Galactosyl Prodrug of Ketorolac: Synthesis, Stability, and Pharmacological and Pharmacokinetic Evaluations

Annalisa Curcio,[‡,||] Oscar Sasso,[§,||] Daniela Melisi,*[,‡] Maria Nieddu,[#] Giovanna La Rana,[§] Roberto Russo,[§] Elisabetta Gavini,[†] Gianpiero Boatto,[#] Enrico Abignente,[‡] Antonio Calignano,[§] and Maria Grazia Rimoli[‡]

Department of Pharmaceutical and Toxicological Chemistry and Department of Experimental Pharmacology, Faculty of Pharmacy, Federico II University of Naples, Via Domenico Montesano 49, 80131, Naples, Italy, and Department of Drug, Chemistry and Toxicology and Department of Drug Sciences, Faculty of Pharmacy, University of Sassari, Via Muroni 23/a, 07100, Sassari, Italy

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Although ketorolac is one of the most potent anti-inflammatory and analgesic drugs, its use has been strongly limited owing to the high incidence of adverse effects reported, particularly in the gastrointestinal tract. Using the prodrug approach, which allows the reduction of toxicological features of the parent drug without altering its pharmacological properties, we synthesized an orally administrable prodrug of ketorolac by means of its reversible conjugation to D-galactose (ketogal). In a single dose study, its pharmacokinetic profile was compared with that of ketorolac. Moreover, we found that this prodrug was able to maintain the anti-inflammatory and the analgesic activity of the drug without giving rise to gastric ulcer formation. Thus, these results indicate that ketogal is a highly effective and valid therapeutic alternative to ketorolac itself.

Introduction

Ketorolac, a racemic mixture of pyrrolizine carboxylic acids, is one of the most effective nonsteroidal anti-inflammatory drugs (NSAIDs[a]). Because its analgesic efficacy is comparable to that of morphine, it is often administrated in postoperative pain treatment.[1] As indicated in a recent study on NSAIDs, ketorolac is among those drugs that present the highest risk of toxic reactions, a feature that has drastically limited its use despite its analgesic and anti-inflammatory efficacy.[2] Accordingly, nowadays its administration is limited to very short periods of time in the treatment of renal colic and moderate and severe postoperative pain.[3] Mild and severe gastrointestinal adverse events are ascribable to the drug's direct impact on the gastrointestinal mucosa and to its indirect effect, via blood circulation, following absorption.[4–6]

In general, a prodrug approach consists of designing pharmacologically inactive derivatives that undergo biotransformation before exhibiting their pharmacological effects following the release of the active drug. Such a technique has proven to be useful to overcome pharmaceutical and pharmacokinetic barriers in clinical drug application, including toxicity, chemical instability, low oral absorption, lack of site specificity, and poor patient acceptance.[7]

With respect to ketorolac, many efforts have been made to synthesize new prodrugs. For instance, to avert damage to the gastric mucosa while preserving satisfactory pharmacological activity, some scientists have created esteric and amidic derivatives bound to polymeric carriers or amino acids to mask the carboxylic acid group.[8,9]

Since metabolizable carbohydrates, especially hexoses, are gastroprotective agents,[10] being components of the gastric mucus,[11,12] we decided to harness the synergic action of sugar release and the prodrug approach.

On the basis of our past experience in the synthesis of prodrugs,[13–16] we chose D-galactose to design a novel ketorolac prodrug. In this paper, we report the synthesis of a ketorolac−galactose conjugate (ketogal). Both its stability and its pharmacokinetic profile were evaluated in a single dose study. Moreover, in vivo experiments were performed to assess its pharmacological activities and, more important, its potential capability to reduce ulcerogenic activity in comparison with its parent drug ketorolac.

Results

General Procedure for the Synthesis of Ketogal. Esterification of ketorolac (**1**) with 1,2,3,4-di-*O*-isopropylidene-D-α-galactopyranose (DIPG) in the presence of *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 4-(dimethylamino)pyridine (DMAP), dissolved in dry dichloromethane, yielded 75% of **1a**. Ketals were completely removed with trifluoroacetic acid (TFA) dissolved in dry dichloromethane to obtain 65% of ketogal (Scheme 1).

Stability Tests of Ketogal and Ketorolac. Data concerning chemical stability at pH 1 (hydrochloric acid, 0.1 N) and at pH 7.4 and pH 8.0 (buffer solutions) and enzymatic stability in plasma of both ketogal and ketorolac are reported in Table 1. When we evaluated chemical stability of both compounds, the prodrug appeared quite stable at both pH 7.4 and pH 8.0 and less stable at pH 1. To determine whether ketogal was suitable for enzymatic hydrolysis and thus apt to regenerate the parent drug, we incubated the compound in rat plasma. The half-life of enzymatic stability was lower than the one in buffer solutions. On the contrary, ketorolac demonstrated to be extremely stable under all conditions.

* To whom correspondence should be addressed. Phone: +39081678612. Fax: +39081678612. E-mail: melisi@unina.it.
‡ Department of Pharmaceutical and Toxicological Chemistry, Federico II University of Naples.
|| These authors contributed equally to this study.
§ Department of Experimental Pharmacology, Federico II University of Naples.
# Department of Drug, Chemistry and Toxicology, University of Sassari.
† Department of Drug Sciences, University of Sassari.

[a] Abbreviations: AUC, area under the curve; AUMC, area under the first moment curve; CL, clearance; CMC, carboxymethyl cellulose; DIPG, 1,2,3,4-di-*O*-isopropylidene-D-α-galactopyranose; DMAP, 4-(dimethylamino)pyridine; EDC, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride; ip, intraperitoneal; MRT, mean residence time; NMR, nuclear magnetic resonance; NSAIDs, nonsteroidal anti-inflammatory drugs; rpm, revolutions per minute; TLC, thin layer chromatography; TFA, trifluoroacetic acid; TMS, tetramethylsilane.

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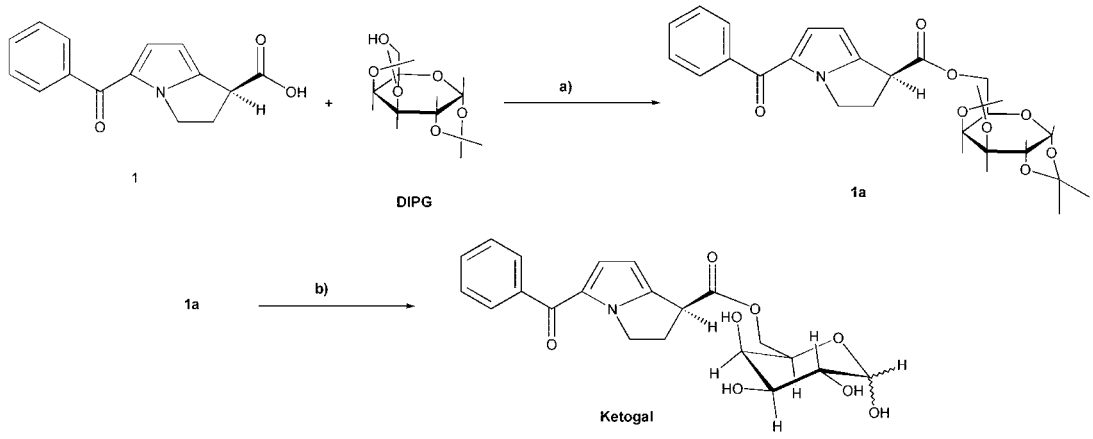
Scheme 1. Ketogal Synthesis[a]



[a] (a) EDC, DMAP/DCM dry, room temp, 12 h; (b) TFA/DCM dry, room temp, 48 h.

Table 1. Chemical and Enzymatic Stability of Ketogal[a]

	$t_{1/2}$ (h)			
compd	pH 1	pH 7.4	pH 8.0	plasma
ketogal	2.1 ± 0.15	>4	>4	1.5 ± 0.80
ketorolac	>4	>4	>4	>4

[a] Data are reported as the mean ± SEM.

Effect of Ketogal on Acetic Acid Induced and Magnesium Sulfate Induced Writhes. The ip administration of an irritating dose of 0.6% acetic acid solution produced a robust visceral pain, which peaked 5−20 min after injection of the acid. The nocifensive behavior was attenuated in a dose-dependent manner by oral administration of ketorolac (0.01−10 mg/kg) (Figure 1A). In particular, the effect was significant at doses of 1 and 10 mg/kg ($p < 0.01$). Synthetic derivative ketogal showed, at equimolecular doses of ketorolac (0.0163−16.3 mg/kg), a dose-dependent analgesic effect (Figure 1A) stronger than that produced by ketorolac. In fact, 0.163 mg/kg ketogal produced a significant ($p < 0.01$) analgesic effect, whereas the equimolecular dose of ketorolac (0.1 mg/kg) had no effect (Figure 1A).

Acetic acid-evoked writhing responses are accompanied by a profound, often lethal peritoneal inflammation. To determine whether ketorolac and ketogal inhibit visceral pain, we tested the effects of these compounds on magnesium sulfate-evoked writhing in experimental models that exhibit a less marked inflammatory component.

Magnesium sulfate produced a reversible nocifensive response when injected intraperitoneally in mice. The administration of magnesium sulfate 120 mg/kg, ip caused a mean of 6 ± 0.9 writhing episodes in mice ($n = 6$). Ketorolac (0.01−10 mg/kg) and ketogal (equimolecular doses of ketorolac), administered 1 h before magnesium sulfate, inhibited this response in a dose-dependent manner (Figure 1B).

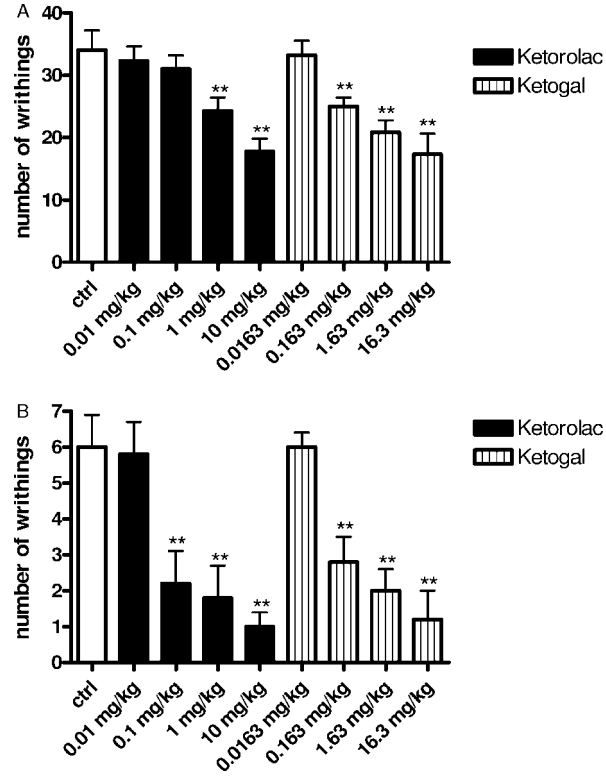


Figure 1. Analgesic effect on acetic acid-induced (A) or magnesium sulfate-induced (B) writhings. Control animals (open bar) received oral doses of CMC, ketorolac (closed bar, 0.01−10 mg/kg), or ketogal (straight bar, 0.0163−16.3 mg/kg) 1 h before ip administration of chemical agents. Data are expressed as mean values ± SEM of six animals for each group: (**) $p < 0.01$ versus control group.

Effect of Ketogal on Carrageenan-Induced Paw Edema. As already reported in a previous characterization of this model,[17] mouse paw edema developed in two distinct phases: an acute first phase peaking at 6 h and a second phase peaking at 72 h after carrageenan challenge. Ketogal treatment (0.163, 1.63, or 16.3 mg/kg), 1 h before carrageenan, markedly reduced paw edema in a time- and dose-dependent manner (Figure 2B). During the first phase (0−6 h), all doses of ketogal significantly ($p < 0.001$) inhibited edema formation (Figure 2B). In the second phase (24−96 h), ketogal remained significantly effective ($p < 0.01$ for 1.63 and 16.3 mg/kg) until 48 h after carrageenan (Figure 2B). Furthermore, at the highest dose (16.3 mg/kg), it was still significantly effective even 72 h after carrageenan challenge (Figure 2B).

To compare the efficacy of ketogal with that of ketorolac, the latter was tested under the same experimental conditions at a doses of 0.1, 1.0, and 10 mg/kg (equimolecular doses of

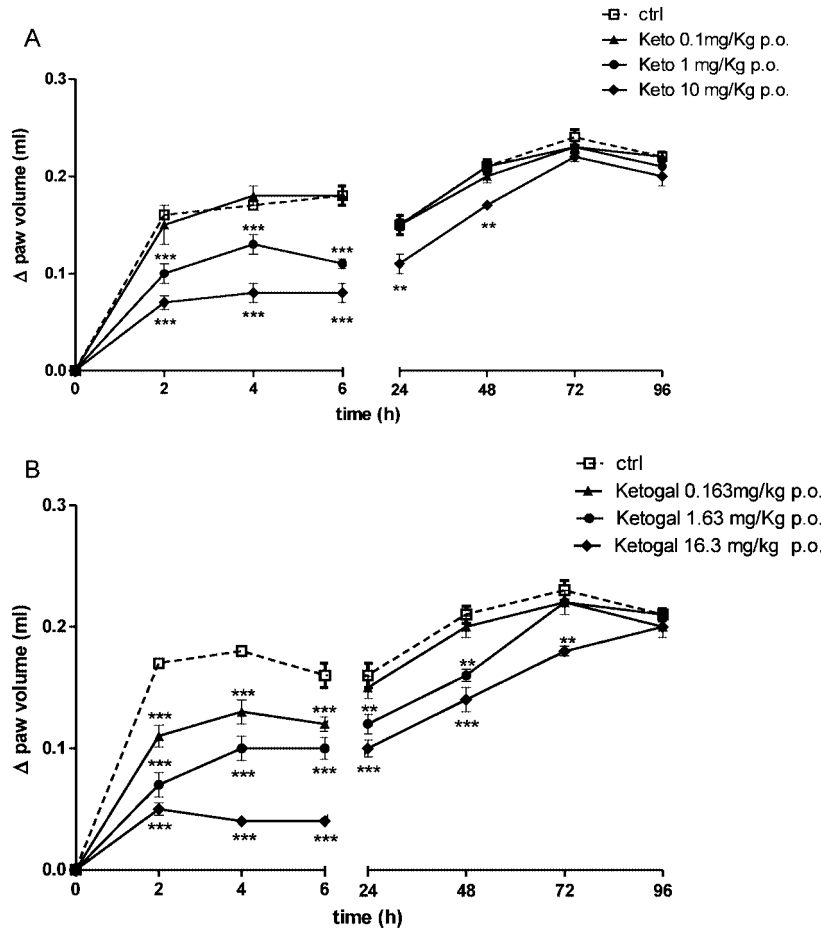


Figure 2. Anti-inflammatory effect of oral administration of ketorolac (A) (0.1−10 mg/kg) and ketogal (B) (0.163−16.3 mg/kg) on mouse carrageenan paw edema. Control animals were treated with CMC, ketorolac, or ketogal 1 h before subplantar injection of carrageenan. Data are expressed as mean values ± SEM of six animals for each group: (***) $p < 0.001$; (**) $p < 0.01$ versus control group.

Table 2. Ulcerogenic Activity

drug	oral dose (mg/kg)	no. of ulcers[a]	gastric lesion score
control (CMC, 0.5%)		0	
ketorolac	0.1	4 ± 0.4	10
ketogal	0.163	1 ± 0.4	1

[a] Data are reported as the mean ± SEM.

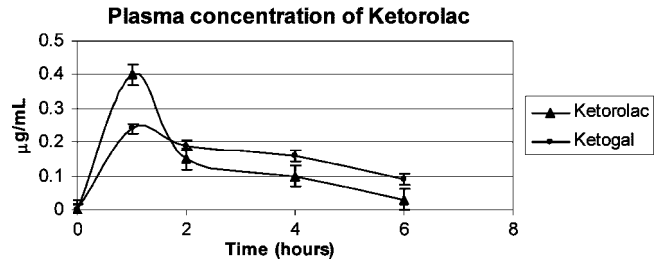


Figure 3. Plasma concentration time profile of ketorolac upon oral administration of ketorolac itself (0.100 mg/kg) and of ketogal (0.163 mg/kg). Data are expressed as mean values ± SD ($n = 3$).

ketogal) (Figure 2A). The lowest dose did not reduce paw edema, whereas 1.0 and 10 mg doses elicited a time- and dose-dependent reduction. During the second phase, only the highest dose (10 mg) was active until 48 h ($p < 0.001$ at 24 h; $p < 0.01$ at 48 h) (Figure 2A).

Effect of Ketogal on Ulcerogenic Activity. Ketogal remarkably decreased ulcerogenic activity compared to ketorolac. Noteworthy, ketogal caused only irritation without ulceration (Table 2). Thus, our results indicate that ketogal remarkably reduced ulcerogenicity compared to ketorolac itself.

Pharmacokinetic Studies. The pharmacokinetic profile of ketogal was compared to that of ketorolac according to plasma, stomach, kidney, and liver concentrations after oral administration of ketorolac (0.100 mg/kg) and ketogal (0.163 mg/kg).

Ketorolac plasma concentration profile versus time is shown in Figure 3, and pharmacokinetic parameters are reported in Table 3. A different pharmacokinetic profile was observed for the ketorolac, resulting from prodrug hydrolysis and the ketorolac itself. A single dose of ketorolac, orally administrated, was rapidly absorbed and eliminated within 6 h (Figure 3). Instead, although plasma concentrations of the drug released from ketogal were lower than those of ketorolac during the first 2 h, they nonetheless remained higher than that of the drug itself throughout the remaining time of the experiments. Therefore, ketogal revealed the typical profile of a controlled release drug system. The pharmacokinetic parameters related to these curves were calculated and are listed in Table 3. The areas under the curve from time 0 to 6 h, AUC_{0-6}, obtained from both curves, were very similar, thus indicating that the prodrug did not modify the bioavailability of ketorolac. On the other hand, the pharmacokinetic parameters pinpointed that the pharmacokinetic behavior of the drug released from ketogal and the drug itself was different, thereby suggesting that the prodrug was responsible for a sustained release of the drug and its longer half-life. Therefore, the introduction of a galactosyl promoiety on ketorolac elicited a slow and sustained release of ketorolac, thus prolonging its activity. Regression analysis was performed, and

Table 3. Pharmacokinetic Parameters[a]

drug	$t_{1/2}$ (h)	t_{max} (h)	C_{max} (μg/mL)	AUC_{0-6} (μg·h/mL)	$AUMC_{0-6}$ (μg·h/mL)	MRT[b] (h)	$CL_{(area)}$ [c](mL/h)	$t_{1/2}$ [d](h)	$t_{1/2}$ [e](h)
ketorolac	1.65 ± 0.05	1 ± 0	0.40 ± 0.05	0.8550 ± 0.08	3.3 ± 0.14	3.1 ± 0.1	94.23 ± 6.08	1.99 ± 0.01	0.39 ± 0.41
ketorolac released by ketogal	5.23 ± 0.09	1 ± 0	0.24 ± 0.03	0.9350 ± 0.03	5.6 ± 0.75	4.5 ± 0.37	80.36 ± 4.34	2.4 ± 0.32	1.68 ± 0.03

[a] Data are reported as the mean ± SEM. [b] MRT: mean residence time (time for 63.2% of administered dose to be eliminated). [c] CL: clearance. Systemic clearance based on observed data points: AUC_{0-6}. [d] Elimination phase (terminal phase regression analysis, 4−6 h). [e] Absorption phase (first residual regression analysis, 1−2 h).

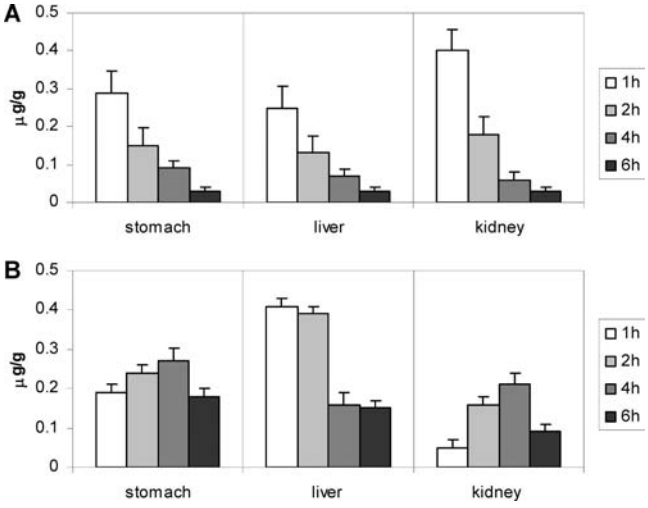


Figure 4. Ketorolac distribution vs time in stomach, liver, and kidney after oral administration of ketorolac itself (0.100 mg/kg) (A) and of ketogal (0.163 mg/kg) (B).

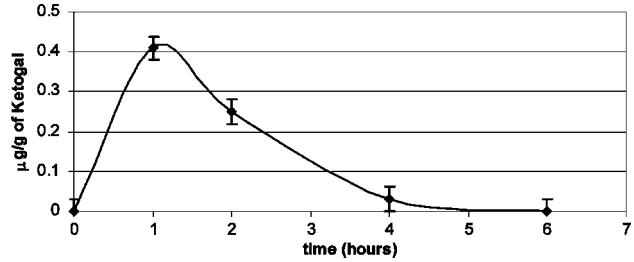


Figure 5. Stomach concentration of ketogal vs time after oral administration (0.163 mg/kg). Data are expressed as mean values ± SD ($n = 3$).

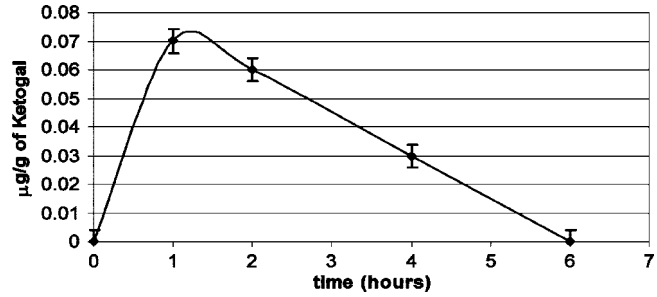


Figure 6. Liver concentration of ketogal vs time after oral administration (0.163 mg/kg). Data are expressed as mean values ± SD ($n = 3$).

pharmacokinetic parameters of absorption and elimination phase were calculated by plotting experimental data as semilog concentration vs time. Our data demonstrated that the $t_{1/2}$ absorption phase of ketorolac released from ketogal was higher than that of the drug alone (Table 3), a finding that demonstrates a slower absorption of the drug when administered as a prodrug. Finally, the values of MRT, CL, and $t_{1/2}$ elimination phase demonstrated that the elimination rate of ketorolac released from the prodrug was slower than that from the drug itself (Table 3). The following data are based on the determination of drug distribution in stomach, liver, and kidney after oral administration of both compounds (Figure 4). Six hours following oral administration of ketorolac, initial peak concentrations were followed by a progressive decrease in drug amount in all three organs analyzed (Figure 4A). By contrast, oral doses of ketogal were slowly hydrolyzed in the stomach, an event that reduced the drug concentration but prolonged its release for up to 2 h after administration. These effects were due to its stability at acidic pH (about 2 h) and its $t_{1/2}$ absorption phase, which was remarkably higher than the value found for ketorolac (Table 3). Remarkably, such behavior was consistent with the scarce ulcerogenic effect of the prodrug. Moreover, the lower absorption rate of the prodrug determined, in turn, a lower concentration of the drug in the plasma (Figure 3) and its accumulation in the stomach (Figure 4B). Figure 5 also illustrates ketogal concentration in the stomach throughout the entire experimental procedure. The presence of ketogal in the liver (Figure 6) and the distribution profile of ketorolac from the prodrug may also indicate that the prodrug was absorbed and accumulated in the liver. Indeed, the rapid and significant hydrolysis of the prodrug in the liver caused an increase in ketorolac levels (Figure 4B). Finally, the distribution profile of the drug and prodrug in the kidney (Figure 4A and Figure 4B) demonstrated that the amount of ketorolac released from the prodrug was eliminated at a slower rate than the drug alone, as demonstrated by the elimination parameters (Table 3). Indeed, concerning ketogal, the drug was found in the kidney after 4 h while ketorolac concentration was significantly high only within the first 2 h. No trace of the prodrug was detected in kidney samples.

Discussion

Several NSAIDs drugs, including ketorolac, cause a wide range of gastrointestinal adverse events. Although ketorolac exerts an excellent therapeutic activity, its chronic use has long been limited owing to the high incidence of these adverse effects. To overcome these undesired drug reactions, new strategies have been investigated to create suitable pharmaceutical formulations that would preserve the powerful anti-inflammatory and analgesic activity of the parent drug while minimizing its negative effects.[18–20]

In this paper, we synthesized for the first time a galactosyl derivative of ketorolac, which we named ketogal. Drug stability experiments were carried out to verify the handling of this new prodrug and its ability to release the parent drug both enzymatically and nonenzymatically in plasma and under physiological conditions (pH 7.4), respectively. As this study entailed oral administration of the drugs, stability analyses were also performed under acidic and basic conditions to mimick gastric and intestinal pH. Under the same experimental conditions, ketorolac showed high stability, thus demonstrating it to be the active drug.

Pharmacological investigations of the synthesized prodrug were done for anti-inflammatory, analgesic, and ulcerogenic activities. The range of ketogal doses (0.0163−16.3 mg/kg) administered was equimolar to the range of doses of ketorolac (0.01−10 mg/kg). Our results clearly showed that ketogal administration minimized the gastrointestinal toxicity while preserving the anti-inflammatory and analgesic activity. Noteworthy, ketogal effectively reduced acetic acid-induced writhings and carrageenan-induced paw edema up to 6 h after administration. Furthermore, the ulcerogenic index of ketogal was significantly lower than the one exhibited by the parent drug. Intriguingly, the reduction in adverse events obtained with the ketorolac−galactose conjugate was most likely attributable to its ability to inhibit the direct contact of carboxyl group of ketorolac with the gastric mucosa, a circumstance that eventually leads to gastric damage.

Altogether, our pharmacokinetic studies of ketogal have demonstrated that this prodrug is a potential candidate for a slower and sustained release form of ketorolac. Equally important is the fact that we were able to demonstrate the capability of this new prodrug to reduce ulcerogenicity while preserving the high pharmacological efficacy of its parent drug, as demonstrated by the fact that the drug reached therapeutic but not toxic plasma concentrations.

On the basis of these results, this prodrug approach may constitute a possible pharmacological solution to minimize gastrointestinal toxicity while preserving the powerful anti-inflammatory and analgesic activities of the ketorolac.

Experimental Section

Drugs and Chemicals. All chemicals used for the synthesis of ketogal were purchased from commercial sources (Sigma-Aldrich). The commercially available ketorolac tris salt was dissolved in water, acidified with an aqueous solution of HCl until a pH level of 4.5 was attained, and extracted with $CHCl_3$. To obtain ketorolac free acid **1**, the organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo. All reagents and solvents used for stability tests were analytical grade. Deionized and distilled water was purified through a Milli Q system (Millipore). For enzymatic stability, mouse plasma (male Swiss mice) was supplied by the Department of Experimental Pharmacology, Faculty of Pharmacy, "Federico II" University of Naples, Italy.

Chemistry. The course of reactions and purity of products were monitored by TLC (silica gel 60F254s, Merck), and spots were detected by UV radiation and after iodine exposition. Flash chromatography was performed on Merck silica gel (0.040−0.063 mm). Melting points (mp) were determined with a Buchi B-540 hot stage microscope. Elemental analyses, indicated by the symbols of the elements, were performed on a Perkin-Elmer model 240 elemental analyzer and were within ±0.4% of theoretical values. ESI mass spectra were recorded with an Applied Biosystems API 2000 spectrometer. Proton and carbon-13 nuclear magnetic resonance (NMR) spectra were recorded on a Varian Mercury 400 spectrometer operating at 400 MHz. Chemical shift values are reported in δ units (ppm) relative to TMS, which was used as the internal standard.

Synthesis of Diacetone 6′-*O*-Ketorolac-D-galactopyranoside (1a). Ketorolac **1** (1.0 g, 3.9 mmol), 1,2,3,4-di-*O*-isopropylidene-D-α-galactopyranose (DIPG) (1.01 g, 3.9 mmol), *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (748 mg, 3.9 mmol), and 4-(dimethylamino)pyridine (DMAP) (24 mg, 0.19 mmol) were dissolved in dry dichloromethane (10 mL). The reaction mixture was kept under continuous stirring at room temperature for 12 h. The organic layer was washed several times with water, dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel and eluted with $CHCl_3$, giving 1.45 g of **1a** as a white solid (75% yield). ^{1}H NMR ($CDCl_3$): δ 1.30, 1.31, 1.40, 1.41 (4s, 12H, ketals); 2.80, 2.90 (m, 2H, 2-H); 3.30 (m, 1H, 1-H); 4.05 (m, 1H, 4′-H); 4.15 (m, 1H, 5′-H); 4.20 (m, 2H, 6′-H); 4.40 (m, 1H, 2′-H); 4.50 (m, 1H, 3′-H); 4.60 (m, 2H, 3-H); 5.35 (m, 1H, 1′-H); 5.90 (d, 1H, 7-H); 6.80 (d, 1H, 6-H); 7.45 (m, 2H, 2,6-Ph); 7.55 (m, 1H, 4-Ph); 7.85 (m, 2H, 3,5-Ph). ^{13}C NMR ($CDCl_3$): δ 20 and 22 ($4CH_3$-ketals); 30 (C-2); 44 (C-3); 48 (C-1); 65 (C-6′); 66.7 (C-4′); 71 (C-5′); 71.5 (C-2′); 72 (C-3′); 97 (C-1′); 105 (C-7); 109 and 111 (C-ketals); 125 (C-6); 127 (C-5); 129 (3,5-Ph); 130 (2,6-Ph); 133 (4-Ph); 140 (C-8); 142 (1-Ph); 175 (CO ketone); 185 (CO ester). *m*/*z*: 498 $(M + H)^+$. Anal. ($C_{32}H_{41}NO_8$) C, H, N.

Synthesis of Ketorolac-D-galactos-6′-yl Ester (Ketogal). Two milliliters of trifluoroacetic acid (TFA) was added to a solution of **1a** (1.45 g, 2.9 mmol) in dry dichloromethane (10 mL). The mixture was continuously stirred at room temperature for 48 h. The solvents were then evaporated and purified by flash chromatography on silica gel. Elution with $CHCl_3$ in a gradient of CH_3OH gave 800 mg of ketogal as a white solid (65% yield). Mp: 195 °C. ^{1}H NMR (CD_3OD): δ 2.85 (m, 2H, 2-H); 3.50 (m, 1H, 1-H); 3.75 (m, 1H, 4′-H); 3.85 (m, 1H, 5′-H); 4.20 (m, 2H, 6′-H); 4.30 (m, 1H, 2′-H); 4.35 (m, 1H, 3′-H); 4.50 (m, 2H, 3-H); 5.15 (m, 1H, 1′-H); 6.15 (d, 1H, 7-H); 6.80 (d, 1H, 6-H); 7.45 (m, 2H, 2,6-Ph); 7.55 (m, 1H, 4-Ph); 7.75 (m, 2H, 3,5-Ph). ^{13}C NMR (CD_3OD): δ 30 (C-2); 44 (C-3); 48 (C-1); 65.5 (C-6′); 68 (C-4′); 70 (C-5′); 73.1 (C-2′); 74.9 (C-3′); 94 and 99 (C-1′); 105 (C-7); 125 (C-6); 127 (C-5); 129 (3,5-Ph); 130 (2,6-Ph); 133 (4-Ph); 140 (C-8); 142 (1-Ph); 175 (CO ketone); 185 (CO ester). *m*/*z*: 418 $(M + H)^+$. Anal. ($C_{21}H_{23}NO_8$) C, H, N.

Stability Tests of Ketogal and Ketorolac. Prodrug solutions (100 μg/mL), maintained at 37 °C, were prepared by dissolving ketogal in pH 7.4 and pH 8.0 phosphate buffers, in hydrochloric acid 0.1 N (pH 1) or in plasma. Aliquots (20 μL) withdrawn hourly during the 24 h incubation period were analyzed by HPLC. To determine enzymatic stability, an aliquot of plasma was extracted with acetonitrile (1:2) every hour and the solutions were vortexed and centrifuged at 3000 rpm (1000*g*) for 10 min. The supernatant (20 μL) was analyzed by HPLC. The same procedure was applied to ketorolac by preparing equimolecular ketorolac tris salt solutions (90.5 μg/mL).

The hydrolysis of the compound was followed by HPLC diode array detection, which will be described later. Pseudo-first-order half-lives ($t_{1/2}$) for the chemical and enzymatic hydrolyses were calculated from the linear slopes of logarithm plots of remaining esters over time.

HPLC System. Chromatographic separations were performed using a 1090 L liquid chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a diode array detector HP 1040A. All separations were accomplished on a Phenomenex Luna C 18 (250 mm × 4.6 mm, particle size 5 μm). The selected wavelength was 313 nm. The mobile phase used in the separation consisted of acetonitrile and aqueous 1 mM phosphoric acid, pH 3 [68:32]. The flow rate was 1 mL/min with an injection volume of 20 μL. All reagents and solvents were analytical grade. Deionized and distilled water was purified through a Milli Q system (Millipore). Retention times were 6.7 min for ketorolac and 3.8 min for ketogal.

Animals. Male Swiss mice weighing 20−25 g were purchased from Harlan Italy (San Pietro al Natisone, UD, Italy). Animals were housed in groups of 5−6 per cage (24 cm × 72 cm × 12 cm). Food and water were given ad libitum. Housing conditions were thermostatically maintained at 24 ± 1 °C with a constant humidity (65%) and a 12:12 light/dark cycle.

All animal experiments complied with the Italian D.L. No. 116 of January 27, 1992, and with associated guidelines in the European Communities Council Directive of November 24, 1986, 86/609/ECC. All surgical procedures were reviewed and approved by the Ministero della Ricerca Scientifica and conformed to the guidelines of the International Association for the Study of Pain.[21]

Analgesic Activity. Acute analgesia produced by the drugs was assessed by the acetic acid- and magnesium sulfate-induced writhing methods in mice.[22,23] Animals were fasted overnight prior to treatment and received free access to water during the experiments.

One hour after oral administration of ketorolac free acid or ketogal, all mice, divided into groups of 6 per cage, received intraperitoneal (ip) injections of acetic acid 0.6%, 0.5 mL of saline, and 120 mg/kg magnesium sulfate, dissolved in 10 mL of saline. For acetic acid, the total number of writhes, a parameter of chemically induced pain detectable by constriction of abdomen, turning of trunk, and extension of hind legs, was counted for 20 min starting 5 min after the administration of the irritant agent; for magnesium sulfate, writhes were counted for 5 min. The analgesic effect was expressed as the number of writhes in comparison with control.

Anti-Inflammatory Activity. The carrageenean induced mice hind paw edema assay described by D'Agostino et al.[24] was used to evaluate the acute anti-inflammatory activity of the conjugate. Mice were divided into control and test groups of six animals each. Initial paw volumes of all animals were measured using a plethysmometer apparatus (Ugo Basile, Milan, Italy) before treatment. Paw edema was induced by a subplantar injection of 50 μL of saline containing 1% λ-carrageenan into the right hind paw. Ketorolac free acid and ketogal were orally administrated 1 h before carrageenan challenge. Paw volume was measured at different time intervals by plethysmometer. The increase in paw volume was evaluated as the difference between the paw volume measured at each time point and the basal paw volume measured immediately before carrageenan injection.

Ulcerogenicity Studies. NSAID-induced gastric damage in mice was evaluated following the procedure described by Chan et al.[25] In fasted (16−18 h) mice ($n = 6$ per group), ketorolac free acid (0.1 mg/kg), ketogal (0.163 mg/kg), or vehicle (CMC, 0.5%) was administered orally. After 4 h of treatment, mice were euthanized and the stomach was excised along its greater curvature and rinsed with normal saline. The mucosa was then examined by means of a magnifying glass for the presence of irritation or frank hemorrhagic lesions (ulcers). Irritation was assigned a score of 1, and ulcerations were scored according to their length (a score of 5 for lesions with a length between 1 and 3 mm; a score of 10 for lesions greater than 3 mm). The sum of total scores was used for comparison.

Data Analysis. Results are expressed as the mean ± SEM of *n* experiments. All analyses were conducted using GraphPad Prism (GraphPad Software Inc., San Diego, CA). Statistically significant differences between the groups were determined by one-way (for writhing tests and for ulcerogenic activity) and two-way (for carrageenan-induced edema) analyses of variance (ANOVA) followed by Bonferroni post hoc test for multiple comparisons.

Extraction Procedure from Biological Matrices for Pharmacokinetic Studies. Male Wistar rats (200 ± 20 g, Harlan, Correzzana, Milan, Italy) were housed under 12 h/12 h light/dark cycle at 22 °C with food and water ad libitum. Rats received oral administration of either ketorolac free acid (0.100 mg/kg) or ketogal (0.163 mg/kg). They were then sacrificed by decapitation 2, 4, and 6 h after administration of drugs. At least three rats were used for each time point, which was measured in triplicate.

Plasma, stomach, kidney, and liver from each animal were collected in glass tubes and stored at −20 °C until analysis. Aliquots of all biological matrixes were deproteinized with acetonitrile. The suspension was vortexed, mixed, and centrifuged at 3000 rpm for 10 min. The organic phase was injected into the HPLC system.

Pharmacokinetic analysis was performed with a PK Solutions software (version 2.0, Summit Research Services, Montrose, CO).

Method of Validation. The method was validated according to international guidelines.[26,27] Linearity was obtained with an average regression coefficient (R^2) greater than 0.998. Calibration curves were prepared in drug-free biological matrixes by spiking with ketorolac and ketogal. Concentrations ranging from 0.1 to 100 μg/mL were prepared and analyzed using the above procedure. Tolmetine was used as internal standard (retention time of 9.3 min under HPLC conditions described above). Sensitivity limits were 0.1 μg/mL for ketogal and 0.05 μg/mL for ketorolac.

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Supporting Information Available: HPLC chromatograms and mass spectrum of ketogal. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Gillis, J. C.; Brogden, R. N. Ketorolac. A reappraisal of its pharmacodynamic and pharmacokinetic properties and therapeutic use in pain management. *Drugs* **1997**, *53* (1), 139–188.
(2) Laporte, J. R.; Ibáñez, L.; Vidal, X.; Vendrell, L.; Leone, R. Upper gastrointestinal bleeding associated with the use of NSAIDs: newer versus older agents. *Drug Safety* **2004**, *27* (6), 411–420.
(3) Dula, D. J.; Anderson, R.; Wood, G. C. A prospective study comparing i.m. ketorolac with i.m. meperidine in the treatment of acute biliary colic. *J. Emerg. Med.* **2000**, *20*, 121–124.
(4) Cioli, V.; Putzolu, S.; Rossi, V.; Barcellona, P. S.; Corradino, C. The role of direct tissue contact in the production of gastro-intestinal ulcers by anti-inflammatory drugs in rats. *Toxicol. Appl. Pharmacol.* **1979**, *50*, 283–289.
(5) Rainsford, K. D. Mechanisms of gastrointestinal toxicity of non-steoridal anti-inflammatory drugs. *Scand. J. Gastroenterol., Suppl.* **1989**, *163*, 9–16.
(6) Hawkey, C.; Laine, L.; Simon, T.; Beaulieu, A.; Maldonado-Cocco, J.; Acevedo, E.; Shahane, A.; Quan, H.; Bolognese, J.; Mortensen, E. Comparison of the effect of rofecoxib (a cyclooxygenase 2 inhibitor), ibuprofen and placebo on the gastroduodenal mucosa of patients with osteoarthritis: a randomized, double blind, placebo-controlled trial. *Arthritis Rheum.* **2000**, *43* (2), 370–377.
(7) Stanczak, A.; Ferra, A. Prodrugs and soft drugs. *Pharmacol. Rep.* **2006**, *58*, 599–613.
(8) Mishra, A.; Veerasamy, R.; Jain, P. K.; Dixit, V. K.; Agrawal, R. K. Synthesis, characterization and pharmacological evaluation of amide prodrugs of ketorolac. *Eur. J. Med. Chem.* **2008**, *43* (11), 2464–2472.
(9) Vyas, S.; Trivedi, P.; Chaturvedi, S. C. Ketorolac−dextran conjugates: synthesis, in vitro and in vivo evaluation. *Acta Pharm.* **2007**, *57*, 441–450.
(10) Rainsford, K. D.; Whitehouse, M. W. Protection of the Gastric Mucosal Lining from Damage by Aspirin and Related Drugs. US 4440762. 1984.
(11) Kauffman, G. L., Jr. Gastric mucus and bicarbonate secretion in relation to mucosal protection. *J. Clin. Gastroenterol.* **1981**, *3* (2), 45–50.
(12) Srikanta, B. M.; Siddaraju, M. N.; Dharmesh, S. M. A novel phenol-bound pectic polysaccharide from *Decalepis hamiltonii* with multi-step ulcer preventive activity. *World J. Gastroenterol.* **2007**, *13* (39), 5196–5207.
(13) Bonina, F.; Puglia, C.; Rimoli, M. G.; Melisi, D.; Boatto, G.; Nieddu, M.; Calignano, A.; La Rana, G.; De Caprariis, P. Glycosyl derivatives of dopamine and L-dopa as anti-Parkinson prodrugs: synthesis, pharmacological activity and in vitro stability studies. *J. Drug Targeting* **2003**, *11*, 25–36.
(14) Melisi, D.; Secondo, A.; Montoro, P.; Piacente, S.; Rimoli, M. G.; Minale, M.; de Caprariis, P.; Annunziato, L. Galactosyl derivatives of L-arginine and D-arginine: synthesis, stability, cell permeation, and nitric oxide production in pituitary GH3 cells. *J. Med. Chem.* **2006**, *49* (16), 4826–4833.
(15) Ruocco, L. A.; Viggiano, D.; Viggiano, A.; Abignente, E.; Rimoli, M. G.; Melisi, D.; Curcio, A.; Nieddu, M.; Boatto, G.; Carboni, E.; Carnevale, U. A.; Sadile, A. G. Galactosylated dopamine enters into the brain, blocks the mesocorticolimbic system and modulates activity and scanning time in Naples high excitability rats. *Neuroscience* **2008**, *152* (1), 234–244.
(16) Ruocco, L. A.; Viggiano, D.; Pignatelli, M.; Iannaccone, T.; Rimoli, M. G.; Melisi, D.; Curcio, A.; De Lucia, S.; Carboni, E.; Carnevale, U. A.; de Caprariis, P.; Sadile, A. G. Galactosilated dopamine increases attention without reducing activity in C57BL/6 mice. *Behav. Brain Res.* **2008**, *187* (2), 449–454.
(17) Posadas, I.; Bucci, M.; Roviezzo, F.; Rossi, A.; Parente, L.; Sautebin, L.; Cirino, G. Carrageenan-induced mouse paw oedema is biphasic, age-weight dependent and displays differential nitric oxide cyclooxygenase-2 expression. *Br. J. Pharmacol.* **2004**, *142* (2), 331–338.
(18) Qandil, A.; Al-Nabulsi, S.; Al-Taani, B.; Tashtoush, B. Synthesis of piperazinylalkyl ester prodrugs of ketorolac and their in vitro evaluation for transdermal delivery. *Drug Dev. Ind. Pharm.* **2008**, *34* (10), 1054–1063.

(19) Bhandari, K. H.; Newa, M.; Yoon, S. I.; Kim, J. S.; Jang, K. Y.; Kim, J. A.; Yoo, B. K.; Woo, J. S.; Lee, J. H.; Kim, D. D.; Choi, H. G.; Yong, C. S. Evaluation of physicochemical properties, skin permeation and accumulation profiles of ketorolac fatty ester prodrugs. *Biol. Pharm. Bull.* **2007**, *30* (11), 2211–2216.

(20) Puglia, C.; Filosa, R.; Peduto, A.; de Caprariis, P.; Boatto, G.; Nieddu, M.; Santagati, N. A.; Bonina, F. Synthesis, physicochemical properties and in vitro permeation studies of new ketorolac ester derivatives. *Curr. Drug Delivery* **2007**, *4* (3), 205–210.

(21) Zimmermann, M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* **1983**, *16*, 109–110.

(22) Jain, N. K.; Kulkarni, S. K.; Singh, A. Differential antinociceptive effect of cyclooxygenase inhibitors in acetic acid-induced chemonociception in mice. *Analgesia* **2001**, *5*, 211–216.

(23) Calignano, A.; La Rana, G.; Piomelli, D. Antinociceptive activity of the endogenous fatty acid amide, palmitylethanolamide. *Eur. J. Pharmacol.* **2001**, *419*, 191–198.

(24) D'Agostino, G.; La Rana, G.; Russo, R.; Sasso, O.; Iacono, A.; Esposito, E.; Raso, G. M.; Cuzzocrea, S.; Lo Verme, J.; Piomelli, D.; Meli, R.; Calignano, A. Acute intracerebroventricular administration of palmitoylethanolamide, an endogenous peroxisome proliferator-activated receptor-alpha agonist, modulates carrageenan-induced paw edema in mice. *J. Pharmacol. Exp. Ther.* 2007*3*, 1137–1143.

(25) Chan, C. C.; Boyce, S.; Brideau, C.; Ford-Hutchinson, A. W.; Gordon, R.; Guay, D.; Hill, R. G.; Li, C. S.; Mancini, J.; Penneton, M.; Praist, P.; Rasori, R.; Riendeau, D.; Roy, P.; Tagari, P.; Vickers, P.; Wong, E.; Rodger, I. W. Pharmacology of a selective cyclooxygenase-2 inhibitor, L-745,337: a novel nonsteroidal anti-inflammatory agent with an ulcerogenic sparing effect in rat and nonhuman primate stomach. *J. Pharmacol. Exp. Ther.* **1995**, *3*, 1531–1537.

(26) Peters, F. T.; Maurer, H. H. Bioanalytical method validation and its implications for forensic and clinical toxicology. *Accredit. Qual. Assur.* **2002**, *7*, 441–449.

(27) Taverniers, I.; de Loose, M.; Van Bockstaele, E. Analytical method validation and quality assurance. *Trends Anal. Chem.* **2004**, *23*, 533–551.

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