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RESEARCH ARTICLE

NSAIDs do not require the presence of a carboxylic acid to exert their anti-inflammatory effect – why do we keep using it?

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Abstract

The carboxylic acid group (–COOH) present in classical NSAIDs is partly responsible for the gastric toxicity associated with the administration of these drugs. This concept has been extensively proven using NSAID prodrugs. However, the screening of NSAIDs with no carboxylic acid at all has been neglected. The goal of this work was to determine if new NSAID derivatives devoid of acidic moieties would retain the anti-inflammatory activity of the parent compound, without causing gastric toxicity. To test this concept, we replaced the carboxylic acid group in ibuprofen, flurbiprofen, and naproxen with three ammonium moieties. We tested the resulting water-soluble NSAID derivatives for anti-inflammatory and ulcerogenic activity *in vitro* and *in vivo*. In this regard, we observed that all non-acidic NSAIDs exerted a potent anti-inflammatory activity, suggesting that the acid group in commercial 2-phenylpropionic acid NSAIDs not be an essential requirement for anti-inflammatory activity. These data provide complementary evidence supporting the discontinuation of ulcerogenic *acidic* NSAIDs.

Keywords

Anti-inflammatory, cyclooxygenase, NSAIDs

History

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Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most extensively used therapeutic agents worldwide. The use of NSAIDs accounts for nearly 5% of all prescribed medications[1] because of their proven efficacy in a wide variety of inflammatory disorders[2–8]. The principal pharmacological mechanism of action exerted by NSAIDs is the inhibition of cyclooxygenase (COX)-1 and COX-2 enzymes, which leads to decreased levels of prostaglandins (PGs) and thromboxanes (TXs) systemically. The scientific literature describes COX-1 as a constitutive enzyme involved in the physiological production of PGs. In contrast, COX-2 has been regarded as an inducible enzyme expressed as a response to different stimuli. However, the ''traditional'' roles attributed to both enzymes are now under scrutiny and constitute a challenging research area. It is now clear that we need to reevaluate the ''traditional'' roles for each enzyme, not only in inflammation but also in the underlying mechanisms of NSAID-induced toxicity[9]. Gastrointestinal (GI) erosions and bleeding are two of the most common toxic side-effects associated with the administration of NSAIDs. These effects have been observed even with small prophylactic doses of aspirin (81 mg/d)[10]. Approximately 50% of patients taking NSAIDs on a long-term basis may develop mucosal damage in the small intestine[11].

It has been observed that the GI toxicity of NSAIDs is partly due the presence of a free carboxylic acid group[12]. Because of this, the scientific literature describes the design of a high number of NSAID *prodrugs* [mainly ester and amide derivatives[13–15]]. However, scientists have neglected the study of drugs entirely devoid of the –COOH moiety. In this regard, we thought of the following two alternatives: (a) to reduce an acid/ester prodrug to an alcohol and (b) to reduce an amide prodrug to an amine. The presence of an amine was more attractive to us considering that we could also form the corresponding polar (and potentially water-soluble) ammonium salt.

Therefore, as part of an ongoing research work aimed to obtain effective and safe anti-inflammatory agents, we report the synthesis and the biological evaluation of nine different ammonium salts derived from naproxen (**1**), ibuprofen (**2**), and flurbiprofen (**3**, Scheme 1). We also conducted docking studies to evaluate and compare the binding interactions exerted by the test compounds on the active site of the COX enzyme.

Materials and methods

Chemistry

We determined melting points for solid products using an Electrothermal Mel-Temp® melting point apparatus (Barnstead, Dubuque, IA) and are uncorrected. We recorded ^{1}H- and ^{13}C-NMR spectra on a Bruker AVANCE 600, or a Bruker AM-300 NMR spectrophotometer (Bruker BioSpin Corporation, Billerica, MA). In this paper, we reported all coupling constants (*J*) in Hertz (Hz), but the corresponding chemical shifts in relative

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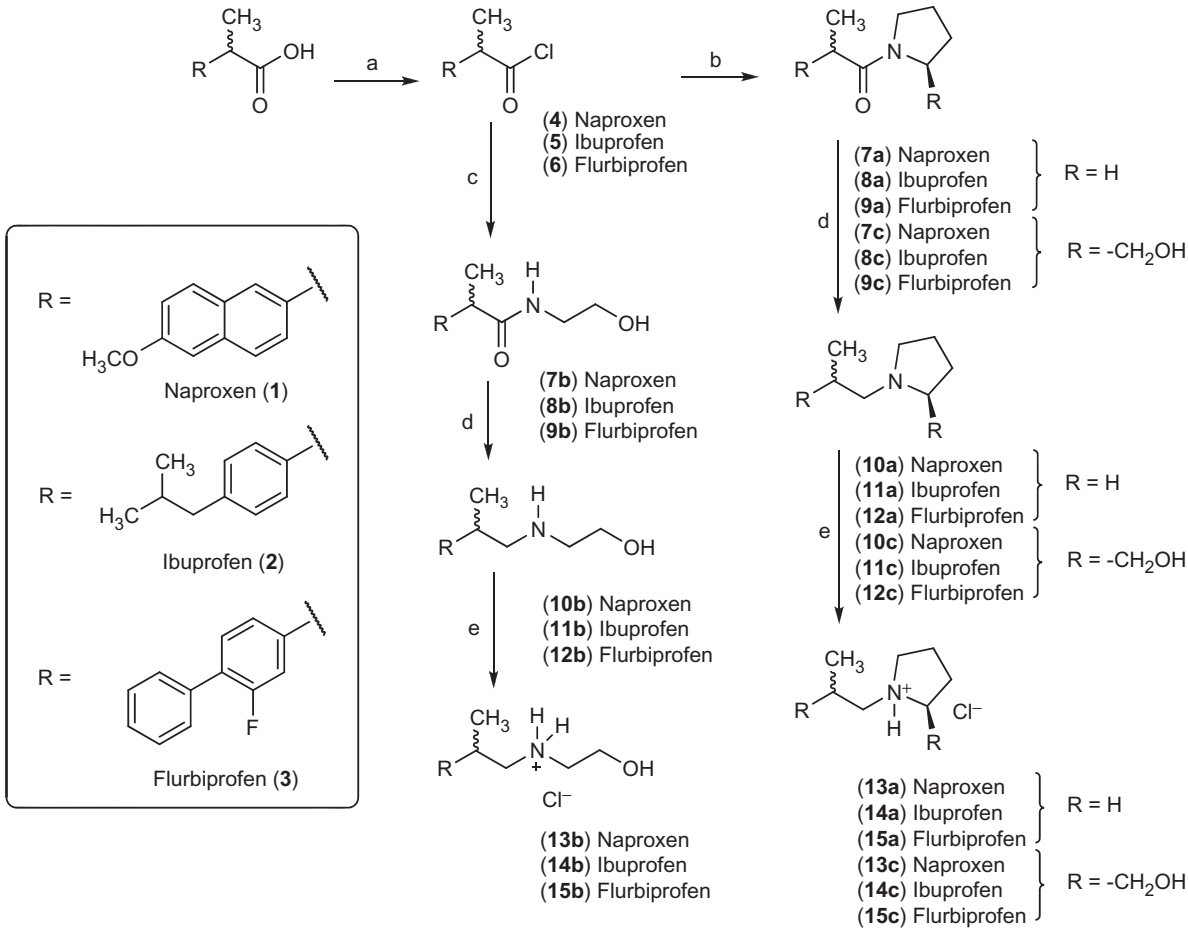

Scheme 1. Chemical synthesis of naproxen (1), ibuprofen (2), and flurbiprofen (3) derivatives possessing a polar pyrrolidinium (**13a**, **14a**, and **15a**), 2-(hydroxymethyl)pyrrolidinium (**13 c**, **14 c**, and **15 c**), or a hydroxyethylammonium chloride moiety (**13 b**, **14 b**, and **15 b**). Reagents and conditions: (a) oxalyl chloride, DCM, 25° C, 3–12 h; (b) pyrrolidine or L-proline, DCM, 25 °C, 3 h; (c) 2-hydroxyethylamine, DCM, 25 °C, 3 h; (d) $LiAlH_4$, THF, 25 °C (compounds **10a**, **10c**, **11a**, **11c**, **12a**, and **12c**) or reflux (compounds **10b**, **11b**, and **12b**) for 3 h; (e) dry HCl, ether, N_2, 25 °C.

units (δ units, or ppm) using TMS as an internal standard. We recorded mass spectra (ESI-MS) using a Water's micro mass ZQ-4000 single quadruple mass spectrometer (Waters Corporation, Milford, MA). We determined elemental analyzes (C, H, N) for some products, accepting those samples that were within ± 0.4 of the theoretical values (Microanalytical Service Laboratory, Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada). We observed that compounds **13**–**15** showed a single spot on RediSep® silica gel glass plates (Axel Semrau GmbH & Co, Sprockhövel, Germany) (UV254, 0.2 mm) when we used a high, medium, and low polarity solvent mixtures. No residue remained after combustion, indicating a purity greater than 95%. We performed column chromatography separations using a CombiFlash Retrieve, or a CombiFlashRf system using RediSepRf silica gel® (40–60 μm) cartridges (Axel Semrau GmbH & Co, Sprockhövel, Germany), or prepacked RediSep Gold® columns (Axel Semrau GmbH & Co, Sprockhövel, Germany). We synthesized the corresponding acid chlorides of naproxen (**4**), ibuprofen (**5**), and flurbiprofen (**6**), following reported literature procedures[16]. We purchased all other reagents from Aldrich Chemical Company (Milwaukee, WI) and were used without further purification.

General procedure for the synthesis of amides (7–9)

We added a solution of the corresponding amine (2 eq.) dissolved in dichloromethane (5 mL), to a solution of naproxen acid chloride (**4**, 1 eq.), ibuprofen acid chloride (**5**, 1 eq.), or flurbiprofen acid chloride (**6,** 1 eq.) in dichloromethane (10 mL) at 25 °C. In all cases, we monitored the reaction progress by TLC while stirring at room temperature (about 25 °C). We detected the formation of amides in all mixtures within 3 h. Then we filtered the corresponding reaction mixture and evaporated the solvent under vacuum. Finally, we purified the crude products by flash chromatography using a mixture of *n*-hexane/ethyl acetate (gradient).

2-(6-*Methoxynaphthalen*-2-*yl*)-1-(*pyrrolidin*-1-*yl*)*propan*-1-*one* (**7a**): 90% yield; m.p. 109–110 °C; ^{1}H-NMR: ($CDCl_3$) δ 7.76–7.65 (m, 3H, naphthyl), 7.43 (dd, *J*: 8.55 Hz, *J*: 1.18 Hz, 1H, naphthyl), 7.16–7.12 (m, 2H, naphthyl), 3.92 (s, 3H, CH_3O), 3.86 (q, *J*: 7.32, 1H, $CHCH_3$), 3.46 (broad singlet, 4H, pyrrolidinyl H-2, H-5), 1.73 (broad singlet, 4H, pyrrolidinyl H-2, H-4), 1.51 (d, *J*: 7.32 Hz, 3H, CH_3CH).

N-(2-*hydroxyethyl*)-2–(6-*methoxynaphthalen*-2-*yl*)*propanamide* (**7b**): 48% yield; m.p. 81–83 °C; ^{1}H-NMR ($CDCl_3$) δ 7.75–7.68 (m, 3H, naphthyl), 7.37 (d, *J*: 7.92 Hz, 1H, naphthyl), 7.18–7.13 (m, 2H, naphthyl), 5.85 (broad singlet, 1H, OH), 4.33 (t, 1H, NH), 3.93 (s, 3H, CH_3O), 3.74 (q, *J*: 6.72 Hz, 1H, $CHCH_3$), 3.65 (t, *J*: 4.26 Hz, 2H, CH_2OH), 3.36 (t, *J*: 4.26 Hz, 2H, CH_2NH), 1.62 (d, *J*: 6.09 Hz, 3H, $CHCH_3$).

1-[2-(6-*Methoxynaphthalen*-2-*yl*)-1-*oxo*-1-*propyl*]*proline* (**7c**): 81% yield; m.p. 156–157 °C; ^{1}H-NMR ($CDCl_3$) δ 7.75–7.61 (m, 3H, naphthyl), 7.35 (dd, *J*: 8.52 Hz, *J*: 1.83 Hz, 1H, naphthyl), 7.17–7.11 (m, 2H, naphthyl), 4.71 (m, 1H, CHCOOH), 3.92

(s, 3H, *C*H_3O), 3.88 (q, *J*: 6.69 Hz, 1H, C*H*CH_3), 3.53 (m, 1H, prolinyl), 3.30 (m, 1H, prolinyl), 2.47 (m, 1H, prolinyl), 1.97–1.84 (m, 3H, prolinyl), 1.54 (d, *J*: 7.32 Hz, 3H, CH_3CH).

2-(4-*Isobutylphenyl*)-1-(*pyrrolidin*-1-*yl*)*propan*-1-*one* (**8a**)*:* 87% yield; ^{1}H-NMR ($CDCl_3$) δ 7.18 (d, *J*: 7.95 Hz, 2H, phenyl H-2, H-6), 7.07 (d, *J*: 7.95 Hz, 2H, phenyl H-3, H-5), 3.71 (q, *J*: 6.72 Hz, 1H, $CHCH_3$), 3.43 (br, 4H, pyrrolidinyl H-2, H-5), 2.43 (d, *J*: 7.32 Hz, 2H, *C*H_2Ar), 1.86 (m, 5H, $CH(CH_3)_2$ and pyrrolidinyl H-3, H-4), 1.43 (d, *J*: 6.72 Hz, 3H, CH_3CH), 0.89 (d, *J*: 6.72 Hz, 6H, $(CH_3)_2$CH).

N-(2-*Hydroxyethyl*)-2–(4-*isobutylphenyl*)*propanamide* (**8b**): 85% yield; ^{1}H-NMR ($CDCl_3$) δ 7.19 (d, *J*: 7.95 Hz, 2H, phenyl H-2, H-6), 7.11 (d, *J*: 7.95 Hz, 2H, phenyl H-3, H-5), 6.04 (br, 1H, NH), 3.64 (t, *J*: 5.49 Hz, 2H, CH_2OH), 3.58 (q, *J*: 7.32 Hz, 1H, $CHCH_3$), 3.34 (m, 2H, CH_2N), 3.08 (s, 1H, OH), 2.44 (d, *J*: 7.32 Hz, 2H, *C*H_2Ar), 1.84 (m, 1H, $CH(CH_3)_2$), 1.51 (d, *J*: 7.32 Hz, 3H, CH_3CH), 0.90 (d, *J*: 6.69 Hz, 6H, $(CH_3)_2$CH).

1-[2-(4-*Isobutylphenyl*)-1-*oxo*-1-*propyl*]*proline* (**8c**): 91% yield; m.p. 113–114 °C; ^{1}H-NMR ($CDCl_3$) δ 7.24 (d, *J*: 7.95 Hz, 2H, phenyl H-2, H-6), 7.09 (d, *J*: 7.95 Hz, phenyl H-3, H-5), 4.72 (m, 1H, prolinyl H-2), 3.53 (q, *J*: 7.32 Hz, 1H, $CHCH_3$), 3.41–3.16 (m, 2H, prolinyl), 2.45 (d, *J*: 7.32 Hz, 2H, CH_2Ph), 1.94–1.69 (m, 5H, prolinyl and $CH(CH_3)_2$), 1.43 (d, *J*: 6.72 Hz, 3H, CH_3CH), 0.88 (d, *J*: 6.72 Hz, 6H, $(CH_3)_2$CH).

2-[4-(*Phenyl*)-3-*fluorophenyl*)-1-(*pyrrolidin*-1-*yl*)*propan*-1*one* (**9a**): 98% yield; m.p. 86–87 °C; ^{1}H-NMR ($CDCl_3$) δ 7.55–7.33 (m, 6H, phenyl), 7.16–7.11 (m, 2H, phenyl), 3.79 (q, *J*: 6.72 Hz, 1H, $CHCH_3$), 3.48 (br, 4H, pyrrolidinyl H-2, H-5), 1.86 (br, 4H, pyrrolidinyl H-3, H-4), 1.49 (d, *J*: 6.72 Hz, 3H, CH_3 CH).

2 -(4-*Phenyl*-3-*fluorophenyl*)-*N*-(2-*hydroxyethyl*)*propanamide* (**9b**): 92% yield; m.p. 82–83 °C; ^{1}H-NMR ($CDCl_3$) δ 7.55–7.36 (m, 6H, phenyl), 7.17–7.11 (m, 2H, phenyl), 6.00 (br, 1H, NH), 3.69 (t, 2H, *J*: 4.8 Hz, CH_2OH), 3.64 (q, *J*: 7.32 Hz, 1H, $CHCH_3$), 3.39 (m, 2H, CH_2N), 2.33 (br, 1H, OH), 1.55 (d, *J*: 7.32 Hz, 3H, CH_3 CH).

1-[2-(4-*Phenyl*-3-*fluorophenyl*)-1-*oxo*-1-*propyl*]*proline* (**9c**). 66% yield; ^{1}H-NMR ($CDCl_3$) δ 7.55–7.34 (m, 5H, phenyl), 7.14–7.09 (m, 3H, phenyl), 4.61 (m, 1H, CH prolinyl, 3.84 (q, *J*: 7.32 Hz, CHAr), 3.69 (m, 1H, pyrrolidin-1-yl), 3.35 (m, 1H, prolinyl), 2.09–1.91 (m, 4H, prolinyl), 1.53 (d, *J*: 7.32 Hz, 3H, CH_3 CH).

General procedure for the preparation of amines (10a–c to 12a–c)

We added (dropwise) a solution of the corresponding amide (**7–9**, 1 eq.) in dry THF (5 mL), to a suspension of lithium aluminum hydride (2–4 eq.) in dry THF (15 mL). Then we stirred the reaction mixture at about 25 °C for 3–4 h (except for compounds **10b**, **11b**, and **12b**, in which case we stirred the mixture under reflux for 3 h). In all cases, we monitored the reaction progress by TLC. After completion, we quenched the reaction by adding water (dropwise), 1 N NaOH solution, and ethyl acetate. After stirring for about 2 min, we separated the organic layer and dried it with sodium sulfate. Finally, we evaporated the solvent under vacuum, and we purified the corresponding amines by flash chromatography. All products (10–12) were used immediately after purification to obtain the corresponding ammonium salts (13–15) described below.

General procedure for the preparation of ammonium salts (13–15)

We passed dry HCl gas through a solution of the corresponding amine (**10–12**; 2 mmol) dissolved in diethyl ether (20 mL), under magnetic stirring until we did not observe any more precipitate coming out of solution. We filtered the resulting insoluble ammonium salt, washed it twice with ether, and dried it under vacuum in a desiccator for 18 h. To generate HCl gas, we produced it *in situ* by adding (dropwise) conc. H_2SO_4 to a commercial 37% solution of HCl under vigorous magnetic stirring. Then we transferred the HCl gas produced in this reaction using Teflon tubing connected to a $CaCl_2$ trap. While passing the HCl gas, we isolated the system (three-necked round bottom flask) by using a septum inlet adapter with stopcock, to which we had attached a balloon to store any excess of HCl gas.

1-(2-(6-*Methoxynaphthalen*-2-*yl*)*propyl*)*pyrrolidinium chloride* (**13a**): 89.36% yield; m.p. 197–198 °C; ^{1}H-NMR (D_2O) δ 7.88 (m, 3H, naphthyl), 7.52 (dd, *J*: 8.55 Hz, 1.83 Hz, 1H, naphthyl), 7.38 (d, *J*: 2.43 Hz, 1H, naphthyl), 7.25 (dd, *J*: 8.55 Hz, 2.43 Hz, 1H, naphthyl), 3.95 (s, 3H, *C*H_3O), 3.62 (m, 2H, *C*H_2N), 3.4 (m, 1H, *C*HCH_3), 3.28 (br, 4H, pyrrolidinyl), 1.95 (br, 4H, pyrrolidinyl), 1.38 (d, *J*: 6.72 Hz, 3H, *CH*$_3$CH). ^{13}C-NMR (D_2O): 156.4, 136.4, 132.9, 128.6, 128.1, 127.2, 125.4, 124.9, 118.1, 105.5, 60.5, 54.7, 53.9, 36.1, 21.8, 18.3. ESI-MS (*m*/*z*); 292 $[M + Na]^+$(100%), 269 $[M + H]^+$.

N-(2-*Hydroxyethyl*)-2–(6-*methoxynaphthalen*-2-*yl*)*propan*-1-*ammonium chloride* (**13b**): 85.0% yield; m.p. 230–231 °C; 1-NMR (D_2O): δ 7.91–7.81 (m, 3H, naphthyl), 7.51 (dd, *J*: 8.55 Hz, *J*: 1.23 Hz, 1H, naphthyl), 7.38 (d, *J*: 2.43 Hz, 1H, naphthyl), 7.23 (dd, *J*: 9.15 Hz, *J*: 2.43 Hz, 1H, naphthyl), 3.95 (s, 3H, *C*H_3O), 3.77 (t, *J*: 5.49 Hz, 2H, CH_2OH), 3.39 (m, 1H, *C*HCH_3), 3.35 (m, 2H, *C*H_2NH), 3.15 (q, *J*: 5.49 Hz, *C*H_2NH), 1.41 (d, *J*: 6.72 Hz, 3H, *CH*$_3$CH). ^{13}C-NMR (D_2O): 156.3, 136.4, 132.8, 128.6, 128.1, 127.1, 125.2, 125.0, 118.1, 105.5, 55.5, 54.6, 52.5, 48.5, 36.1, 18.3. ESI-MS (*m*/*z*); 296 $[M + Na]^+$(100%), 273 $[M + H]^+$.

2-(*Hydroxyethyl*)-1-[2-(6-*methoxynaphtalen*-2-*yl*)-1-*propyl*]-*pyrrolidinium chloride* (**13c**): 85.0% yield, m.p. 153–154 °C; ^{1}H-NMR (D_2O) δ 7.91–7.83 (m, 3H, naphthyl), 7.51 (dd, *J*: 8.55 Hz, *J*: 1.23 Hz, 1H, naphthyl), 7.37 (d, *J*: 2.44 Hz, 1H, naphthyl), 7.24 (dd, *J*: 8.55 Hz, *J*: 2.44 Hz, 1H, naphthyl), 3.94 (s, 3H, *C*H_3O), 3.87 (m, 2H, CH_2OH), 3.66 (m, 1H, *C*HCH_3), 3.59 (m, 2H, CH_2N), 3.42 (m, 2H, pyrrolidinyl), 3.28 (m, 1H, pyrrolidinyl), 2.15 (m, 2H pyrrolidinyl), 1.90–1.67 (two m, 2H, pyrrolidinyl), 1.36 (d, *J*: 6.81 Hz, 3H, *CH*$_3$CH). ^{13}C-NMR (D_2O): 156.4, 136.0, 132.9, 128.6, 128.1, 127.3, 125.4, 124.8, 118.1, 105.5, 69.2, 58.3, 54.6, 53.6, 53.5, 35.9, 24.7, 21.1, 19.5. ESI-MS (*m*/*z*); 322 $[M + Na]^+$ (100%), 299 $[M + H]^+$.

1-(2-(4-*Isobutylphenyl*)*propyl*)*pyrrolidinium chloride* (**14a**)*:* 90.9% yield; m.p. 187–188 °C; ^{1}H-NMR (D_2O) δ 7.33–7.27 (m, 4H, phenyl), 3.59 (m, 4H, pyrrolidinyl), 3.15 (m, 2H, *C*H_2N), 2.98 (m, 1H, CHAr), 2.49 (d, *J*: 6.69 Hz, 2H, CH_2Ar), 2.07–1.80 (two m, 4H, pyrrolidinyl), 1.81 (m, 1H, $CH(CH_3)_3$, 1.30 (d, *J*: 6.72 Hz, 3H, CH_3CH), 0.87 (d, *J*: 6.09 Hz, 6H, $(CH_3)_2$CH). ^{13}C-NMR (D_2O): 141.1, 138.3, 129.3, 126.3, 60.6, 54.5, 53.2, 43.4, 35.7, 28.9, 21.8, 20.8, 18.9. ESI-MS (*m*/*z*); 268 $[M + Na]^+$(100%), 245 $[M + H]^+$.

N-(2-*Hydroxyethyl*)-2-(4-*isobutylphenyl*)*propan*-1-*ammonium chloride* (**14b**): 89.6% yield; m.p. 137–138 °C; ^{1}H-NMR (D_2O) δ 7.32–7.26 (m, 4H, phenyl), 3.78 (t, *J*: 4.89 Hz, 2H, CH_2OH), 3.32 (d, *J*: 7.32 Hz, 2H, CH_2N), 3.22–3.09 (m, 3H, CH_2N and *C*HAr), 2.49 (d, *J*: 7.32 Hz, 2H, CH_2Ar), 1.85 (m, 1H, *C*$H(CH_3)_2$), 1.32 (d, *J*: 7.32 Hz, 3H, CH_3), 0.87 (d, *J*: 6.72 Hz, 6H, $(CH_3)_2$CH). ^{13}C-NMR (D_2O): 141.1, 138.2, 129.3, 126.3, 55.5, 52.7, 48.5, 43.4, 35.7, 28.9, 20.8, 18.3. ESI-MS (*m*/*z*); 258 $[M + Na]^+$(100%), 235 $[M + H]^+$.

2-(*Hydroxymethyl*)-1-[2-(4-*isobutylphenyl*)-1-*propyl*]*pyrrolidinium chloride* (**14c**)*:* 67.5% yield; m.p. 145–146 °C; ^{1}H-NMR (D_2O) δ 7.35–7.25 (m, 4H, phenyl), 3.88 (m, 2H, CH_2OH), 3.77 (m, 2H, CH_2N) 3.42–3.21 (m, 3H, pyrrolidinyl), 2.99 (m, 1H, CHAr), 2.49 (d, *J*: 6.72 Hz, 2H, CH_2Ar), 2.22–2.11

(m, 4H, pyrrolidinyl), 1.83 (m, 1H, $CHCH_2$), 1.31 (d, *J*: 6.69 Hz, 3H, CH_3CH), 0.87 (d, *J*: 6.72 Hz, 6H, $(CH_3)_2CH$). ^{13}C-NMR (D_2O): 140.8, 139.1, 129.5, 129.2, 126.4, 126.2, 69.3, 60.8, 60.3, 58.1, 43.4, 35.7, 28.9, 24.4, 21.2, 20.7, 19.5, 19.3. ESI-MS (*m/z*); 298 $[M + Na]^+$ (100%), 275 $[M + H]^+$.

1-[2-(4-*phenyl*-3-*fluorophenyl*)-1-*propyl*]*pyrrolidinium chloride* (**15a**)*:* 70% yield; m.p. 197–198 °C; ^{1}H-NMR (D_2O) δ 7.60–7.43 (m, 6H, phenyl), 7.27–7.22 (m, 2H, phenyl), 3.56–3.39 (m, 6H, 4H pyrrolidinyl, and CH_2N), 3.24 (m, 1H, $CHCH_3$), 2.00 (br, 4H, pyrrolidinyl), 1.33 (d, *J*: 6.72 Hz, 3H, CH_3 CH). ^{13}C-NMR (D_2O): δ 160.4, 146.0, 137.4, 133.8, 131.2, 130.5, 130.0, 126.0, 117.4, 117.1, 63.4, 57.7, 57.0, 38.9, 24.9, 21.8. ESI-MS (*m/z*); 306 $[M + Na]^+$(100%), 283 $[M + H]^+$.

N-(2-*Hydroxyethyl*)-2-(4-*phenyl*-3-*fluorophenyl*)*propan*-1-*ammonium chloride* (**15b**): 65% yield; m.p. 157–158 °C; ^{1}H-NMR (D_2O) δ 7.63–7.44 (m, 6H, phenyl), 7.26 (m, 2H, phenyl), 3.83 (t, *J*: 4.89 Hz, 2H, CH_2OH), 3.37 (d, *J*: 7.95 Hz, 2H, CH_2N), 3.31 (m, 1H, $CHCH_3$), 3.19 (m, 2H, CH_2N), 1.37 (d, *J*: 6.72 Hz, 3H, CH_3 CH). ^{13}C-NMR (D_2O): 160.4, 146.1, 146.0, 137.5, 133.8, 131.6, 130.6, 129.3, 117.4, 117.1, 58.7, 55.5, 51.8, 38.9, 21.4. ESI-MS (*m/z*); 296 $[M + Na]^+$(100%), 273 $[M + H]^+$.

1-[2-(4-*Phenyl*-3-*fluorophenyl*)*propan*-1-*yl*]-2-*hydroxymethyl-pyrrolidinium chloride* (**15c**)*:* 70% yield; m.p. 54 °C; ^{1}H-NMR (D_2O) δ 7.76–7.47 (m, 8H, phenyl), 3.90 (m, 2H, CH_2OH), 3.69 (m, 3H, CH_2CH and CH pyrrolidinyl), 3.47 (m, 2H, CH_2 pyrrolidinyl), 3.32 (m, 1H, *C*HAr), 2.15–1.87 (m, 4H, pyrrolidinyl), 1.38 (d, *J:* 6.72 Hz, 3H, *CH*$_3$ CH). ^{13}C-NMR (D_2O): δ 160.1, 146.5, 137.4, 133.6, 131.4, 130.6, 130.1, 127.1, 117.3, 117.1, 70.0, 60.9, 59.0, 54.4, 36.3, 25.1, 21.8, 20.1. ESI-MS (*m/z*); 336 $[M + Na]^+$ (100%), 313 $[M + H]^+$.

Cyclooxygenase inhibition assay

We determined the ability of the test compounds (listed in Table 1) to inhibit ovine COX-1 and human recombinant COX-2 by using a fluorescent inhibitor screening assay kit (Catalog no. 700100, Cayman Chemicals Inc., Ann Arbor, MI). We followed the procedure suggested by the manufacturer (the excitation wavelength = 530–540 nm, the emission wavelength of 585–595 nm).

Table 1. Cyclooxygenase (COX)-1 and COX-2 inhibition by water soluble NSAID derivatives lacking a carboxylic acid group (**13a–13c**, **14a–14c**, and **15a–15c**).

	IC_{50} (μM)*		
Compd.	COX-1	COX-2	COX-2 S.I.†
13a	>100	1.6	>62
13b	>100	8.1	>12
13c	>100	2.9	>34
14a	>100	22.2	>4
14b	>100	28.1	>3
14c	>100	20.7	>5
15a	>100	60.4	1.6
15b	>100	19.2	>5
15c	>100	29.0	>3
Celecoxib	>100	0.02¶	>5000

*The test compound concentration required to produce (*in vitro*) 50% inhibition of ovine COX-1 or human recombinant COX-2. The result (IC_{50}, μM) is the mean of two determinations acquired using the enzyme immunoassay kit (Catalog no. 560131, Cayman Chemicals Inc., Ann Arbor, MI) and the deviation from the mean is <10% of the average value.

†*In vitro* COX-2 selectivity index (COX-1 IC_{50}/COX-2 IC_{50}).

¶Data acquired using ovine COX-2 (Catalog No. 56101, Cayman Chemical Inc., Ann Arbor, MI).

Carrageenan-induced paw edema anti-inflammatory assay

We evaluated the test compounds, as well as the reference drugs naproxen, ibuprofen, and flurbiprofen using the *in vivo* carrageenan-induced rat foot paw edema model reported previously[17]. We administered all compounds orally, suspended in methylcellulose (1% aqueous solution) as the vehicle. We carried out this assay using protocols approved by the Health Sciences Animal Welfare Committee at the University of Alberta.

Docking studies

We performed the molecular docking experiments using crystal coordinates from the X-ray crystal structure of COX-2 (murine, 6COX, SC558 bound in the active site), obtained from the protein data bank. All the test compounds were built using the builder toolkit of the software package ArgusLab 4.0.1 (Mark, A. ArgusLab, Version 4.0.1; Thompson Planaria Software LLC, Seattle, WA). We carried out an energy minimization using the semi-empirical quantum mechanical method p.m.3. We chose the monomeric structure of the enzyme, and we defined the active site around the ligand. Then we inserted the molecule to be docked in the active site of the enzyme in the workspace, carrying the structure of the protein. The docking program implements an efficient grid-based docking algorithm that approximates an exhaustive search within the free volume of the binding site cavity. We surveyed the conformational space by geometry optimization of the flexible ligand (where we considered the rings as rigid), in combination with the incremental construction of the ligand torsions. Thus, the docking occurred between the flexible ligand parts of the compound and enzyme. We determined the ligand orientation by a shape scoring function based on A score, and we ranked the final positions by the lowest interaction energy values. The $E_{interaction}$ is the sum of the energies involved in H-bond interactions, hydrophobic interactions, and van der Waal's interactions. Finally, we determined the H-bond and hydrophobic interactions between the test compounds and the enzyme by distance measurements.

Ulcerogenic assay

We evaluated the ulcerogenic activity of naproxen (40 mg/kg) and the test compounds **13a**, **13b**, and **13c** (equimolar dose to 40 mg/kg of naproxen), after oral administration of a single dose of the drug, suspended in 1.0 mL of a 1% methylcellulose solution. Control rats received only the vehicle (1.0 mL of 1% methylcellulose solution). We removed food 24 h before the administration of test compounds, but we allowed the animals to have a regular water intake. We euthanized the animals (CO_2 chamber) 6 h after oral administration of the drug; then we removed the stomachs, we cut them along their greater curvature, we gently rinsed them with water, and we finally placed on ice. We determined the number, and the length of ulcers observed in each stomach using a magnifier lens. We assessed the severity of each gastric lesion along its greatest length (1 mm = rating of 1, 1–2 mm = score of 2, >2 mm = score according to their length in mm). We calculated the ''ulcer index'' (UI) for each test compound by adding the total length (*L*, in mm) of individual ulcers in each stomach, and averaging over the number of animals in each group ($n = 4$): $UI = (L_1 + L_2 + L_3 + L_4)/4$.

Arthritic index assay

We randomly divided the rats into different groups ($n = 4$/group). On day one, we injected all rats with 0.2 mL of *Mycobacterium butyricum* in squalene (50 mg/mL). We injected the control animals 0.2 mL of normal saline solution. We started the drug

administration (1 mg/kg) on the first day of the experiment as a preventive measure. During the experiment, we measured daily the body weight gain and paw thickness using a caliper (sensitivity = 25 μm; Mitutoyo Canada Inc., Toronto, ON). On day 18 after injection of the adjuvant, we calculated the arthritis index according to a previously described method[18]. Briefly, (a) for hinds (0–4 score): 0 = no virtual sign of arthritis; 1 = involved a single joint; 2 = more than one joint and/or ankle with swelling; 3 = several joints and ankle with moderate swelling; 4 = several joints and ankle with severe swelling. (b) for paws (0–3 score): 0 = no inflammation in any joint, 1 = involved inflammation a single joint, 2 = more than one joint and/or wrist with moderate inflammation, and 3 = wrist and joints with moderate-to-severe swelling.

Statistics

We presented the results as the mean values ± SEM. We determined statistical differences between treatment groups using one-way ANOVA, followed by the Student–Newman–Keuls *post hoc* test using SigmaStat 3.5 program for Windows, Systat Software Inc. (San Jose, CA).

Results and discussion

Chemistry

We describe the synthesis of polar NSAID derivatives possessing a pyrrolidinium, 2-(hydroxymethyl)pyrrolidinium, or a 2-hydroxyethylammonium chloride in Scheme 1. In this regard, we reacted the corresponding NSAID with oxalyl chloride in dichloromethane to form acid chlorides (**4–6**), which we then stirred with pyrrolidine, L-proline, or 2-hydroxylamine to afford the amide intermediates **7a–7c**, **8a–8c**, and **9a–9c**. The subsequent reduction of the carbonyl group using lithium aluminum hydride in dry THF afforded the corresponding amines **10a–10c**, **11a–11c**, and **12a–12c**. The final step was the formation of ammonium chloride salts by reacting solutions of the corresponding amine in ether with dry hydrochloric acid. Following this procedure, we obtained the final products (**13a–13c**, **14a–14c**, and **15a–15c**) in moderate to good yields.

In vitro COX inhibition

The mechanism of action of classical NSAIDs is one of the most well-established drug targets in Medicinal Chemistry. Therefore, the obligated step toward the biological evaluation of any investigational drug with potential anti-inflammatory activity involves an *in vitro* screening for COX inhibition. In this regard, the ammonium salts obtained from naproxen (**13a–13c**) showed a significant inhibitory profile when we incubated them with human recombinant COX-2, exhibiting IC_{50} values in the 1.6–8.1 μM range (Table 1). We observed the same inhibitory profile for the ibuprofen derivatives **14a–14c** (COX-2 IC_{50}'s in the 20.7–28.1 μM range), and the flurbiprofen derivatives **15a-15c** (COX-2 IC_{50}'s in the 19.2–60.4 μM range). Under the experimental conditions used in our laboratory, we could not detect a

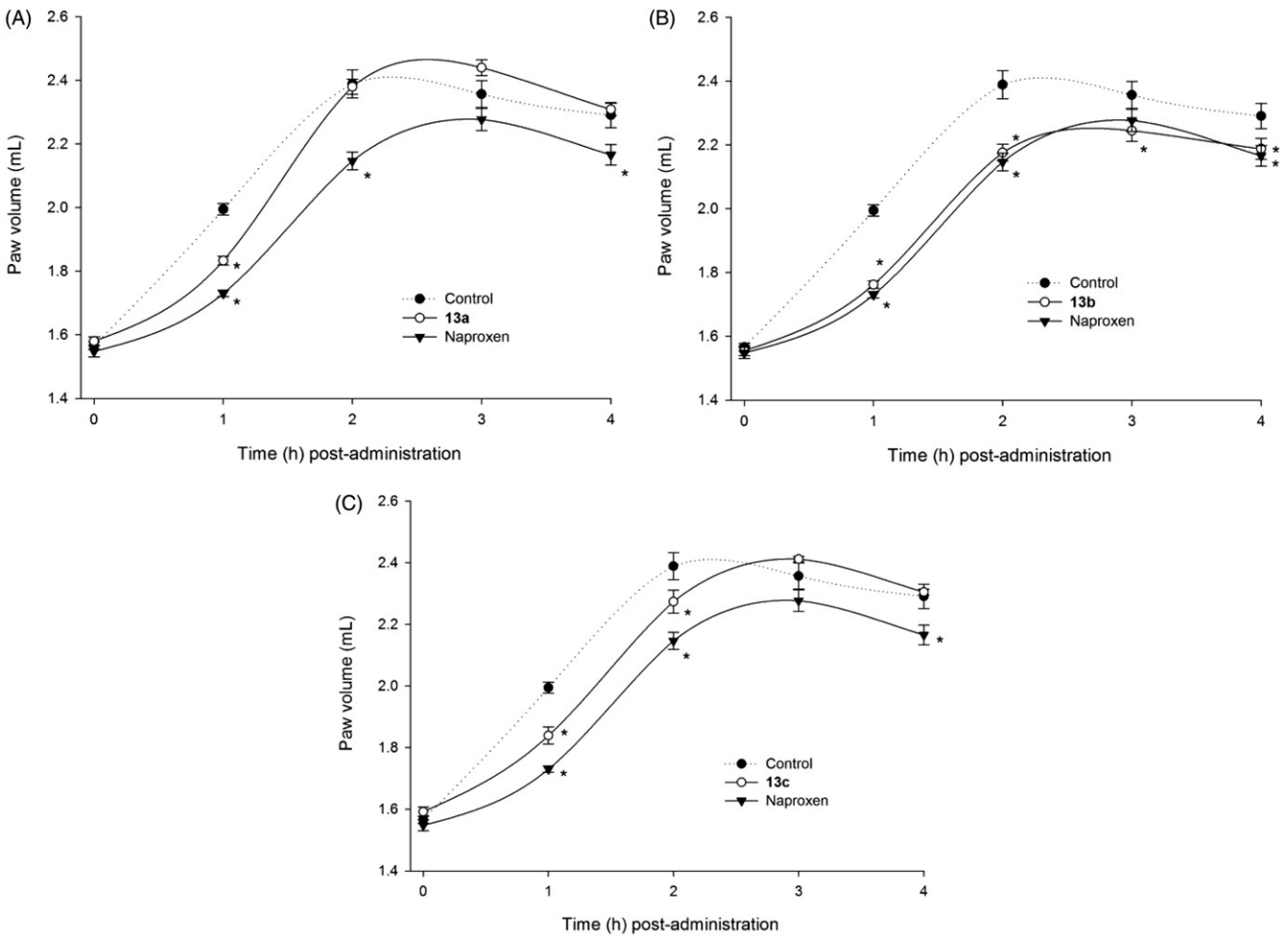

Figure 1. Time-dependent anti-inflammatory effect exerted by naproxen derivatives. (A) Compound **13a**, (B) compound **13b**, (C) compound **13c**; four rats were used in each group ($n = 4$); individual values represent the average of 40 measurements (10 measurements per animal) ± SEM; (*) = $p < 0.05$ versus the control (vehicle) group for the time points marked.

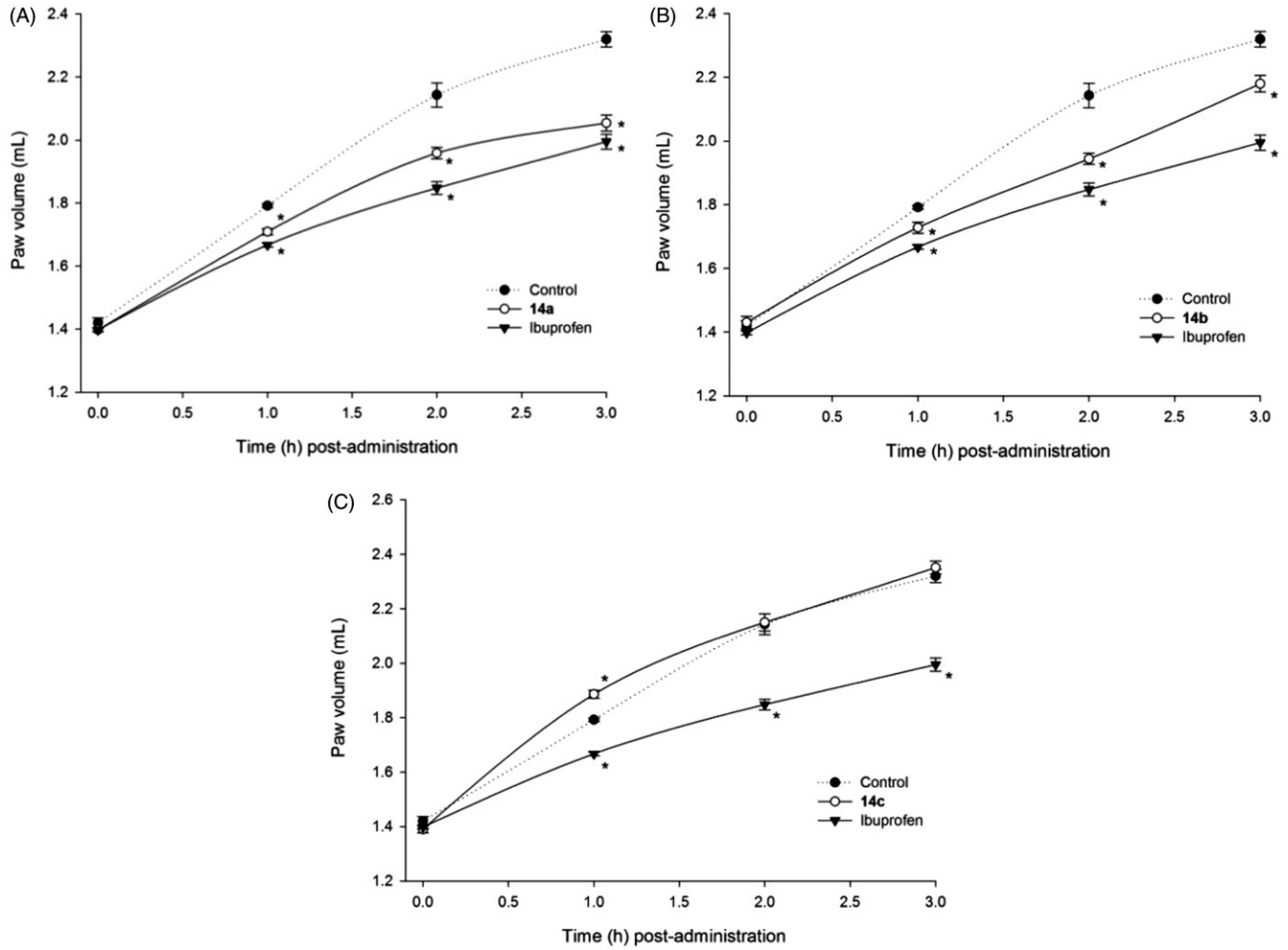

Figure 2. Time-dependent anti-inflammatory effect exerted by ibuprofen derivatives. (A) Compound **14a**, (B) compound **14b**, (C) compound **14c**; four rats were used in each group ($n = 4$); individual values represent the average of 40 measurements (10 measurements per animal) ± SEM; (*) = $p < 0.05$ versus the control (vehicle) group for the time points marked.

significant inhibition of the COX-1 (bovine) isoform by any of the test compounds, at the maximum compound concentration (100 μM). The relative potency of all ammonium salts tested *in vitro* was 13 (naproxen derivatives) >14 (ibuprofen derivatives) >15 (flurbiprofen derivatives). Moreover, we observed that all compounds were relatively weaker COX inhibitors compared to the reference drug celecoxib (COX-2 IC_{50} value = 0.02 μM; COX-1 IC_{50} value >100 μM).

These results suggest a selective and relatively potent inhibitory activity of the COX-2 enzyme, while sparing the enzymatic activity of the COX-1 isoform (the extent at which the test compounds inhibited COX-1 activity at the highest test compound concentration of 100 μM was in the 17–44% range).

In vivo anti-inflammatory activity (carrageenan-induced inflammatory model)

To evaluate the potential *in vivo* anti-inflammatory profile exerted by the ammonium salts derived from naproxen, ibuprofen, and flurbiprofen, we carried out the conventional carrageenan-induced rat paw edema assay[17] using male Sprague–Dawley rats.

When we administered (orally) the ammonium salts derived from naproxen (**13a–13c**), we observed a significant and time-dependent anti-inflammatory effect exerted by the test drugs (at a dose equivalent to 5 mg/kg of naproxen). In this regard, derivative **13a** showed a statistically significant ($p < 0.05$) anti-inflammatory effect 1 h after the injection of the carrageenan reagent, but unlike naproxen, this compound was inactive from the second time point (2 h) onward; this effect can be observed in the corresponding dose–response curve (Figure 1A). However, compound **13b** significantly decreased inflammation at all time-points (1–4 h, Figure 1B), but derivative **13c** showed a significant anti-inflammatory effect (compared with the control group), only during the first 2 h (Figure 1C).

Importantly, when we compared the anti-inflammatory profile of derivatives **13a–13c**, to that obtained with the parent drug naproxen, at the same molar dose, it is evident that the carboxylic acid group present in the NSAID is not an essential requirement for the therapeutic effect. At least one derivative (compound **13b**), maintained the desired *in vivo* anti-inflammatory activity (Figure 1B).

The results obtained with the ammonium salts derived from ibuprofen (**14a–14c**), provided additional evidence to support the non-essential role of the carboxylic acid group. Even though compounds **14a** and **14b** were not as potent as ibuprofen, they exerted a statistically significant ($p < 0.05$) anti-inflammatory effect compared with the control group (Figure 2A and B). Nevertheless, compound **14c** (oral dose equivalent to 60 mg/kg of ibuprofen) was not active at any time point.

Finally, when we determined the anti-inflammatory activity exerted by the flurbiprofen ammonium salts **15a–15c** (oral dose equivalent to 0.5 mg/kg of flurbiprofen), we observed that these

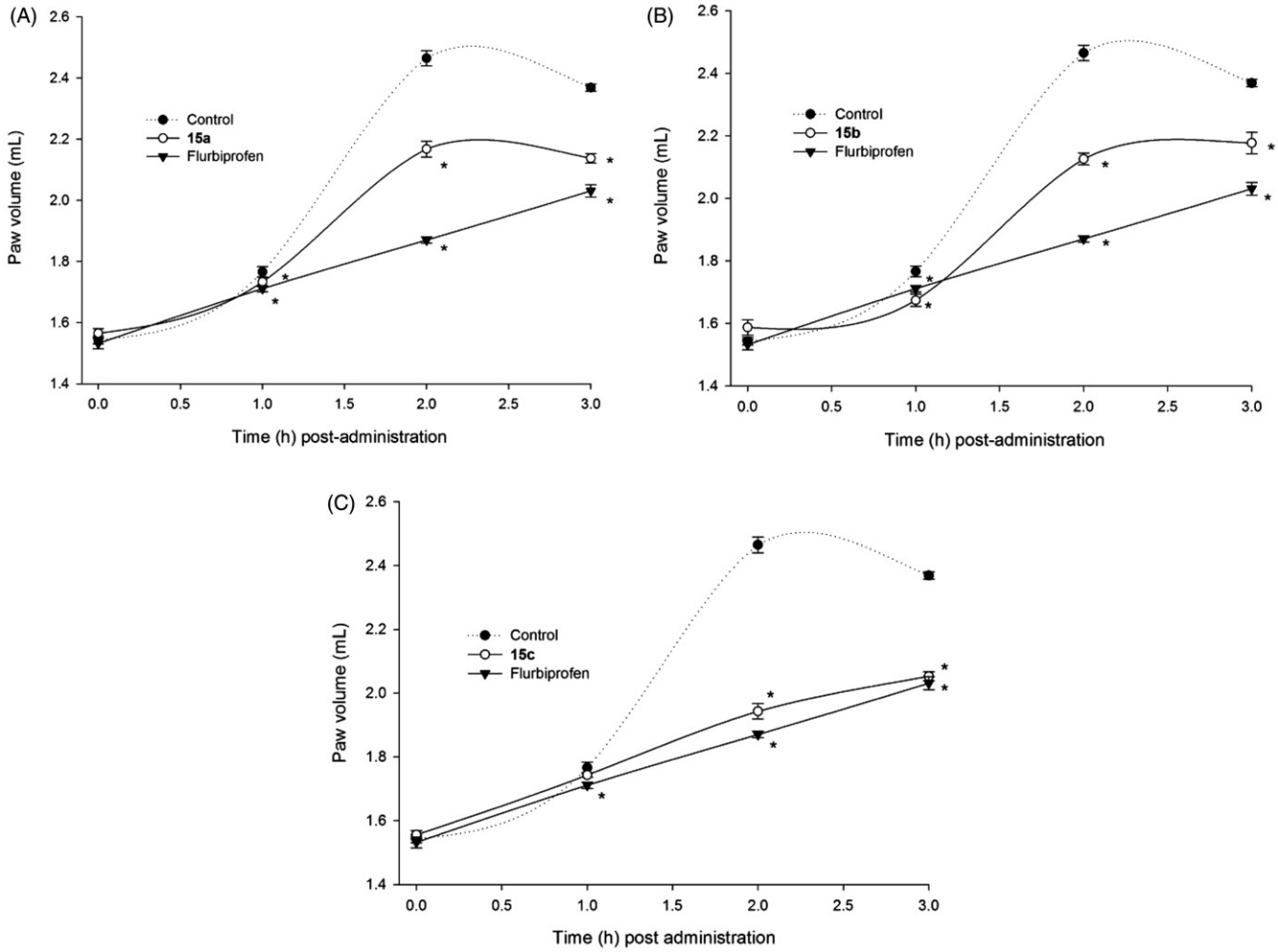

Figure 3. Time-dependent anti-inflammatory effect exerted by flurbiprofen derivatives. (A) Compound **15a**, (B) compound **15b**, (C) compound **15c**; four rats were used in each group ($n = 4$); individual values represent the average of 40 measurements (10 measurements per animal) ± SEM; (*) = $p < 0.05$ versus the control (vehicle) group for the time points marked.

drugs exerted a significant anti-inflammatory effect ($p < 0.05$) compared to control animals. This difference was bigger at 2 h and 3 h post-injection of the carrageenan reagent (Figure 3), suggesting that the ammonium salts **15a–15c** are absorbed at low rates from the gastrointestinal tract of rats (''slow-acting'' drugs). In this regard, derivative **15c** showed a very similar anti-inflammatory profile compared with the parent drug flurbiprofen (Figure 3A–C).

Docking studies

To find out if the NSAID derivatives **13–15** can bind to the active site of the COX enzyme, despite not having the carboxylic acid group present in their parent counterparts, we conducted a molecular modeling (docking) study using the active naproxen derivative **13b**. According to our docking studies, compound **13b** oriented favorably within the COX-2 active site, in such a way that the terminal hydroxyl group (OH) is close to Gln192, Phe518, Arg513, and Val523 (active site residues; Figure 4). The hydroxyl group of compound **13b** formed hydrogen bonding interactions with a nitrogen atom in Phe518 (OH–N distance = 2.63 Å). At the same binding pose, the hydroxyl group also formed a hydrogen bond with the carbonyl oxygen of Gln192 (OH–O distance = 2.07 Å; COX-2 secondary pocket residue). Additionally, we observed that the nitrogen atom in compound **13b** formed an additional hydrogen bond with Leu352 (COOH–N distance = 2.23 Å), and it is close to Gln192 (the distance between the carbonyl oxygen and the OH = 3.01 Å). Interestingly, the two conjugated aromatic rings of the naproxen derivative **13b** were located near the hydrophobic region of the COX-2 active site formed by Trp387, Phe518, and Tyr385.

An interesting case is that observed with compound **15c**, which is a flurbiprofen derivative that also showed significant efficacy *in vivo*. The best binding mode obtained from the molecular docking study, showed that compound **15c** resided at the center of the COX-2 active site (Figure 5), where the fluorine atom present at one of the phenyl rings is close to both the Phe518 (1.98–2.89 Å range), and the Val523 (2.01–3.02 Å range) residues. Notably, the hydroxyl group (–OH) of the prolinol ring is located in the vicinity of Leu352, Ala527, and Gly526 residues. Also, the hydroxyl group showed hydrogen bonding interactions with the carbonyl oxygen of Val523 (2.46 Å) and the NH group of Ala527 (2.56 Å). In the best binding mode calculated for compound **15c**, the unsubstituted phenyl ring is located in the vicinity of Gln192, Phe518, Ala516, Arg513, and Val523 amino acid residues (Figure 5).

Previous reports describe a particular role for the carboxylic acid group in the overall binding modes of classical NSAIDs within the COX active site. For example, Ermondi et al.[19] described the binding interaction between the carboxylate group present in flurbiprofen and a cavity in the enzyme formed by Leu531, Val116, Val349, Leu359, and Tyr355. Authors also

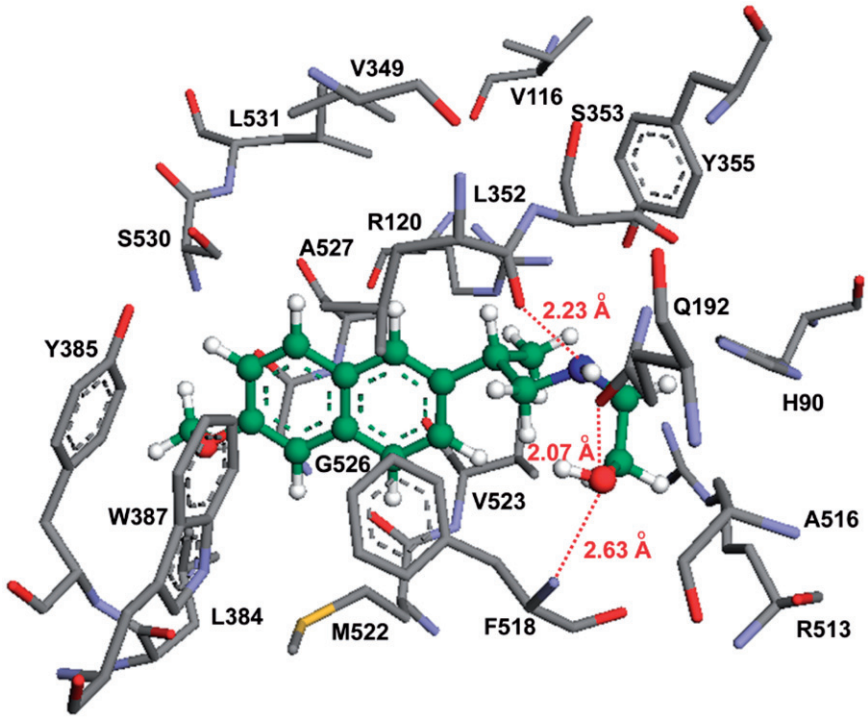

Figure 4. Molecular modeling (docking) of compound **13b** (carbon atoms in green color) in the binding site of COX-2 (PDB ID: 6COX; $E_{\text{intermolecular}} = -12.23$ kcal/mol). Hydrogen atoms of amino acid residues have been omitted for clarity.

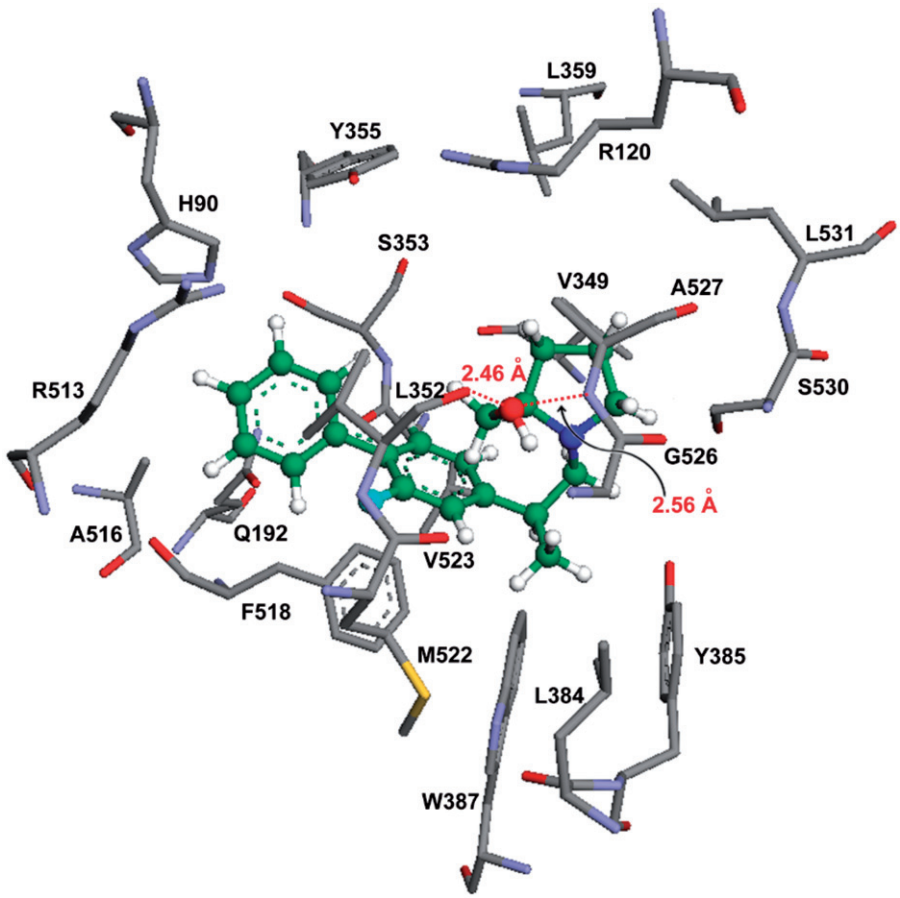

Figure 5. Molecular modeling (docking) of compound **15c** (carbon atoms in green color) in the binding site of COX-2 (PDB ID: 6COX; $E_{\text{intermolecular}} = -12.16$ kcal/mol). Hydrogen atoms of amino acid residues have been omitted for clarity.

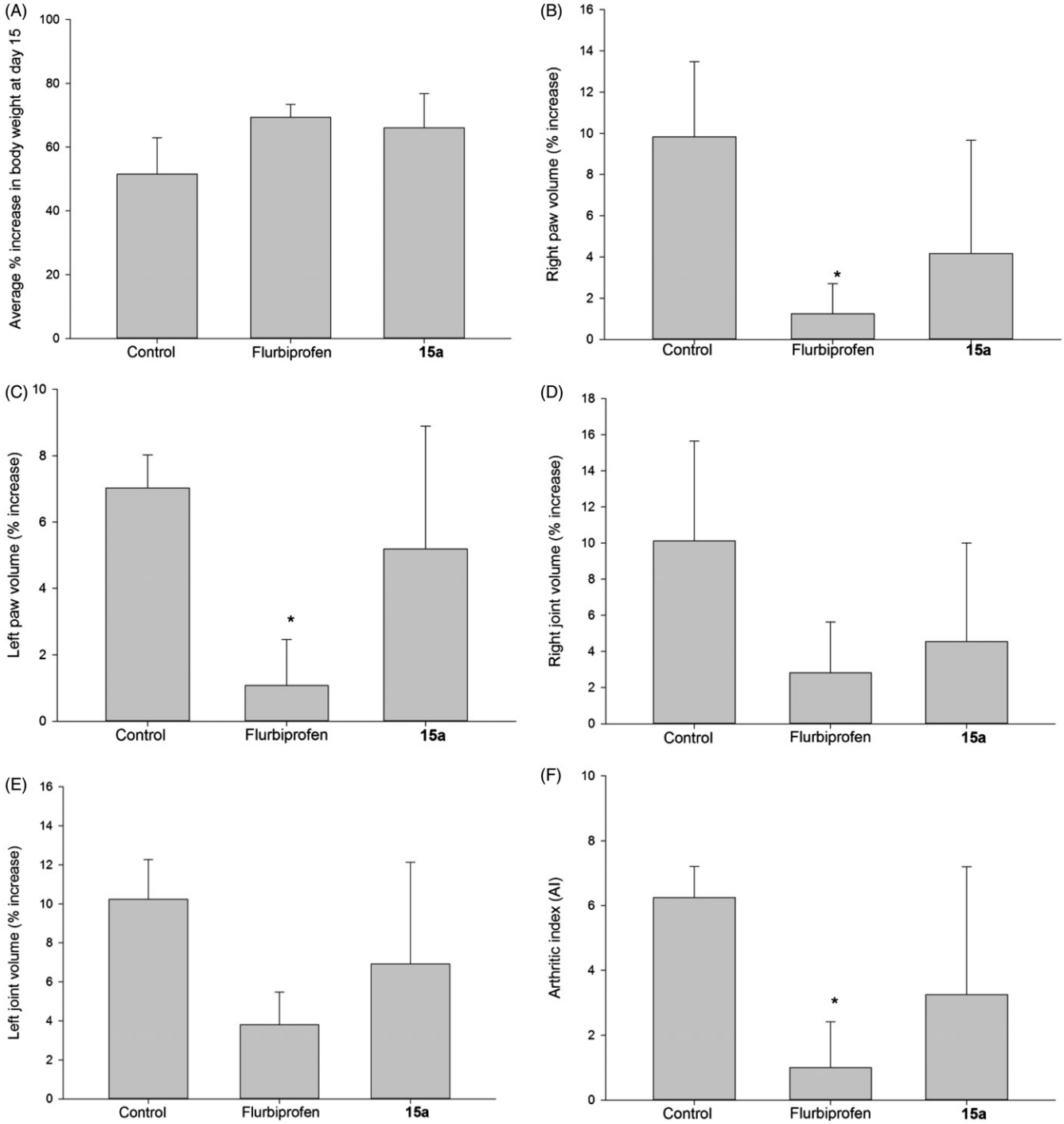

Figure 6. 15-Day anti-inflammatory study comparing flurbiprofen with compound **15a**. (A) Percent increase in body weight; (B) percent increase of right paw volumes; (C) percent increase of left paw volumes; (D) percent increase of right joint volumes; (E) percent increase in left joint volume; (F) the arthritic index calculated for compound **15a** and flurbiprofen. All values are expressed as percent increase at day 15 compared to day 1; $N = 4$; doses: flurbiprofen (1 mg/kg orally), **15a** (equimolar dose) treated groups. (*) $= p < 0.05$ versus the control (vehicle) group.

suggest that the –COOH group in NSAIDs limits their selectivity toward COX-1 or COX-2, due to decreased ''freedom of movement'' created by a binding interaction with Arg120. Additional binding interactions with Ser530 and Tyr385 were also considered significant, accounting for some enzyme–ligand interactions that are also relevant to the inhibition of COX-2[19].

Therefore, considering that derivatives **13a**–**13c**, **14a**–**14c**, and **15a**–**15c** do not have a carboxylic acid functional group, and still exert *in vitro* inhibition of the COX-2 isoform, while producing significant *in vivo* anti-inflammatory activity, provides evidence to argue against the importance of the COOH group in the chemical structure of NSAIDs. Furthermore, the complete entry of compound **13b** and **15c** into the COX-2 active site, and the significant electronic interactions observed with these compounds within the COX-2 active site, is consistent with the significant anti-inflammatory activity determined experimentally.

In vivo anti-inflammatory activity (arthritic index)

We decided to challenge one of the new NSAID derivatives (**15a**) using a longer-term *in vivo* assay, in which Sprague–Dawley rats

are injected with *M. butyricum*, inducing an arthritis-like state. As shown in Figure 6, the effects produced by compound **15a** were modest; we evaluated the increase in both right and left paw volume in animals treated with the test drug (1 mg/kg p.o.), and compared them to those obtained with vehicle-treated (control). We also tested the standard (flurbiprofen, equimolar dose to **15a**). In this regard, we observed that only the animal group receiving flurbiprofen showed a statistically significant difference ($p < 0.05$) compared with control animals. The change in volume recorded for the right paw in animals dosed with **15a** (Figure 6B), also experienced a significant decrease. However, due to the high variability associated with this experiment, we could not establish a potential anti-inflammatory associated to drug **15a**. We observed a similar case when we compared the left and right *joints* of animals dosed with drug **15a**; the decrease in joint volume suggested a potential anti-inflammatory effect exerted by both flurbiprofen (Figure 6D), and **15a** (Figure 6E), but this effect was not statistically significant when we compared it with that obtained with the control group.

We observed an encouraging result when we recorded the change (increase) in body weight in animals dosed with flurbiprofen and compound **15a**. This observation suggests that arthritic animals dosed with drug **15a** and flurbiprofen, may have experienced some relief from pain and inflammation, which presumably, allowed these animals to feed better (hence the increase in body weight) than those in the control group (Figure 6A).

These results seem to suggest that the removal of the carboxylic acid group present in naproxen, ibuprofen, and flurbiprofen does not abolish their desirable anti-inflammatory activity, and therefore, the acid group *may not be an essential requirement for these drugs*. Moreover, these results showed that *in vitro* screening should not be the only parameter by which a new anti-inflammatory drug is are evaluated experimentally. For example, despite the weak *in vitro* COX-2 enzyme inhibition exerted by compound **15a** (only 34% inhibition at 100 μM), this compound significantly decreased inflammation when tested *in vivo* (it displayed 50% of the anti-inflammatory effect exerted by flurbiprofen at 2 h, and almost the same effect at 3 h; see Figure 3A).

One of the potential repercussions of this observation is the fact that NSAIDs are regarded as potential cancer chemopreventive drugs[20,21] and consequently, in addition to complementary experiments aimed at validating the anti-inflammatory activity of these ammonium salts, we are also interested in exploring the effects of these compounds on cancer-related targets commonly associated with classical NSAIDs (future work).

Table 2. Ulcer index (UI) determined for NSAID derivatives **13a–13c**.

Compd.	Ulcer index*
Control†	0
Naproxen‡	48
13a¶	0
13b¶	0
13c¶	0

*The overall average length (in mm) of individual ulcers in each stomach ± SEM, $n = 4$, at 6 h after oral administration of the test compound.
†Vehicle treated (1.0% methylcellulose solution).
‡40 mg/kg dose.
¶Equivalent amount to 40 mg of naproxen/kg.

Ulcer index assay

To complement our study, we needed to evaluate the potential gastric toxic effects exerted by the test compounds using an acute NSAID-induced ulcerogenic assay[22]. In this regard, we dosed animals with oral naproxen (40 mg/kg), or an equimolar amount of compounds **13a**, **13b**, or **13c** (single oral dose). After 6 h, we euthanized all animals, and we analyzed their stomachs looking for ulcers. We observed that only the rats dosed with the parent naproxen developed gastric lesions (ulcer index [UI] = 48), whereas the naproxen derivatives **13a–13c** did not exert visually detectable gastric toxicity (UI = 0; see Table 2 and Figure 7). This provides strong (but, we know, preliminary) evidence supporting the use of carboxyl-free molecules as effective and *safe* NSAIDs. Furthermore, it also provides evidence suggesting that inhibition of prostaglandin synthesis does not necessarily correlates with gastric damage, an issue that has been described previously in the literature[23–25].

We realize that to provide conclusive evidence demonstrating the gastrointestinal safety of compounds **13–15**, we will need to use complementary approaches outlined in the literature[23]. Some of these complementary assays include (a) the use of sub-chronic administrations of the drug (i.e. 25 mg/kg for 3 d); (b) an animal model of impaired mucosal defense; (c) detect hemoglobin in feces; (d) collect blood from animals to measure hematocrit; and (e) assess the effects of these new drugs on myeloperoxidase activity[26,27]. All these assays extend beyond the scope of this report and constitute the object of at least one research project being carried out in our group.

Conclusion

NSAID-induced gastropathy is still a significant clinical concern, and this work presents data to propose a new framework to reconsider the chemical features that are essential/required in classical non-steroidal anti-inflammatory drugs (NSAIDS). As it has been demonstrated previously with the vast number of

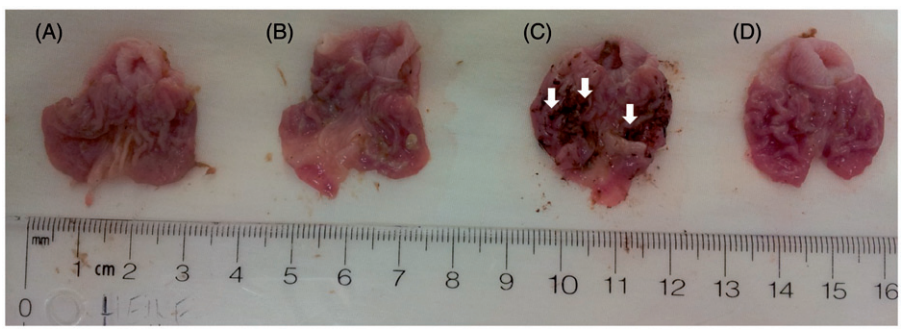

Figure 7. Ulcerogenicity assay data illustrating the extent of NSAID-induced gastric ulcers exerted by a single oral dose of naproxen (40 mg/kg), compared with that observed with derivatives **13a** (''A''), and **13c** (''B''). Experimental drugs were administered using equimolar doses to that of naproxen (''C''). Control animals (''D'') received methylcellulose as vehicle. Only one (representative) example from each group is presented. White arrows point to areas where we observed significant gastric bleeding.

publications describing ester and amide NSAID derivatives, we have shown that the carboxylic acid group present in these drugs *may not be needed at all*, and it can be replaced by an ammonium salt, which maintains the *in vitro* COX-2 selectivity with relatively high potency, and considerable *in vivo* efficacy (carrageenan assay).

Declaration of interest

The authors report that they have no conflict of interest. The authors alone are responsible for the content and writing of this article. The authors gratefully acknowledge the financial support provided by the Faculty of Pharmacy and Pharmaceutical Sciences at the University of Alberta (startup funds), the Higher Education Commission (HEC) of Pakistan for a graduate scholarship to N. U., the Saudi Cultural Bureau in Canada for a graduate scholarship to F. S. A., and the National Council of Science and Technology (CONACYT, Mexico) for a graduate scholarship to A. R. D.

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