaction mixture was added under stirring to the mixture of ethyl acetate (60 mL), water (200 mL), and sodium thiosulfate (2 g), and the mixture was stirred vigorously for 10 min at pH 10.75 and at 25 °C. The organic layer was separated, washed with brine solution (250 mL), filtered, and the solvent was evaporated completely under vacuum to get very viscous oil at 40 °C, which on cooling to −15 to −10 °C for 4 h solidified to the colorless desired solid product **6** (5.1 g, 85%), mp 71.2 °C. Chromatographic purity (HPLC): 98.15%. MS (ESI+) m/z = 382.1 $(M + H)^+$. UV max (methanol) 276 nm (48 mM^{-1} cm^{-1}). IR (KBr) cm^{-1} 3304 (N−H), 1750 (C=O), 1723 (C=O)cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.02 (s, 3H, $-COCH_3$), 3.83 (s, 2H, $-Ph-CH_2-CO-$), 4.28−4.37 (2 m, 4H, $-O-CH_2-CH_2-O$), 6.55 (d, 1H, J = 8.0 Hz, Ar−H), 6.85 (bs, 1H, −NH), 6.94 (m, 2H, Ar−H), 7.11 (t, 1H, J = 7.5, Ar−H), 7.23 (d, 1H, J = 7.5, Ar−H), 7.33 and 7.35 (2s, 2H, Ar−H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.88 ($-CH_3$), 38.53 (2C, $-CH_2-$Ph), 67.03 (C, $-OCH_2CH_2O-$), 62.14 (2C, $-OCH_2CH_2$ O−),118.46−142.85 (10C, Ar),170.93 ($-OCOCH_3$), 172.30 (ArCH_2CO-).

GC Analysis. *a. Analysis Method for Promoities **1** and **2**.* GC analysis was performed using a Perkin-Elmer GC model Clarus 500, column DB-1, 30 m × 0.53 mm, 1.5 μm, by maintaining the following chromatographic parameters: 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, 1:20.

*b. Analysis Method for Promoity **3**.* A Perkin-Elmer GC, model Clarus 500, column DB-5, 30 m × 0.53 mm, 5 μm, was used. Chromatographic parameters were as follows: 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, up to 110 °C for 6 min; rate 2, 35 °C/min up to 250 °C for 10 min; injection temperature, 125 °C; detector temperature, 270 °C; flow (carrier), 5.0 mL/min; injection volume, 0.2 μL; split, 1:10.

HPLC Analysis. In-process analysis and determination of chromatographic purity and partition coefficients of prodrugs **4**, **5**, **6** were performed by HPLC instrument (Waters alliance), pump 2695, and UV detector 2487 with the following chromatographic parameters: wavelength 254 nm; column, YMC-Pack C_8, 100 mm × 4.6 mm, 3 μm; injection volume, 20 μL; run time, 20 min. Separation was performed as isocratic elution. Mode of operation was isocratic. Mobile phase was as follows. Solution A: mix of 2.0 mL of glacial acetic acid in 1000 mL of water (pH 3.0). Solution B: acetonitrile (filtered and degassed). Mobile phase mix ratio was 30 volumes of solution A and 70 volumes of solution B. Flow rate was 0.8 mL/min at 25 °C.

Metabolic Stability Analysis. Analysis was performed by using an HPLC instrument (Waters alliance), pump 2695, and PDA detector 2996 with the following chromatographic parameters: wavelength, 275 nm; column, Inertsil C_{18}, 3V, 250 mm × 4.6 mm, 5 μm; injection volume, 20 μL; run time, 20 min. Mode of operation was isocratic. Solution A was as follows: mix of 2.0 mL of glacial acetic acid in 1000 mL of water (pH 3.0), Solution B was as follows: acetonitrile (filtered and degassed). Mobile phase was a mixture of 30 volumes of solution A and 70 volumes of solution B. Flow rate was 1.0 mL/min at 25 °C.

Aqueous Solubility. Diclofenac sodium and prodrug solubility was determined in various pH buffers at pH 1.0, pH 3.0, pH 5.2, pH 7.4, and pH 9.0 at 25 °C. An excess compound was added to buffer solutions, and the suspension was shaken for 5 h at 25 °C on mechanical shaker at 350 rpm. The solution was filtered through a Millipore filter (0.22 μm) and analyzed quantitatively by HPLC.

Partition Coefficient. The partition coefficient of diclofenac sodium and prodrugs was determined by the HPLC method. Before a partition coefficient, 1-octanol and buffer solution were mutually saturated for 24 h by stirring vigorously on a mechanical shaker at 25 °C, and the phases were allowed to separate for 24 h at 25 °C. A stock solution of test compound was prepared at 25 °C, filtered through 0.22 μm membrane filter, and the percentage of test compound was determined by HPLC. Three tests were carried out in duplicate with various volume ratios of octanol (stock solution) to presaturated buffer,

i.e., 1:1, 1:2, 2:1. The test vials were shaken on a mechanical stirrer at 350 rpm for 60 min, centrifuged for 5 min at 3500 rpm at room temperature. The concentration of the test compounds in both phases was determined precisely by HPLC at a wavelength of 254 nm. The partition coefficient (log P) was determined by the logarithm of the ratio of concentrations of un-ionized compound (solute) in octanol to aqueous solution.

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

Rat Plasma. Rat plasma was harvested from in-house rats. Fresh blood was collected from the male rat using a retro-orbital bleeding method in a 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 the further experiment.

In Vitro Physiological Stability of Prodrugs 4, 5, 6 and Diclofenac Sodium (1a) 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 (75 μL) were removed and added to ice-cold acetonitrile (75 μL) and mixed well by vortexing for 2 min. The mixture was centrifuged at 14 000 rpm for 10 min, and the supernatant was diluted with acetonitrile and analyzed by HPLC. After 0 min, the remaining sample was incubated at 37 °C for 60 and 120 min. After 60 and 120 min, the sample (75 μL) was treated with ice cold acetonitrile (75 μL) and centrifuged at 14 000 rpm for 10 min. The supernatant was diluted with acetonitrile and injected into HPLC instrument. The percentage of prodrug remaining was calculated according to the following equation.

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

Anti-Inflammatory Assay. Anti-inflammatory assay of the test compounds **4**, **5**, **6** and reference drug diclofenac sodium **1a** was evaluated by using in vivo rat carrageenan foot paw edema model reported previously.[24]

Chemicals. Carrageenan was purchased from Sigma (St. Louis, MO, U.S.). Diclofenac sodium (EP) was procured from a local bulk drug manufacturer.

Animals. All the experiments were approved by the Institutional Animal Ethics Committee (IAEC) and complied with the NIH guidelines on handling of experimental animals. Male Wistar rats weighing 150−200 g were used for the study (n = 6). 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 left paw. Animals were divided into groups, namely, normal control, carrageenan, and carrageenan treated with diclofenac prodrug (100 mg/kg) as well as parent drug diclofenac sodium at dose of 50 mg/kg, 1 h prior to carrageenan administration. Paw volume was measured using the plethysmograph after 3 h of carrageenan administration.

The percentage inhibition was calculated by using following equation:

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

Acute Ulcer Index. The ability to produce gastric damage was evaluated according to a reported procedure.[25] Animals (n = 6/group) were fasted for 18 h with free access to water. Prodrugs at 100 mg/kg and diclofenac sodium at 50 mg/kg were administered orally and evaluated for ulcer potential. Animals were sacrificed under ether anesthesia after a 6 h dosing of drug compounds. The stomach was removed, opened along the greater curvature, washed, and mounted on a thermostat sheet and examined for ulcers. Ulcerative lesions were scored as follows: (1) The length of all lesions were measured using Vernier caliper. (2) The ulcers were classified as level I, ulcer area of <1 mm diameter; level II, ulcer area of 1−3 mm diameter; and level III, ulcer area of >3 mm diameter. (3) The ulcerative lesion index (UI) was calculated as 1(number of ulcer level I) + 2(number of ulcer level II) + 3(number of ulcer level III). The sum of six animal ulcer readings was reported as the ulcer index (UI).

Rat Liver Microsomes. Rat liver microsomes were prepared in-house by previously published method[26] and used immediately in the experiments. Protein concentrations were determined by the Biorad protein assay (Bio-Rad, Hercules).

Microsomal Stability. *Materials.* Materials 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); test compounds.

Requirements. Requirements 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 solution (concentration, 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. Sample (75 μL) was immediately taken out (0 min) and transferred to the centrifuge tube containing ice cold acetonitrile (75 μL). The assay mixture was incubated in a water bath at 37 °C for 60 min and 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 all the tubes were vortexed and centrifuged at 14000g for 10 min. Aliquots of the supernatant were separated and used for analysis by HPLC. The percentage of prodrug remaining was calculated as per 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 **1**, **2**, **3**; ^{1}H NMR, ^{13}C NMR, IR, chromatographic purity (HPLC), UV, and mass data for all 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(hydroxylmethyl)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; GC, gas chromatograph; DMAc, dimethylacetamide; PXRD, powder X-ray diffraction; ND, not detected; RT, room temperature; EP, European Pharmacopoeia; Ar, aromatic; BDL, below detection limit

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