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Original article

Synthesis and pharmacological characterization of a novel nitric oxide-releasing diclofenac derivative containing a benzofuroxan moiety

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ABSTRACT

1-Oxy-benzo[1,2,5]oxadiazol-5-ylmethyl [2-(2,6-dichloro-phenylamino)-phenyl]-acetate, a new diclofenac derivative bearing a benzofuroxan heterocyclic moiety in its structure, was prepared by the reaction of sodium diclofenac and 5-bromomethyl-benzo[1,2,5]oxadiazole 1-oxide. Pharmacological characterization of this modified diclofenac maintained the anti-inflammatory activity similar to its parent compound assayed *in vitro* and *in vivo*. The ulcerogenic properties of native diclofenac were not observed with this modified compound, despite the inhibition of prostaglandin E_2 gastric content. The better gastric tolerability seems to be related to nitric oxide release ability.

1. Introduction

The beneficial effects of non-steroidal anti-inflammatory drugs (NSAIDs) are always balanced by their side effects; the major undesirable effect being related to the gastrointestinal system. In recent years, a number of novel approaches have been taken to develop gastrointestinal-sparing NSAIDs. One of these was the establishment of selective cyclooxygenase (COX)-2 inhibitors in the therapeutic routine. The ability to inhibit the COX-2 isoform provides these compounds with anti-inflammatory properties that are comparable to standard NSAIDs in many situations. In contrast to the standard NSAIDs, these compounds present a reduced capacity to induce gastric ulceration since they lack the ability to inhibit prostaglandin derived-COX-1, which is physiologically expressed in the stomach [1]. Nevertheless, selective COX-2 drugs interfere strongly with ulcer healing, since COX-2 is induced in the stomach during injury and adaptive cytoprotection mechanisms [2]. Another important and recent finding is the increased incidence of thrombotic and cardiovascular events when COX-2 selective drugs are prescribed long term [3]. A second approach is the addition of a nitric oxide (NO)-releasing moiety to classical NSAIDs, producing the NO-NSAIDs. The NO released by these NSAIDs exerts beneficial effects on the mucosa by modulating gastric functions such as mucosal blood flow and, consequently, the mucus production. Nitric oxide is also able to interact with caspases, resulting in a decreased synthesis of Th-1 derived cytokines, such as IL-1 β, IL-18 and IFN-γ [4]. The advantage of this approach is the maintenance of the original activity of classical NSAIDs that do not modify the dynamics of prostanoid synthesis combined with reduced gastric side effects [5].

Sodium diclofenac (**1**) (Fig. 1), a NSAID widely used in chronic treatments, has a very reactive carboxylate group that can be used to incorporate new substituents to its structure. Taking into consideration the beneficial effects of the nitric oxide described above, it would be of interest to synthesize and investigate the pharmacological properties of a diclofenac derivative containing a heterocyclic ring system, such as benzofuroxan, that is able to release NO (**2**).

In this study, we describe the synthesis and pharmacological characterization of a novel modified diclofenac, which has a benzofuroxan heterocyclic moiety on its structure joined to the parent drug by an ester linkage.

2. Results and discussion

2.1. Chemistry

Because of their ability to release nitric oxide molecules under physiological conditions, much attention has been recently paid to furoxans and benzofuroxans [6–9]. These heterocyclics can be

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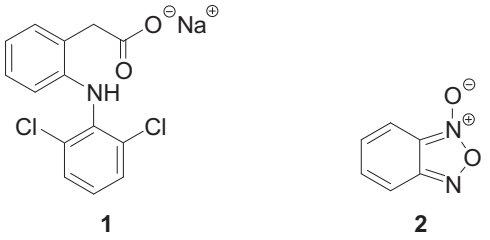


Fig. 1. Chemical structures of sodium diclofenac (**1**) and benzofuroxan heterocyclic system (**2**).

prepared by various synthetic routes, including oxidation of 1,2-dioximes [10], thermal decomposition of 2-nitro-arylazides [11,12] and oxidation of 2-nitroanilines with sodium hypochlorite [12,13] or iodobenzene diacetate [14].

With the aim of preparing a new derivative of diclofenac that contains a heterocyclic moiety with NO-releasing properties, we hypothesized that a nucleophilic substitution reaction could be used to link a benzofuroxan moiety to the reactive carboxylate group of diclofenac. As shown in Scheme 1, we first prepared the benzofuroxan **4** from 4-methyl-2-nitro-aniline (**3**) through an oxidation promoted by sodium hypochlorite. This reaction proceeded smoothly and furnished the desired heterocycle with a 79% yield after recrystallization. The conversion of **4** to the bromide **5** was carried out in refluxing carbon tetrachloride by the action of *N*-bromosuccinimide in the presence of a catalytic amount of benzoyl peroxide. After purification by flash chromatography, **5** was obtained with an 85% yield. We were also able to isolate a small amount (7% yield) of the corresponding dibromo derivative, as indicated by GCMS analysis.

With the bromide **5** in hands, we had a suitable substrate to prepare the desired diclofenac derivative. The nucleophilic substitution was performed by mixing sodium diclofenac and **5** in stoichiometric amounts using DMF as solvent. The reaction was completed after 2 h at room temperature, and the modified diclofenac **6** was isolated in 89% yield after purification by flash chromatography.

The ester **6** was chemically characterized by its IR, NMR and mass spectra. The IR spectrum showed bands at 3266 cm^{-1} (N–H stretch), 1716 cm^{-1} (C=O stretch), 1256 and 1151 cm^{-1} (C–O stretches). An interesting feature observed in its ^1H NMR spectrum is the fact that all the three hydrogens attached to the benzofuroxan system are viewed as a broad multiplet between δ 7.20–7.60. Broad multiplets were also observed in the spectra of benzofuroxan **4** (δ 7.00–7.60) and bromide **5** (δ 7.10–7.70). A tautomeric equilibrium between the two benzofuroxan isomeric structures (Scheme 2), that is very fast at room temperature, is responsible for the coalescence of the individual signals into a very broad multiplet. The occurrence of this phenomenon has been previously observed in many benzofuroxan derivatives [15]. In the ^{13}C NMR spectrum of **6**, the signals related to the benzofuroxan aromatic carbons were not observed, while in the spectra of **4** and **5**, the aromatic carbons originated two broad multiplets (**4**: δ 114.0 and 135.0; **5**: δ 115.0 and 131.0) because of the very fast tautomeric equilibrium. The mass spectrum of **6** showed a peak at *m*/*z* 277, corresponding to the radical-ion formed by loss of a hydroxymethyl-benzofuroxan molecule from the molecular-ion; the base peak was viewed at *m*/*z* 214, presumably originated from subsequent losses of chlorine atom and carbon monoxide (Scheme 3).

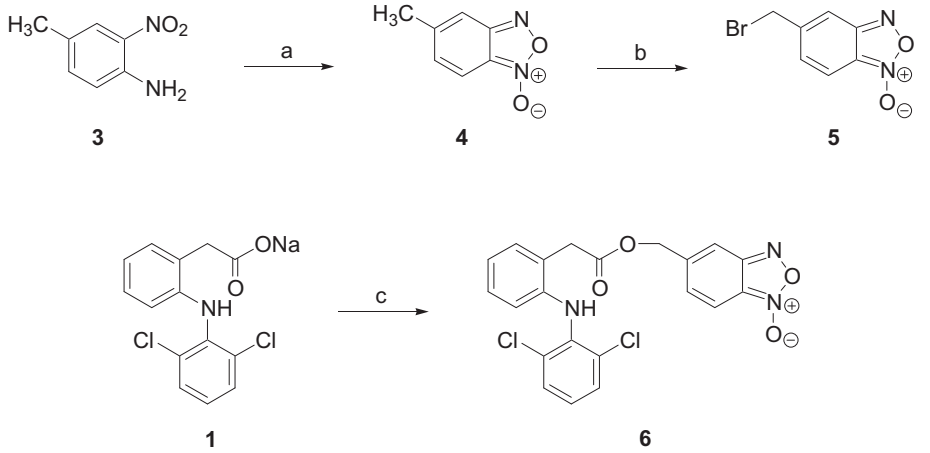
2.2. Pharmacology

The compound **6** was compared to the diclofenac *in vitro* for COX-1 and COX-2 inhibitory effect using the human whole blood assay. The anti-inflammatory activities of the two drugs were then studied, *in vivo*, using the rat paw edema test, and gastric tolerability to the drugs was accessed by measuring gastric prostaglandin, following oral administration to rats.

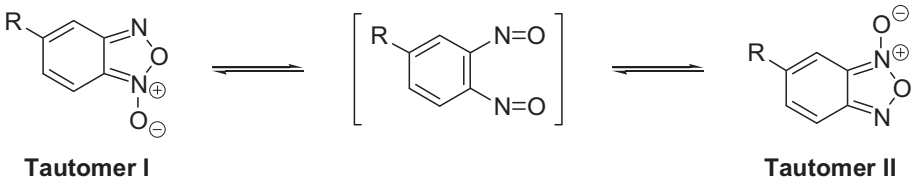
2.2.1. Anti-inflammatory activity

The novel modified diclofenac (compound **6**) was prepared as described above and tested *in vitro* and *in vivo* to evaluate its pharmacological activity. As shown in Fig. 2, incubation of heparinized whole blood with 1 and 100 μM of diclofenac and compound **6**, before LPS challenge, resulted in a significant reduction in PGE_2 production. When the TXB_2 production was assessed in serum samples, clot formation was obtained from whole blood previously incubated with the test drug. At a concentration of 1 and 100 μM, the original diclofenac promoted a significant inhibition, while the compound **6** was only effective at 100 μM, suggesting that compound **6** was more selective for COX-2, *in vitro* (Fig. 3).

The use of a classical experimental model of inflammation demonstrated that the subplantar injection of carrageenan caused a dose-dependent increase in rat hind paw oedema, compared to that observed in the saline-injected contralateral paw (data not shown). When rats received 100 mg kg^{-1} of diclofenac or compound **6** intraperitoneally, both treatments evoked



Scheme 1. Reagents and conditions: a. 12% NaClO, EtOH/H_2O, 4 °C, 40 min (79%); b. NBS (1.2 equivalent), $(PhCO)_2O_2$ (0.1 equivalent), CCl_4, refl., 5 h (85%); c. Bromide **5** (1.0 equivalent), DMF, 25 °C, 2 h (89%).



Scheme 2. Rearrangement occurring in benzofuroxan systems.

a pronounced decreased in paw volume at 2, 3 and 4 h after the carrageenan injection (Fig. 4). The AUC for diclofenac was 2.01 ± 0.31, while for compound **6** the AUC was 2.00 ± 0.45, indicating that there were no differences between the two compounds. The AUC obtained for the control was 4.01 ± 0.42, demonstrating that the structural modification maintained the original pharmacological activity *in vivo*.

2.2.2. Acute ulcerogenicity and PGE_2 gastric content

The usual side effects of these compounds were evaluated by their oral administration. These procedures, applied with native diclofenac (30 mg Kg^{-1}) caused damage in the gastric mucosa that consisted of hemorrhagic lesion that was significantly different from that of the control animals that received only vehicle. In contrast, the administration of an equimolar dose of compound **6** provoked lesions in the rat stomach that were much less extensive than those induced by the original compound (Fig. 5), confirming the previous literature reports regarding the ability of NO-releasing NSAIDs to demonstrate anti-inflammatory properties, but not ulcerogenic side effects [4,5,16]. The PGE_2 content in the gastric mucosa was reduced in both the original diclofenac and compound **6** groups, presenting different values from the control group, showing that the gastric sparing is due to NO release rather than the maintenance of the local production of PGE_2 (Fig. 6). Finally, in order to evaluate the involvement of nitric oxide in the reduction of ulcerogenicity of compound **6**, its ability to release NO was ensured by measuring nitric oxide-releasing properties in phosphate buffer, pH 7.4, in the presence of L-cysteine, relative to nitric oxide released from standard sodium nitrite solution. Compound **6** (10^{-4} M) produces *in vitro* 372 ± 11 nM of nitrite. NOR-3, a potent NO-donnor, at same experimental condition produces 2706 ± 412 nM [17]. Vehicle and native diclofenac were not able to release any nitrite at same experimental condition.

3. Conclusions

The present data demonstrates that the new diclofenac, obtained from the native compound by adding a benzofuroxan moiety on its structure, possesses the expected anti-inflammatory activity, without the deleterious gastrointestinal effects which is normally present in typical NSAIDs. This approach could really be a good alternative to gastro sparing anti-inflammatory non-steroidal drugs.

4. Experimental protocols

4.1. Chemistry

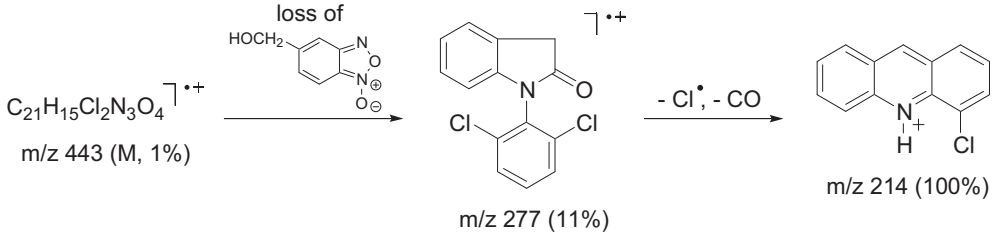
All reagents were obtained from commercial sources and used without further purification. Carbon tetrachloride and *N*,*N*-dimethylformamide were dried according to literature procedures [18]. Column chromatography (flash chromatography) [19] was performed on silica gel 60 (Aldrich, 230–400 mesh). Thin layer chromatography was carried out on TLC aluminium sheets (silica gel 60 Merck). Melting points were determined on a Fisher–Johns apparatus and are uncorrected. IR spectra were recorded on an ABB FTLA2000 spectrophotometer. NMR spectra were obtained on a GEMINI 300 BB instrument at 300 MHz for ^{1}H and 75.5 MHz for ^{13}C spectra. Low resolution mass spectra were determined on a Shimadzu GCMS-QP5050A apparatus at 70 eV. High resolution mass spectra were obtained on a VG-Autospec spectrometer at 70 eV.

*4.1.1. 5-Methyl-benzo[1,2,5]oxadiazole 1-oxide (**4**)*

4-Methyl-2-nitro-aniline (1.52 g, 10.0 mmol) was added to a stirred solution of sodium hydroxide (0.44 g, 11.0 mmol) in 96% ethanol (15 mL). The resulting red solution was cooled to 4 °C and a sodium hypochlorite solution (12%, 20 mL) was added dropwise. The reaction mixture was stirred at 4 °C for 40 min and the precipitate was filtered under reduced pressure and washed with cold water (20 mL). The crude material was purified by recrystallization from 70% ethanol to furnish **4** (1.18 g, 79%) as small yellow needles: mp 94–95 °C (lit. [20] mp 97 °C); IR (KBr) 1619, 1595, 1528, 1490, 793, 570 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.41 (s, 3 H), 7.00–7.60 (br m, 3 H); ^{13}C NMR ($CDCl_3$) δ 22.1, 114.0 (br m), 135.0 (br m); MS *m*/*z* (%) 150 (M, 87), 134 (38), 105 (27), 89 (69), 77 (59), 65 (100).

*4.1.2. 5-Bromomethyl-benzo[1,2,5]oxadiazole 1-oxide (**5**)*

N-Bromosuccinimide (1.34 g, 7.52 mmol) and benzoyl peroxide (151 mg, 0.62 mmol) were added to a stirred solution of **4** (939 mg, 6.25 mmol) in dry carbon tetrachloride (60 mL). The reaction mixture was heated under reflux (77 °C) for 5 h and then cooled to room temperature. The precipitate (succinimide) was filtered and washed with carbon tetrachloride (10 mL). The solvent was removed under reduced pressure to produce a brown oil, which was submitted to flash chromatography (10% ethyl acetate: hexanes) to yield **5** (1.22 g, 85%) as an amorphous yellow solid: TLC



Scheme 3. MS fragments of **6**.

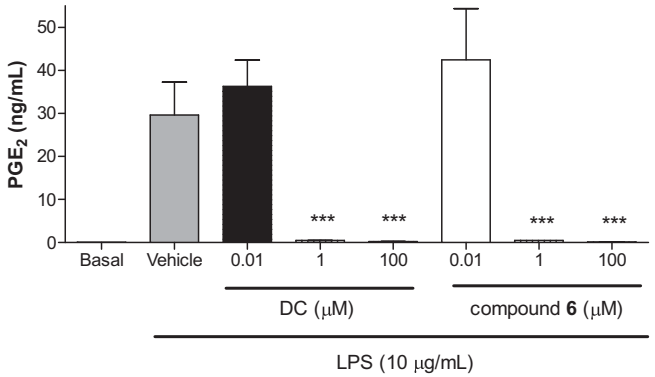


Fig. 2. Prostaglandin E_2 concentrations in plasma samples from blood aliquots stimulated with LPS (10 μg/mL) previously incubated with different concentrations of diclofenac (0.01–100 μM) or compound **6** (0.01–100 μM). Data are mean ± S.E.M of 3 experiments. $p < 0.01$ when compared with blood aliquot previously incubated with DMSO (0.1%).

Rf: 0.25 (10% ethyl acetate:hexanes); mp 73–74 °C (lit. [21] mp 75 °C); IR (KBr) 1620, 1594, 1538, 1015, 655, 574 cm^{-1}; ^1H NMR ($CDCl_3$) δ 4.44 (s, 2H), 7.10–7.70 (br m, 3H); ^{13}C NMR ($CDCl_3$) δ 31.6, 115.0 (br m), 131.0 (br m); MS *m*/*z* (%) 230 (12), 228 (M, 12), 149 (100), 133 (60), 89 (76), 76 (30).

4.1.3. 1-Oxy-benzo[1,2,5]oxadiazol-5-ylmethyl [2-(2,6-dichlorophenylamino)-phenyl]-acetate (**6**)

Bromide **5** (180 mg, 0.79 mmol) was added to a solution of sodium diclofenac (250 mg, 0.79 mmol) in dry *N*,*N*-dimethylformamide (7 mL). The reaction mixture was stirred at room temperature (25 °C) for 2 h. After this time, water (20 mL) was added and the mixture was extracted with dichloromethane (three portions of 15 mL). After the combination of the organic layers, the resulting solution was washed with saturated sodium chloride (10 mL) and dried over anhydrous sodium sulfate. The solvent was evaporated to give a brown oil which was submitted to flash chromatography (20% ethyl acetate:hexanes) to yield **6** (312 mg, 89%) as a crystalline yellow solid: TLC *Rf*: 0.23 (20% ethyl acetate: hexanes); mp 108–109 °C; IR (KBr) 3266, 1716, 1627, 1601, 1256, 1151 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.91 (s, 2H), 5.17 (s, 2H), 6.55 (d, J = 8.0 Hz, 1H), 6.65 (s, 1H), 6.94–7.01 (m, 2H), 7.15 (td, J_1 = 8.0 Hz, J_2 = 1.5 Hz, 1H), 7.24 (d, J = 8.0 Hz, 1H), 7.32 (d, J = 8.0 Hz, 2H), 7.20–7.60 (br m, 3H); ^{13}C NMR ($CDCl_3$) δ 38.4, 65.2, 118.5, 122.3, 123.7, 124.2, 128.3, 128.9, 129.4, 130.9, 137.6, 142.5, 171.6 (benzofuroxan aromatic carbons were not observed due to very fast tautomeric equilibrium); MS *m*/*z* (%) 443 (M, 1), 429 (13), 427 (18), 277 (11), 242 (26), 214 (100); HRMS *m*/*z* calc for $C_{21}H_{15}Cl_2N_3O_4$ 443.0440, found 442.9870, *m*/*z* calc for $C_{13}H_9ClN^+$ 214.0423, found 213.9983.

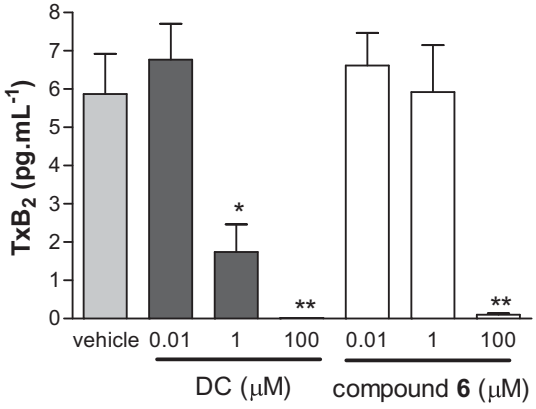


Fig. 3. Thromboxane B_2 concentrations in serum samples from blood clotted aliquots previously incubated with different concentrations of diclofenac (0.01–100 μM) or compound **6** (0.01–100 μM). Data are mean ± S.E.M of 3 experiments. $^*p < 0.05$ and $^{**}p < 0.01$ when compared with blood aliquot previously incubated with DMSO (0.1%).

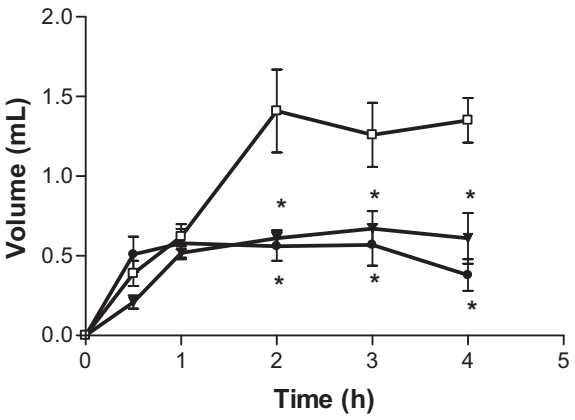


Fig. 4. Effect of intraperitoneally administration of diclofenac (●; 100 mg kg^{-1}) and compound **6** (▼; equivalent molar dose) in volume of rat paw oedema induced by intraplantar injection of carrageenan. Data are mean ± S.E.M of 6 animals. $^*p < 0.05$ when compared with group treated with DMSO (□; 0.1%).

4.2. Pharmacology

4.2.1. Human whole blood assay (WBA)

Whole blood was collected by venupuncture from healthy subjects who had not taken any NSAIDs during the 2 weeks preceding the study. For COX-1 assays, blood was immediately aliquoted in 1 mL volumes into individual glass tubes containing test agents or vehicle (0.1% vol/vol dimethyl sulfoxide; DMSO) and allowed to clot at 37 °C for 60 min. Serum was separated by centrifugation (10 min at 2000 rpm) and kept at −20 °C until assay for TXB_2.

For COX-2 assays, 1 mL whole blood samples containing heparin (IU/mL) were incubated both in the absence and in the presence of LPS (10 μg/mL) plus test agents or vehicle. Incubation was then continued for a further 18 h, after which the tubes were centrifuged (10 min at 2000 rpm) and the plasma was removed and kept at −20 °C until assay for PGE_2 [22].

Concentrations of TXB_2 and PGE_2 in samples were determined using ELISA kits (GE Healthcare, UK).

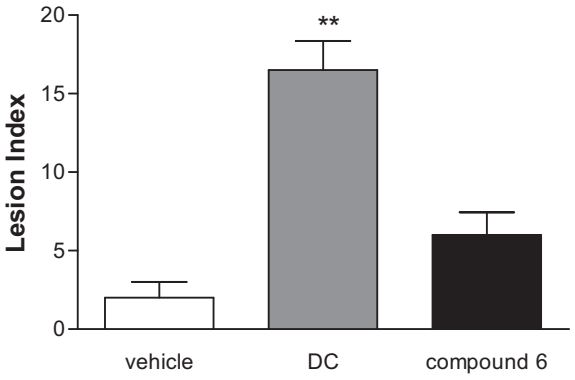


Fig. 5. Mucosal ulcerogenic effects of orally administered diclofenac (50 mg kg^{-1}) and compound **6** (equivalent molar dose). Data are mean ± S.E.M of 5 animals. $^*p < 0.05$ when compared with group treated with DMSO (0.1%). The lesion index was described in Methods.

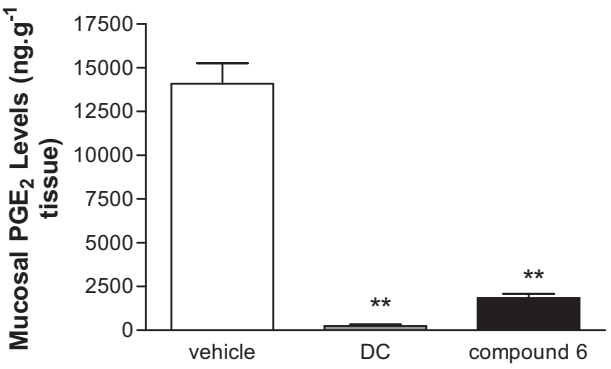


Fig. 6. Effects of orally administered diclofenac (50 mg kg^{-1}) and Compound **6** (equivalent molar dose) on gastric mucosal prostaglandin E_2 contents in rat stomachs. Data are mean ± S.E.M of 5 animals. $^{**}p < 0.01$ when compared with group treated with DMSO (0.1%).

4.2.2. Animals

Male Wistar rats (180–200 g) free of specific pathogens were obtained from CEMIB (State University of Campinas, Campinas, SP, Brazil). The experiments were performed in accordance with the principles outlined by the Brazilian College for Animal Experimentation (COBEA).

4.2.3. Carrageenan-induced paw edema model

Initially, the animals received the anti-inflammatory compounds or vehicle intraperitoneally (i.p.). Thirty minutes later, the animals were anaesthetized with halothane (inhaled). A subplantar injection of carrageenan (1 mg/paw) in 0.1 mL of saline was made into one paw of the rat. The contralateral paw received only 0.1 mL of saline. The paw volume was assessed immediately before the carrageenan injection and was considered as the basal measurement, and thereafter at 0.5, 1, 2, 3 and 4 h, using a hydroplethysmometer (model 7150, Ugo Basile). The results are expressed as the increase in paw volume (mL) calculated by subtracting the basal volume. The area under the time-course curve (AUC_{0-2h}) was also calculated using the trapezoidal rule and the results expressed as total edema volume (mL h) by comparison with the vehicle rats [23].

4.2.4. Evaluation of gastric ulcerogenic activity and mucosal prostaglandin E_2 content

The animals were orally treated with vehicle (1 mL of DMSO) or the anti-inflammatory compounds. After 4 h, the animals were killed and their stomachs were removed and opened along the greater curvature. The ulcerative lesion index of each animal was calculated by adding the following values [24]: loss of normal morphology (1 point), discoloration of mucosa (1 point), hemorrhages (1 point), petechial points (1–3 points), ulcers up to 1 mm (n × 2 points), ulcers > 1 mm (n × 3 points). The stomachs were then weighed and put in a tube with phosphate buffer (pH 7.0) and minced with scissors, homogenized and centrifuged for 10 min at 15000 g at 4 °C. The supernatant of each sample was used for determination of PGE_2 by EIA using a PGE_2 kit.

4.2.5. Assay of in vitro NO release

Solutions of test agents (compound **6**, native diclofenac and NOR-3) were prepared in DMSO and 10 μL was added to 1 mL of 50 mM phosphate buffer (pH 7.4) in presence of L-cysteine (5 mM), for 1 h, at 37 °C. The final drug concentration was 10^{-4} M. Each sample was used for determination of nitrite content using a Nitrate/Nitrite Colorimetric Assay kit.

4.2.6. Statistical analysis

All data are expressed as the mean ± S.E.M. Comparisons among groups were performed using one-way analysis of variance followed by the Student's *t* test or Dunnett multiple comparisons test, when convenient. An associated probability (*p* value) of less than 5% was considered significant.

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