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Original article

Synthesis of substituted benzamides as anti-inflammatory agents that inhibit preferentially cyclooxygenase 1 but do not cause gastric damage

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Abstract – Parsalmide (5-amino-*N*-butyl-2-(2-propynyloxy) benzamide) (**5a**), is a non-steroidal anti-inflammatory drug (NSAID), commercialised in Italy until 1985 with the brand name of Synovial®, that has been widely used to treat arthritic patient. In addition, it was shown to spare gastric mucosa. Here we have synthesised a series of novel substituted benzamides, related to Parsalmide, and have evaluated their activity in vitro on COX-1 and COX-2 as well as in vivo in the carrageenin-induced rat paw edema, a classical in vivo anti-inflammatory assay. Compounds **5b**, **11a** and **11b**, which showed a favourable profile in vitro and in vivo, were screened in comparison with Parsalmide for gastrointestinal (GI) tolerability in vivo in the rat. Results obtained showed that Parsalmide and compound **11b** inhibited both COX-1 and COX-2 in vitro as well as they were active in vivo. Both compounds were devoid of gastric effect at the efficacious dose. In addition, both prevented indomethacin-induced gastric damage. Thus, these compounds may guide the definition of a new leading structure with anti-inflammatory activity that may allow designing new safer NSAIDs. © 2001 Éditions scientifiques et médicales Elsevier SAS

anti-inflammatory agents / COX-2/COX-1 inhibition / benzamide derivatives / gastric damage

1. Introduction

Non-steroidal anti-inflammatory drugs are a mainstay in the treatment of inflammation and they owe their therapeutic and side effects in large part to the inhibition of cyclooxygenase (COX). The separation of the therapeutic effects from the side effects has been a major goal in the design and synthesis of these drugs. The discovery of a second isoform of cyclooxygenase, namely COX-2, has opened a new line of research based on the assumption that pathological prostaglandins are produced by the inducible isoform COX-2 while physiological prostaglandins are produced by the constitutive isoform COX-1. On this premise several new inhibitors have been developed and some are now commercially available [1]. However, there are clearly identified areas of the body where COX-2 expression is beneficial and in some cases COX-1 is relevant for pathological effects [2]. Parsalmide (5-amino-*N*-butyl-2-(2-propynyloxy) benzamide) is a non-steroidal anti-inflammatory drug (NSAID), originally synthesised in Italy in the 1970s [3–5]. Parsalmide has been commercially available since 1985 with the brand name Synovial® and it has

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been widely used to treat arthritic patients [6–10]. This drug has a peculiar basic structure and it has been also shown to spare gastric mucosa and also to prevent gastric lesions induced by other NSAIDs [11–16]. Due to these peculiar actions we have synthesised Parsalmide and designed a new series of analogues in the attempt to obtain COX inhibitors sparing the gastrointestinal (GI) tract. In particular, the structures synthesised bear in the place of the propynyloxy residue in 2-position present in Parsalmide a few aromatic or non-aromatic pharmacophoric portions present in some drugs that have been shown to be COX-2 selective (i.e. Nimesulide, NS-398, RWJ-63356). The aim of this approach was to verify if these structural changes would have shifted selectivity towards COX-2. In addition we investigated if the pharmacological activity was modulated when the amino group in 5-position was transformed into an acidic group by protecting it with the methanesulfonyl- or, in neutral group, through protection with the acetyl-moiety. Indeed, the methanesulfonylamide moiety was fundamental for the activity of the above-mentioned drugs, as well as for celecoxib.

All synthesised compounds were screened in vitro for COX-1 and COX-2 inhibitory effect and then tested in vivo in carrageenin-induced rat paw edema. The more active compounds were screened for GI tolerability in vivo in the rat.

2. Chemistry

The general procedure used for the synthesis of the *N*-(*n*-butyl)-benzamide derivatives **4a**,**b**, **5a**,**b**, **6a**,**b** is shown in *figure 1* while the physical and ^{1}H-NMR data are reported, respectively, in *tables I and II*. Commercially, 5-amino-salicylic acid (**1**) was acylated with acetic hydride to give the intermediate **2**. Condensation of 5-acetamide-2-hydroxy-benzoic acid (**2**) with *n*-butylamine in the presence of 1-hydroxybenzotriazole (HOBt) and *N*,*N*′-dicyclohexylcarbodiimide (DCC) in anhydrous dimethylformamide (DMF) gave 5-acetamide-*N*-butylsalicylamide (**3**), which was alkylated with the appropriate alkyl halide in the presence of sodium metal in anhydrous isopropyl alcohol to provide the desired compounds **4a**,**b**. Compound **4a** was converted into the corresponding amine **5a** (Parsalmide) by treatment with sulphuric acid. The last step did not give the corresponding amine when a 3-methyl-2-butenyloxy residue (**4b**) was present in the 2-position. In fact, in this case the decomposition of the compound was observed. Therefore, the hydrolysis of **4b** to the corresponding amine **5b** was conducted employing 3 N NaOH solution. Finally, the amines **5a** and **5b** were converted into the methanesulfonamides **6a** and **6b** by treatment with methanesulfonyl chloride and pyridine in toluene.

The general strategy for the synthesis of amines **11a**–**e**, acetamides **12a**–**e** and methanesulfonamides **13a**–**e** is summarised in *figure 2* while the physical

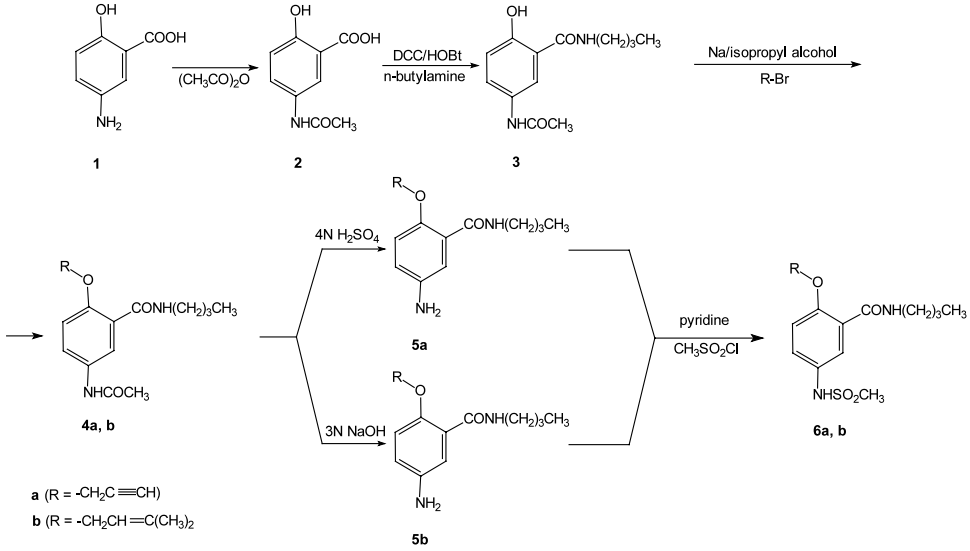
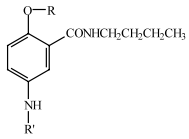
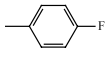
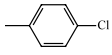
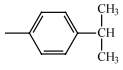
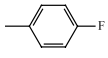
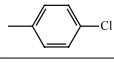


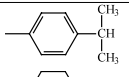
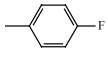
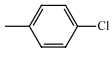
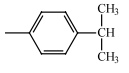
Figure 1. Synthetic procedure of compounds **4a**,**b**; **5a**,**b**; **6a**,**b**.

Table I. Physical data for *N*-(-*n*-butyl)-benzamide derivatives.



compd	R	R'	formula	M.W.	yield (%)	m.p. (°C)	recryst. solvent *	analysis **
4a	-$CH_2C{\equiv}CH$	-$COCH_3$	$C_{16}H_{20}N_2O_3$	288.34	80	130-131	a	C, H, N
4b	-CH_2-$CH{=}C(CH_3)_2$	-$COCH_3$	$C_{18}H_{26}N_2O_3$	318.41	80	151-152	a	C, H, N
5a	-$CH_2C{\equiv}CH$	H	$C_{14}H_{18}N_2O_2$	246.30	90	86-87	a	C, H, N
5b	-CH_2-$CH{=}C(CH_3)_2$	H	$C_{16}H_{24}N_2O_2$	276.37	65	96-97	a	C, H, N
6a	-$CH_2C{\equiv}CH$	-SO_2CH_3	$C_{15}H_{20}N_2O_4S$	324.39	68	142-143	b	C, H, N
6b	-CH_2-$CH{=}C(CH_3)_2$	-SO_2CH_3	$C_{17}H_{26}N_2O_4S$	354.46	65	127-128	b	C, H, N
11a		H	$C_{17}H_{26}N_2O_2 \cdot HCl$	326.86	89	209-210	b	C, H, Cl, N
11b		H	$C_{17}H_{20}N_2O_2 \cdot HCl$	320.81	92	166-167	b	C, H, Cl, N
11c		H	$C_{17}H_{19}FN_2O_2 \cdot HCl$	338.80	78	176-177	b	C, H, Cl,F, N
11d		H	$C_{17}H_{19}ClN_2O_2 \cdot HCl$	355.26	75	157-158	b	C, H, Cl, N
11e		H	$C_{20}H_{26}N_2O_2 \cdot HCl$	362.89	84	157-158	b	C, H, Cl, N
12a		-$COCH_3$	$C_{19}H_{28}N_2O_3$	332.44	71	173-174	b	C, H, N
12b		-$COCH_3$	$C_{19}H_{22}N_2O_3$	326.39	60	142-143	b	C, H, N
12c		-$COCH_3$	$C_{19}H_{20}FN_2O_3$	344.38	64	126-127	b	C, H,F ,N
12d		-$COCH_3$	$C_{19}H_{20}ClN_2O_3$	360.84	69	139-140	b	C, H, Cl, N

Continue table I

12e		-$COCH_3$	$C_{22}H_{28}N_2O_3$	368.47	63	132-133	b	C, H, N
13a		-SO_2CH_3	$C_{18}H_{28}N_2O_4S$	368.49	59	151-152	b	C, H, N
13b		-SO_2CH_3	$C_{18}H_{22}N_2O_4S$	362.44	56	133-134	b	C, H, N
13c		-SO_2CH_3	$C_{18}H_{21}FN_2O_4S$	380.43	58	132-133	b	C, H, F, N
13d		-SO_2CH_3	$C_{18}H_{21}ClN_2O_4S$	396.88	61	147-148	b	C, H, Cl, N
13e		-SO_2CH_3	$C_{21}H_{28}N_2O_4S$	404.52	50	154-155	b	C, H, N

* Crystallization solvents: (a) Diethyl ether; (b) Diethyl ether/ethanol (8:2; v/v).
** Satisfactory analyses for C, H, Cl, F and N were obtained for all these compounds within ± 0.4% of the theoretical values.

Table II. ^{1}H-NMR and MS data of *N*-(-*n*-butyl)-benzamide derivatives.

	^{1}H-NMR ($CDCl_3$); δ (ppm); J (Hz)	MS m/z
4a	8.77 (s, 1H, ArNH); 8.22 (dd, 1H, $J = 2.9$ Hz, ArH); 7.97 (d, 1H, $J = 2.9$ Hz, ArH); 7.89 (br, 1H, CONH); 7.00 (d, 1H, $J = 7.9$ Hz, Ar–H); 4.79 (s, 2H, OCH_2); 3.47 (q, 2H, $J = 6.9$ Hz, CH_2NH); 2.60 (s, 1H, C≡CH); 2.18 (s, 3H, $COCH_3$); 1.57 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.44 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 0.95 (t, 3H, $J = 6.9$ Hz, CH_2CH_3)	289.0 (base, $M^+ + 1$); 249.9 ($M^+ - CH_2C{\equiv}CH$); 215.8 ($M^+ - NH(CH_2)_3CH_3$); 189.9 ($M^+ - CONH(CH_2)_3CH_3$); 173.8 ($M^+ - NH(CH_2)_3CH_3$; $-COCH_3$); 147.8 ($M^+ - CONH(CH_2)_3CH_3$; $-COCH_3$)
4b	8.57 (br, 1H, CONH); 8.22 (dd, 1H, $J = 2.9$ Hz, ArH); 8.20 (br, 1H, CONH); 7.98 (d, 1H, $J = 2.9$ Hz, ArH); 6.96 (d, 1H, $J = 7.9$ Hz, ArH); 5.51 (t, 1H, $J = 5.5$ Hz,CH=C); 4.62 (d, 2H, $J = 3.5$ Hz, OCH_2); 3.44 (q, 2H, $J = 6.9$ Hz, CH_2NH); 2.18 (s, 3H, $COCH_3$); 1.82 (s, 3H, $C(CH_3)_2$; 1.79 (s, 3H, $C(CH_3)_2$); 1.55 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.42 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 0.95 (t, 3H, $J = 6.9$ Hz, CH_2CH_3)	319.0 (base, $M^+ + 1$); 251.0 ($M^+ - CH_2$–CH=$C(CH_3)_2$); 177.5 ($M^+ - CH_2CH{=}C(CH_3)_2$; $-NH(CH_2)_3CH_3$); 151.8 ($M^+ - CH_2$–CH=$C(CH_3)_2$; $-CONH(CH_2)_3CH_3$)
5a	7.83 (br, 1H, CONH); 7.50 (d, 1H, $J = 2.9$ Hz, ArH); 6.83 (d, 1H, $J = 7.9$ Hz, ArH); 6.72 (dd, 1H, $J = 2.9$ Hz, ArH); 4.67 (s, 2H, OCH_2); 3.57 (br, 2H, $ArNH_2$); 3.41 (q, 2H, $J = 6.9$ Hz, CH_2NH); 2.55 (s, 1H, C≡CH); 1.54 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 0.91 (t, 3H, $J = 6.9$ Hz, CH_2CH_3)	247.0 (base, $M^+ + 1$); 207.8 ($M^+ - CH_2C{\equiv}CH$); 173.7 ($M^+ - NH(CH_2)_3CH_3$); 147.8 ($M^+ - CONH(CH_2)_3CH_3$); 134.8 ($M^+ - CH_2C{\equiv}CH$; $-NH(CH_2)CH_3$)
5b	8.20 (br, 1H, CONH); 7.58 (d, 1H, $J = 2.9$ Hz, ArH); 6.92 (d, 1H, $J = 7.9$ Hz, ArH); 6.78 (dd, 1H, $J = 2.9$ Hz, ArH); 5.50 (t, 1H, $J = 5.6$ Hz, CH=C); 4.55 (d, 2H, $J = 3.5$ Hz, OCH_2); 3.44 (q, 2H, $J = 6.9$ Hz, CH_2NH); 3.15 (br, 2H, $ArNH_2$); 1.82 (s, 3H, $C(CH_3)_2$); 1.75 (s, 3H, $C(CH_3)_2$); 1.55 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.40 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 0.95 (t, 3H, $J = 6.9$ Hz, CH_2CH_3)	277.0 (base, $M^+ + 1$); 208.9 ($M^+ - CH_2$–CH=$C(CH_3)_2$); 177.8 ($M^+ - CONH(CH_2)_3CH_3$); 135.7 ($M^+ - CH_2$–CH=$C(CH_3)_2$; $-NH(CH_2)_3CH_3$)
6a	8.54 (s, 1H, ArNH); 8.36 (d, 1H, $J = 2.9$ Hz, ArH); 7.98 (br, 1H, CONH); 7.67 (dd, 1H, $J = 2.9$ Hz, ArH); 7.06 (d, 1H, $J = 7.9$ Hz, ArH); 4.82 (s, 2H, OCH_2); 3.56 (q, 2H, $J = 6.9$ Hz, CH_2NH); 2.93 (s, 3H, SO_2CH_3); 2.62 (s, 1H, C≡CH); 1.62 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.46 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 0.97 (t, 3H, $J = 6.9$ Hz, CH_2CH_3)	325.0 (base, $M^+ + 1$); 251.8 ($M^+ - NH(CH_2)_3CH_3$); 245.9 ($M^+ - SO_2CH_3$); 206.9 ($M^+ - CH_2C{\equiv}CH$; $-SO_2CH_3$); 172.9 ($M^+ - NH(CH_2)_3CH_3$; $-SO_2CH_3$); 145.6 ($M^+ - CONH(CH_2)_3CH_3$; $-SO_2CH_3$); 117.8 ($M^+ - OCH_2C{\equiv}CH$; $-NH(CH_2)_3CH_3$; $-SO_2CH_3$)
6b	8.90 (s, 1H, ArNH); 8.52 (d, 1H, $J = 2.9$ Hz, ArH); 8.33 (br, 1H, CONH); 7.68 (dd, 1H, $J = 2.9$ Hz, ArH); 7.00 (d, 1H, $J = 7.9$ Hz, ArH); 5.55 (t, 1H, $J = 5.5$ Hz, CH=C); 4.65 (d, 2H, $J = 3.5$ Hz, OCH_2); 3.58 (q, 2H, $J = 6.9$ Hz, CH_2NH); 2.90 (s, 3H, $COCH_3$); 1.90 (s, 3H, $C(CH_3)_2$); 1.78 (s, 3H, $C(CH_3)_2$); 1.59 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.48 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 0.98 (t, 3H, $J = 6.9$ Hz, CH_2CH_3)	355.0 (base, $M^+ + 1$); 286.9 ($M^+ - CH_2$–CH=$C(CH_3)_2$); 214.8 ($M^+ - CH_2CH{=}C(CH_3)_2$; $NH(CH_2)_3CH_3$); 207.6 ($M^+ - CH_2$–CH=$C(CH_3)_2$; $-SO_2CH_3$); 135.8 ($M^+ - CH_2$–CH=$C(CH_3)_2$; $-NH(CH_2)_3CH_3$; $-SO_2CH_3$)

Table II. (*Continued*)

	^{1}H-NMR ($CDCl_3$); δ (ppm); J (Hz)	MS m/z
11a [a]	8.68 (br, 1H, CONH); 7.95 (d, 1H, $J = 2.9$ Hz, ArH); 7.50 (d, 1H, $J = 7.9$ Hz, ArH); 7.37 (dd, 1H, $J = 2.9$ Hz, ArH); 4.72 (m, 1H, cyclohexyl); 3.54 (br, 2H, $ArNH_2$); 3.50 (q, 2H, $J = 6.9$ Hz, CH_2NH); 2.10–1.83 (m, 8H, cyclohexyl); 1.69 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.58 (m, 2H, cyclohexyl); 1.50 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 1.02 (t, 3H, $J = 6.9$ Hz, CH_2CH_3)	291.0 (base, $M^+ + 1$); 208.9 ($M^+ - C_6H_{11}$); 190.8 ($M^+ - CONH(CH_2)_3CH_3$); 135.8 ($M^+ - C_6H_{11}$; $-NH(CH_2)_3CH_3$)
11b	8.35 (br, 1H, CONH); 7.75 (d, 1H, $J = 2.9$ Hz, ArH); 4.44 (d, 1H, $J = 7.9$Hz, ArH); 7.30–6.95 (m, 5H, ArH); 3.35 (q, 2H, $J = 6.9$ Hz, CH_2NH); 1.44 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.28 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 0.89 (t, 3H, $J = 6.9$ Hz, CH_2CH_3)	284.9 (base, $M^+ + 1$); 211.9 ($M^+ - NH(CH_2)_3CH_3$)
11c [a]	8.50 (br, 1H, CONH); 7.84 (d, 1H, $J = 2.9$ Hz, ArH); 7.50 (d, 1H, $J = 7.9$ Hz, ArH); 7.10–7.28 (m, 4H, ArH); 7.06 (dd, 1H, $J = 2.9$ Hz, ArH); 3.40 (q, 2H, $J = 6.9$ Hz, CH_2NH); 3.35 (br, 2H, $ArNH_2$); 1.56 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.40 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 0.95 (t, 3H, $J = 6.9$ Hz, CH_2CH_3)	303.0 (base, $M^+ + 1$); 229.9 ($M^+ - NH(CH_2)_3CH_3$)
11d	8.25 (br, 1H, CONH); 7.22 (d, 1H, $J = 2.9$ Hz, ArH); 7.60 (d, 1H, $J = 7.9$ Hz, ArH); 7.29–6.96 (m, 4H, ArH); 6.83 (d, 1H, $J = 2.9$ Hz, ArH); 3.32 (q, 2H, $J = 6.9$ Hz, CH_2NH); 3.01 (br, 2H, $ArNH_2$); 1.46 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.25 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 0.82 (t, 3H, $J = 6.9$ Hz, CH_2CH_3)	319.0 (base, $M^+ + 1$); 245.9 ($M^+ - NH(CH_2)_3CH_3$); 210.8 ($M^+ - NH(CH_2)_3CH_3$; $-Cl$)
11e	8.37 (br, 1H, CONH); 7.84 (d, 1H, $J = 2.9$ Hz, ArH); 7.72 (d, 1H, $J = 7.9$ Hz, ArH); 7.25–6.96 (m, 4H, ArH); 6.82 (dd, 1H, $J = 2.9$ Hz, ArH); 3.38 (q, 2H, $J = 6.9$ Hz, CH_2NH); 2.92 (m, 1H, $CH(CH_3)_2$); 1.47 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.30 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 1.27 (d, 6H, $CH(CH_3)_2$); 0.85 (t, 2H, $J = 6.9$ Hz, CH_2CH_3)	327.0 (base, $M^+ + 1$); 211.9 ($M^+ - NH(CH_2)_3CH_3$; $-CH(CH_3)_2$)
12a	9.07 (s, 1H, ArNH); 8.25 (br, 1H, CONH); 8.14 (dd, 1H, $J = 2.9$ Hz, ArH); 7.98 (d, 1H, $J = 2.9$ Hz, ArH); 6.92 (d, 1H, $J = 7.9$ Hz, ArH); 4.38 (m, 1H, cyclohexyl); 3.43 (q, 2H, $J = 6.9$ Hz, CH_2NH); 2.15 (s, 3H, $COCH_3$); 2.01–1.73 (m, 8H, cyclohexyl); 1.57 (q, 2H, CH_2CH_2NH); 1.50 (m, 2H, cyclohexyl); 1.39 (m, 2H, $J = 7.9$ Hz, CH_2CH_3; 0.92 (t, 3H, $J = 6.9$ Hz, CH_2CH_3)	333.0 (base, $M^+ + 1$); 251.0 ($M^+ - C_6H_{11}$); 177.7 ($M^+ - C_6H_{11}$; $-NH(CH_2)_3CH_3$)
12b	8.40 (s, 1H, ArNH); 8.20 (dd, 1H, $J = 2.9$ Hz, ArH); 8.00 (d, 1H, $J = 2.9$ Hz, ArH); 7.70 (br, 1H, CONH); 7.38–7.00 (m, 5H, ArH); 6.15 (d, 1H, $J = 7.9$ Hz, ArH); 3.48 (q, 2H, $J = 6.9$ Hz, CH_2NH); 2.40 (s, 3H, $COCH_3$); 1.40 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.33 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 0.95 (t, 3H, $J = 6.9$ Hz, CH_2CH_3)	327.0 (base, $M^+ + 1$); 253.9 ($M^+ - NH(CH_2)_3CH_3$); 211.6 ($M^+ - NH(CH_2)_3CH_3$; $-COCH_3$)
12c	8.70 (s, 1H, ArNH); 8.17 (dd, 1H, $J = 2.9$ Hz, ArH); 8.00 (d, 1H, $J = 2.9$ Hz, ArH); 7.69 (br, 1H, CONH); 7.08–6.97 (m, 4H, ArH); 6.82 (d, 1H, $J = 7.9$ Hz, ArH); 3.43 (q, 2H, $J = 6.9$ Hz, CH_2NH); 2.18 (s, 3H, $COCH_3$); 1.50 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.31 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 0.87 (t, 3H, $J = 6.9$ Hz, CH_2CH_3)	345.0 (base, $M^+ + 1$); 271.8 ($M^+ - NH(CH_2)_3CH_3$); 229.9 ($M^+ - NH(CH_2)_3CH_3$; $-COCH_3$)

Table II. (*Continued*)

	^{1}H-NMR ($CDCl_3$); δ (ppm); J (Hz)	MS m/z
12d	9.10 (s, 1H, ArNH); 8.24 (dd, 1H, $J = 2.9$ Hz, ArH); 8.04 (d, 1H, $J = 2.9$ Hz, ArH); 7.77 (br, 1H, CONH); 7.31–6.94 (m, 4H, ArH); 6.86 (d, 1H, $J = 7.9$ Hz, ArH); 3.42 (q, 2H, $J = 6.9$ Hz, CH_2NH); 2.18 (s, 3H, $COCH_3$); 1.48 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.29 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 0.87 (t, 3H, $J = 6.9$ Hz, CH_2CH_3)	361.0 (base, $M^+ + 1$); 287.8 ($M^+ - NH(CH_2)_3CH_3$); 245.8 ($M^+ - NH(CH_2)_3CH_3$; $-COCH_3$)
12e	8.86 (s, 1H, ArNH); 8.18 (d, 1H, $J = 2.9$ Hz, ArH); 8.04 (d, 1H, $J = 2.9$ Hz, ArH); 7.84 (br, 1H, CONH); 7.27–6.89 (m, 4H, ArH); 6.85 (d, 1H, $J = 7.9$ Hz, ArH); 3.43 (q, 2H, $J = 6.9$ Hz, CH_2NH); 2.92 (m, 1H, $CH(CH_3)_2$); 2.19 (s, 3H, $COCH_3$); 1.52 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.32 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 1.27 (d, 6H, $CH(CH_3)_2$); 0.89 (t, 3H, $J = 6.9$ Hz, CH_2CH_3)	369.0 (base, $M^+ + 1$); 253.9 ($M^+ - NH(CH_2)_3CH_3$; $-CH(CH_3)_2$); 211.9 ($M^+ - NH(CH_2)_3CH_3$; $-CH(CH_3)_2$; $-COCH_3$)
13a	8.68 (s, 1H, ArNH); 8.57 (br, 1H, CONH); 8.40 (d, 1H, $J = 2.9$ Hz, ArH); 7.62 (dd, 1H, $J = 2.9$ Hz, ArH); 6.98 (d, 1H, $J = 7.9$ Hz, ArH); 4.61 (m, 1H, cyclohexyl); 3.56 (q, 2H, $J = 6.9$ Hz, CH_2NH); 2.91 (s, 3H, $COCH_3$); 1.79–1.78 (m, 8H, cyclohexyl); 1.58 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.57 (m, 2H, cyclohexyl); 1.46 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 0.96 (t, 3H, $J = 6.9$ Hz, CH_2CH_3)	369.0 (base, $M^+ + 1$); 286.9 ($M^+ - C_6H_{11}$); 213.9 ($M^+ - C_6H_{11}$; $-NH(CH_2)_3CH_3$); 206.8 ($M^+ - C_6H_{11}$; $-SO_2CH_3$); 132.7 ($M^+ - C_6H_{11}$; $-NH(CH_2)_3CH_3$; $-SO_2CH_3$)
13b	8.40 (s, 1H, ArNH); 7.92 (d, 1H, $J = 2.9$ Hz, ArH); 7.85 (br, 1H, CONH); 7.58 (dd, 1H, $J = 2.9$ Hz, ArH); 7.42–7.00 (m, 5H, ArH); 6.92 (d, 1H, $J = 7.9$ Hz, ArH); 3.58 (q, 2H, $J = 6.9$ Hz, CH_2NH); 2.97 (s, 3H, SO_2CH_3); 1.55 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.30 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 0.95 (t, 3H, $J = 6.9$ Hz, CH_2CH_3)	363.0 (base, $M^+ + 1$); 289.9 ($M^+ - NH(CH_2)_3CH_3$); 209.9 ($M^+ - NH(CH_2)_3CH_3$; $-SO_2CH_3$)
13c	8.77 (s, 1H, ArNH); 8.40 (d, 1H, $J = 2.9$ Hz, ArH); 7.95 (br, 1H, CONH); 7.65 (dd, 1H, $J = 2.9$ Hz, ArH); 7.18–6.98 (m, 4H, ArH); 6.85 (d, 1H, $J = 7.9$ Hz, ArH); 3.62 (q, 2H, $J = 6.9$ Hz, CH_2NH); 3.00 (s, 3H, $COCH_3$); 1.62 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.35 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 0.96 (t, 3H, $J = 6.9$ Hz, CH_2CH_3)	381.0 (base, $M^+ + 1$); 307.8 ($M^+ - NH(CH_2)_3CH_3$); 227.8 ($M^+ - NH(CH_2)_3CH_3$; $-SO_2CH_3$)
13d	8.62 (s, 1H, ArNH); 8.31 (d, 1H, $J = 2.9$ Hz, ArH); 7.73 (br, 1H, CONH); 7.54 (dd, 1H, $J = 2.9$ Hz, ArH); 7.30–6.91 (m, 4H, ArH); 6.79 (d, 1H, $J = 7.9$ Hz, ArH); 3.64 (q, 2H, $J = 6.9$ Hz, CH_2NH); 2.91 (s, 3H, $COCH_3$); 1.46 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.25 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 0.81 (t, 3H, $J = 6.9$ Hz, CH_2CH_3)	397.0 (base, $M^+ + 1$); 323.9 ($M^+ - NH(CH_2)_3CH_3$); 318.0 ($M^+ - SO_2CH_3$); 243.9 ($M^+ - NH(CH_2)_3CH_3$; $-SO_2CH_3$); 209.9 ($M^+ - NH(CH_2)_3CH_3$; $-SO_2CH_3$; $-Cl$)
13e	8.77 (s, 1H, ArNH); 8.46 (d, 1H, $J = 2.9$ Hz, ArH); 8.06 (br, 1H, CONH); 7.61 (dd, 1H, $J = 2.9$ Hz, ArH); 7.27–6.98 (m, 4H, ArH); 6.86 (d, 1H, $J = 7.9$ Hz, ArH); 3.55 (q, 2H, $J = 6.9$ Hz, CH_2NH); 2.98 (s, 3H, $COCH_3$); 2.93 (m, 1H, $CH(CH_3)_2$); 1.57 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.34 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 1.28 (d, 6H, $CH(CH_3)_2$); 0.89 (t, 3H, $J = 6.9$ Hz, CH_2CH_3)	405.0 (base, $M^+ + 1$); 289.9 ($M^+ - NH(CH_2)_3CH_3$; $-CH(CH_3)_2$); 211.9 ($M^+ - NH(CH_2)_3CH_3$; $-CH(CH_3)_2$; $-SO_2CH_3$)

[a] These compounds were determined using CD_3OD as solvent.

and ^{1}H-NMR data are reported, respectively, in *tables I and II*. The condensation reaction of the commercially available 2-chloro-5-nitrobenzoic acid (**7**) with *n*-butylamine performed in anhydrous DMF in the presence of DCC/HOBt produced the corresponding intermediate **8**. Subsequent dissolution of **8** in anhydrous dioxane and treatment with a solution of the appropriate alcohol and NaH in anhydrous dioxane gave the corresponding intermediates **9a**–**e**. Reduction of the nitro group of intermediates **9a**–**e**, carried out with sodium borohydride reagent, gave the corresponding intermediates **10a**–**e**. Free bases **10a**–**e** were converted into the corresponding hydrochloride salts **11a**–**e** by treatment with an excess of diethyl ether saturated with dry, gaseous HCl. The acetamides **12a**–**e** were prepared by reaction of the corresponding amines **10a**–**e** in anhydrous DMF with acetic anhydride. Finally, the methanesulfonamides **13a**–**e** were also prepared by reaction of the corresponding intermediates **10a**–**e** with methanesulfonyl chloride and pyridine in toluene. All compounds had satisfactory elemental analyses, and their ^{1}H-NMR and/or mass spectra were consistent with the proposed structures. The pK_a values of compounds **5b**, **11a** and **11b** were performed potentiometrically using a pH meter: micro pH 2002 — Crison Instruments, S.A.

3. Biological assays

All synthesised compounds (series **4**–**6**, **11**–**13**) were screened in vitro for COX-1 and COX-2 in-

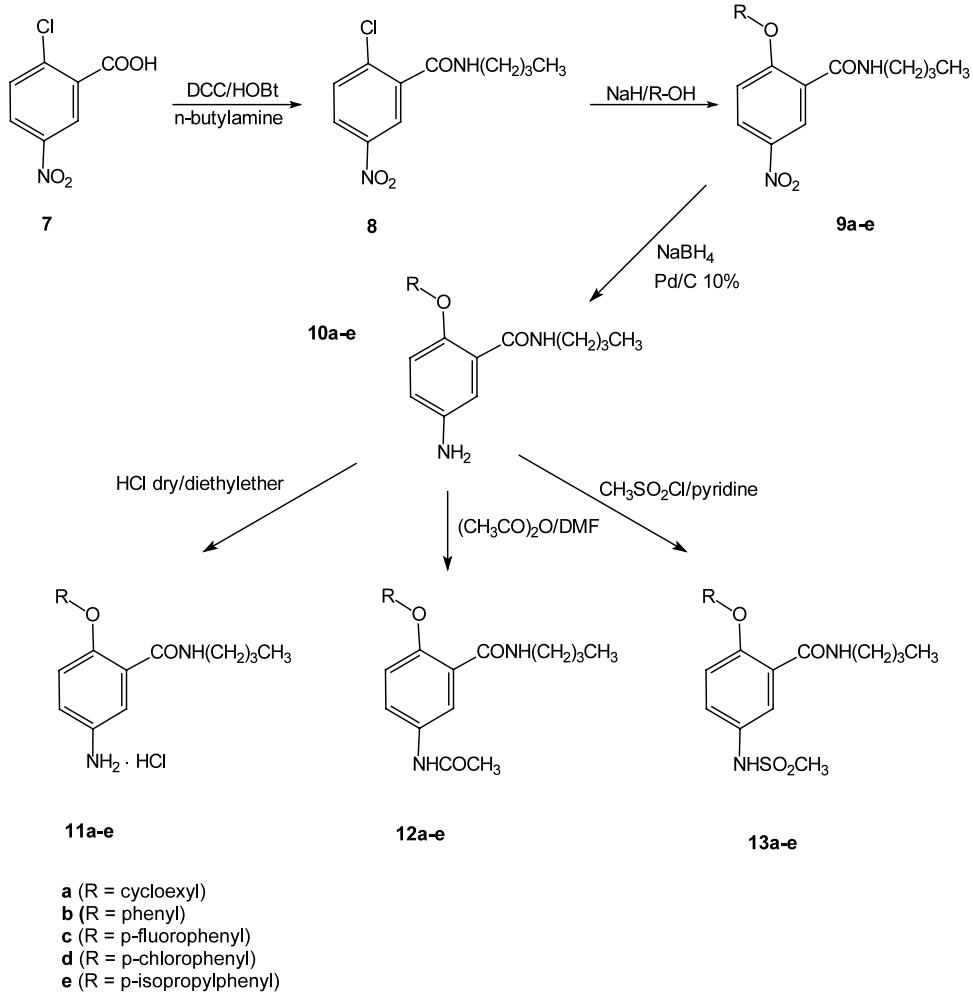


Figure 2. Synthetic procedure of compounds **11a**–**e**, **12a**–**e**, **13a**–**e**.

hibitory effect by evaluating the ability of the compounds to inhibiting COXs in a purified enzyme assay. Then all compounds were tested in vivo in carrageenin-induced rat paw edema, a classical in vivo assay used to characterise NSAIDs. The reference compounds used were indomethacin (3, 10, 30 mg/kg) and celecoxib (3, 10, 30 mg/kg) given orally. Following the preliminary screening, the compounds that showed a favourable profile in vitro and in the paw edema assay (**5a**, **5b**, **11a** and **11b**) were screened for GI tolerability in vivo in the rats as acute gastric damage and indomethacin-induced gastric damage. A dose response in carrageenin oedema was performed also for compounds **11a**, **11b** and Parsalmide (10, 30, 100 mg/kg).

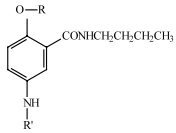
4. Results and discussion

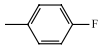
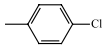
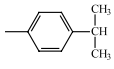
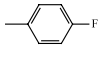
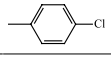
Parsalmide is a 5-amino-*N*-butyl-2-(2-propynyloxy) benzamide derivative with anti-inflammatory properties that has a peculiar basic structure ($pK_a = 4.6$ [5]). Parsalmide, which displayed the same chemical profile originally described [3–5], was synthesised and tested in vitro to assess the COX-2/COX-1 selectivity as well as in vivo to evaluate the anti-inflammatory activity. Parsalmide (**5a**) inhibited about 83% edema formation in vivo and showed in vitro a COX-2/COX-1 ratio of 16 (*table III*). This ratio shows that Parsalmide is more selective for COX-1. In order to acquire new insights into the structure–activity relationships in Parsalmide, we synthesised and screened a new series of derivatives where the propynyloxy moiety in the 2-position of the phenyl ring was replaced by a few alkyl- and (un) substituted aryl-oxy residues bearing electron-donating or -withdrawing substituents. In addition, the amine moiety was free or protected by acetyl- or methanesulfonyl-group. In vitro data showed that the presence of a methanesulfonamide group in 5-position (**6a**) caused a loss of the in vitro COX-2/COX-1 activity that paralleled a reduction of the anti-inflammatory activity in vivo (*table III*). Similarly, protection of the amino group by acetyl **4a** resulted in a loss of activity both in vitro and in vivo (*table III*). Replacing the propynyloxy group with a 3-methyl-2-butenyloxy moiety (**5b**) slightly modified the COX-2/COX-1 ratio, which was 13, but the in vivo anti-inflammatory activity was reduced by about 50% (*table III*). Also, in compound **5b** modifications of the amino group leading to compounds **4b** and **6b** caused a loss of the effect both in vivo and in vitro (*table III*). On the basis of these results we synthesised a few analogues where the propynyloxy residue in 2-position was replaced by a cyclohexyloxy- **11a**, **12a**, **13a**, phenyloxy- **11b**, **12b**, **13b** and phenyloxy- *para*-substituted with fluorine **11c**, **12c**, **13c**, chlorine **11d**, **12d**, **13d** or isopropyl group **11e**, **12e**, **13e**. Modulation in the amino group was performed by inserting an acetyl group **12a–e** or the methanesulfonyl moiety **13a–e**. The presence of a cyclohexyloxy group (**11a**) caused a reduction of the in vitro ratio COX-2/COX-1 but the in vivo activity was comparable to Parsalmide (*table III*). Modification in compound **11a** of the amino group either by inserting an acetyl (**12a**) or a methanesulfonyl group (**13a**) caused a loss of activity both in vivo and in vitro (*table III*). Insertion of an unsubstituted aromatic ring in the place of the cyclohexyl moiety (**11b**) slightly reduced both the COX-2/COX-1 ratio and the anti-inflammatory activity in vivo (*table III*). Again, modification of the amino group (**12b**, **13b**) caused a loss of effect both in vitro and in vivo. The presence of substituent in the aromatic ring such as fluorine (**11c**, **12c**, **13c**), chlorine (**11d**, **12d**, **13d**) or isopropyl group (**11e**, **12e**, **13e**) caused a loss of activity in vivo (*table III*). It is of interest to note that compounds **11c**, **11e**, and **12e** displayed in vitro a COX-2/COX-1 ratio of 19, 30 and 67, respectively, but they were ineffective in the in vivo assay (*table III*).

On the basis of the in vivo and in vitro results we selected basic compounds **5b**, **11a** and **11b** (pK_a values 4.20, 3.80 and 3.15, respectively) and they were tested in comparison with Parsalmide in an in vivo model of gastric damage. The results obtained (*figure 3A*) showed that Parsalmide and compound **11b** were devoid of gastric effect while compounds **5b** and **11a** showed some gastric damage. The ED_{50} for Parsalmide, indomethacin, celecoxib and compounds **11a** and **11b** were 82 ± 7, 6.5 ± 1.4, 15 ± 3.1, 75 ± 6, 68 ± 6 mg/kg, respectively.

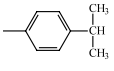
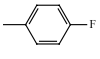
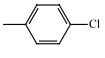
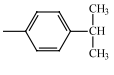
Since Parsalmide has been shown to protect from gastric damage induced by NSAIDs [11–13] we compared compound **11b** with Parsalmide in an assay of gastric damage induced by indomethacin. Both Parsalmide and compound **11b** clearly and significantly prevented the gastric damage induced by indomethacin (*figure 3B*). These data indicated that modification in the 2-position with a phenyl ring (**11b**) preserved activity in vitro and in vivo and left intact the gastric sparing properties. The other modification with a 3-methyl-2-butenyloxy (**5b**) or with a cyclo-

Table III. Enzyme and in vivo activities of *N*-(-*n*-butyl)-benzamide derivatives.



compd	R	R'	IC$_{50}$ (μM)		COX-2 COX-1 ratio	Inhibition paw edema (%) * 10 mg/kg	Inhibition paw edema (%) 100 mg/kg
			COX-1	COX-2			
4a	-$CH_2C{\equiv}CH$	-$COCH_3$	>1000	>1000	ND	3±1.5	13±5.8
4b	-CH_2-$CH{=}C(CH_3)_2$	-$COCH_3$	>1000	>1000	ND	1±0.75	30±9.5
5a	**-$CH_2C{\equiv}CH$ parsalmide**	**H**	**9.92**	**155**	**16**	**9±3.2**	**83±9.5*****
5b	-CH_2-$CH{=}C(CH_3)_2$	H	37.10	478.0	13	1±0.80	46±4.7***
6a	-$CH_2C{\equiv}CH$	-SO_2CH_3	>1000	>1000	ND	6±6.2	25±3.8**
6b	-CH_2-$CH{=}C(CH_3)_2$	-SO_2CH_3	>1000	>1000	ND	5±2.4	27±1.8**
11a		H	1.42	8.35	6	7±3.1	78±5.0***
11b		H	18.0	66.0	4	20±3.2	63±6.0***
11c		H	18.20	353.0	19	12±3.7	25±9.5
11d		H	9.30	14.70	1.6	7±2.7	5±2.1
11e		H	14.30	435.0	30	3±2.0	5±1.8
12a		-$COCH_3$	>1000	>1000	ND	5±2.2	13±3.9
12b		-$COCH_3$	>1000	>1000	ND	22±4.1	25±8.2
12c		-$COCH_3$	17.20	>1000	ND	12±6.0	22±8.4
12d		-$COCH_3$	89.70	>1000	ND	3±1.5	18±3.6

continue table III

12e		-$COCH_3$	0.50	33.60	67	5±3.1	7±2.6
13a		-SO_2CH_3	>1000	>1000	ND	7±1.8	5±1.9
13b		-SO_2CH_3	42.90	>1000	ND	22±5.3	22±7.1
13c		-SO_2CH_3	29.90	79.40	2.6	5±3.5	17±6.5
13d		-SO_2CH_3	41.80	>1000	ND	5±3.4	7±3.7
13e		-SO_2CH_3	1.81	>1000	ND	3±1.2	17±8.3
	Indomethacin	-	0.013	0.46	35	-	-
	Celecoxib	-	1.4	0.80	0.57		

* Paw volume at the 3rd hour was 0.42±0.04: data are expressed as percent of inhibition;
** $P < 0.05$;
*** $P < 0.01$: data were analysed by ANOVA followed by Dunnett test.
ND= not determinable.

hexyloxy (**11a**) caused a loss of the gastric sparing properties.

5. Conclusions

The present data further suggest that to inhibit COX-1 and COX-2 an acidic structure is not necessary. However, structures inhibiting COX-1 selectively, such as ketorolac, which is highly selective for COX-1 [17], have an acidic nature. In this respect, our data shed a new light on the chemical requisite to design new COX inhibitors. Indeed, the paradigm of an acidic structure that has been already challenged by the development of COX-2 inhibitors is further challenged by the present data. In fact, Parsalmide and compound **11b** are both basic structures that preferentially inhibit COX-1 and COX-2 in vitro and are active in vivo in the paw edema assay. The better inhibitory activity on the COX-isoforms of the compounds with a free aniline function is probably mediated by non-covalent bonds to the enzyme [18]. In addition and most importantly, both compounds appear to be not only devoid of gastric effects but are also able to prevent GI damage induced by indomethacin. Thus, the data obtained imply that the introduction in 2-position of structural features possessed by the reference drugs (Nimesulide, NS-398, RWJ-63356) allowed to maintain COX inhibitory effects and in vivo activity. Thus, biological activity was retained when the amine group in 5-position was free.

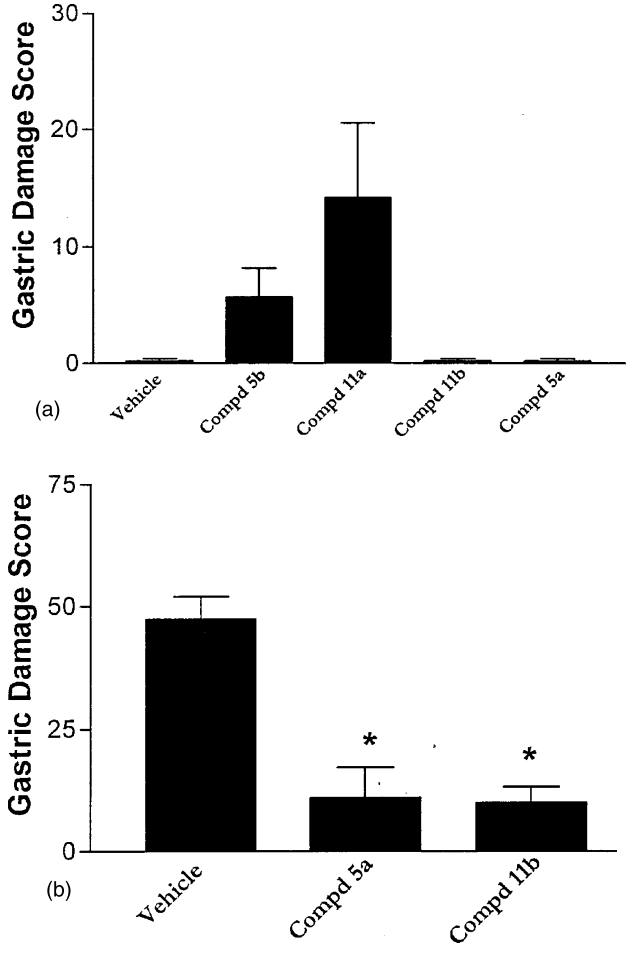


Figure 3. (A) Gastric damage induced by a single oral administration of 100 mg/kg per os of compounds **5a**, **5b**, **11a** and **11b**. (B) Protective effect of compounds **5a** and **11b** given orally at the dose of 100 mg/kg on gastric damage induced by indomethacin.

In conclusion we have demonstrated that Parsalmide is 16 times more selective for COX-1 and that structural modification performed in its structure by inserting groups known to increase the anti-inflammatory activity led to compound **11b**. Compound **11b**, even while displaying a reduced COX-2/COX-1 ratio, showed a comparable anti-inflammatory effect. Parsalmide (**5a**) and compound **11b** may guide the definition of a new leading structure to design GI safe drugs that will not lack the COX-1 component. Indeed, powerful NSAIDs such as indomethacin are already available in the market as well as efficacious COX-2 selective inhibitors but preferential NSAIDs that will be GI safe but still inhibiting COX-1 may turn to be important for all the beneficial biological activities, such as the anti-aggregatory effect, where COX-1 inhibition is required.

6. Experimental protocols

Capillary melting points were determined on a Buchi SPM-20 apparatus and are reported uncorrected. Elemental analyses were performed on a Perkin–Elmer 2400 and were within ±0.4% of the theoretical values unless indicated otherwise. The ^{1}H-NMR measurements were performed on a Bruker AMX 500 spectrometer using $CDCl_3$ or CD_3OD as solvents and tetramethylsilane as internal standard. Chemical shifts (δ) are expressed in ppm downfield from tetramethylsilane and coupling constants J are expressed in Hertz. Significant ^{1}H-NMR data are reported in order: multiplicity (s, singlet; d, doublet; dd, double doublet; t, triplet; q, quartet; m, multiplet; br, broad). Mass spectrometric detection was per-

formed using a MicroMass (Manchester, UK) QuatroII LC triple-quadrupole mass spectrometer, equipped with a heated nebuliser as the APCI source. Thin layer chromatography was performed on precoated silica gel plates Kieselgel 60F254 (Merck, A.G., Darmstadt, Germany) and the spots were detected by UV light.

Silica gel column chromatography was performed on Kieselgel 60 (230–400 mesh). Extraction in usual manner refers to washing the organic layer with water, drying it over anhydrous magnesium sulphate, and evaporating the solvent under reduced pressure.

Dimethylformamide (DMF) was distilled immediately before use over CaH_2. All solvents were purchased from Carlo Erba Reagents. Reagent grade materials were purchased from Aldrich Chemical Co. and from Fluka and were used without further purification.

6.1. Chemistry

6.1.1. 5-Acetamide-2-hydroxy-benzoic acid (2)

To a solution of 5-amino-salicylic acid (15 g, 98.0 mmol) (**1**) in water (40 mL) heated at 50 °C, was added acetic anhydride (11.25 mL, 120.0 mmol). After the mixture was stirred for 15 min, the insoluble residue containing compound **2** was filtered and washed with ice water. The residue was crystallised from water to yield **2** (15 g, 79%) as a white solid, m.p. 229–230 °C. ^{1}H-NMR ($CDCl_3$, δ, ppm): 9.28 (br, 1H, OH); 8.10 (br, 1H, CONH); 8.08 (d, 1H, $J = 2.9$ Hz, ArH); 7.61 (dd, 1H, $J = 2.9$ Hz, ArH); 6.91 (d, 1H, $J = 7.9$ Hz, ArH). Anal. ($C_9H_9NO_4$): C, H, N.

6.1.2. 5-Acetamide-N-butylsalicylamide (3)

To a solution of **2** (15 g, 76.9 mmol) in DMF (50 mL) was added 1-hydroxybenzotriazole (HOBt) (11.28 g, 83.5 mmol) and *N*,*N*′-dicyclohexylcarbodiimide (DCC) (17.21 g, 83.5 mmol) in the above order at 0 °C. After the mixture was stirred for 30 min, *n*-butylamine (7.6 mL, 76.9 mmol) was added. The resulting reaction mixture was stirred at 0 °C for 2 h and overnight at room temperature (r.t.). *N*,*N*′-Dicyclohexylurea (DCU) was filtered off and the DMF was evaporated in vacuum. The residue was dissolved in CH_2Cl_2, washed consecutively with brine, 1 N NaOH, brine, 1 N HCl and brine. The organic phase was dried over magnesium sulphate, filtered, and evaporated to dryness. The obtained residue was purified by chromatography on a silica gel column (elution with diethyl ether–ethanol (8:2), v/v) to yield pure **3** (15.6 g, 86%) as a white solid, m.p. 150–151 °C. ^{1}H-NMR ($CDCl_3$, δ, ppm): 12.35 (br, 1H, OH); 8.02 (s, 1H, ArNH); 7.47 (d, 1H, $J = 2.9$ Hz, ArH); 7.09 (dd, 1H, $J = 2.9$ Hz, ArH); 6.90 (d, 1H, $J = 7.9$ Hz, Ar-H); 6.73 (br, 1H, CONH); 3.39 (q, 2H, $J = 6.9$ Hz, CH_2NH); 2.15 (s, 3H, $COCH_3$); 1.57 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.38 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 0.94 (t, 3H, $J = 6.9$ Hz, CH_2CH_3). Anal. ($C_{13}H_{18}N_2O_3$): C, H, N.

6.1.3. 5-Acetamide-N-butyl-2-(2-propynyloxy) benzamide (4a) and 5-acetamide-N-butyl-2-(3-methyl-2-butenyloxy) benzamide (4b)

Sodium metal (1.75 g, 76.5 mmol) was dissolved in warm anhydrous isopropanol (40 mL). To this freshly made solution was added **3** (9 g, 38.2 mmol) and propargyl bromide or isopenthenyl bromide (49.7 mmol). The resulting reaction mixture was stirred under reflux overnight. The sodium bromide was filtered off and the solvent was removed in vacuum. Recrystallisation of the crude product from diethyl ether gave, respectively, **4a** or **4b** as a white solid.

6.1.4. 5-Amino-N-butyl-2-(2-propynyloxy) benzamide (5a)

A mixture of **4a** (2.02 g, 7.0 mmol) and 4 N sulphuric acid solution (30 mL) was heated at 95 °C for 2 h. The reaction solution was cooled to ambient temperature and the pH adjusted to 10 with 2 N NaOH solution. The resulting white precipitate was filtered off, washed with water, and dried. Recrystallisation from diethyl ether afforded 1.5 g (90%) of the pure product **5a** as a white solid.

6.1.5. 5-Amino-N-butyl-2-(3-methyl-2-butenyloxy) benzamide (5b)

A mixture of **4b** (10 g, 31 mmol) and 3 N NaOH solution (60 mL) was heated at 95 °C for 2 h. The reaction solution was cooled to ambient temperature. The resulting white precipitate was filtered off, washed with water, and dried. Recrystallisation from diethyl ether afforded 5.5 g (65%) of the pure product **5b** as a white solid.

6.1.6. 5-Methanesulfonamide-N-butyl-2-(2-propynyloxy) benzamide (6a) and 5-methanesulfonamide-N-butyl-2-(3-methyl-2-butenyloxy) benzamide (6b)

To a solution of **5a** or **5b** (5.4 mmol) and pyridine (0.5 mL) in toluene (30 mL) was added dropwise methanesulfonyl chloride (0.4 mL, 5.0 mmol). The resulting reaction mixture was stirred at 70 °C for 4 h and then cooled to ambient temperature and concentrated in

vacuum to afford a solid. The crude residue was dissolved in CH_2Cl_2, washed with brine and the organic phase was dried over magnesium sulphate and then concentrated in vacuum. The crude products were further purified by chromatography on a silica gel column (elution with diethyl ether–ethanol (8:2), v/v) followed by recrystallisation from diethyl ether–ethanol to give, respectively, **6a** (68%) or **6b** (65%) as white solid.

*6.1.7. 2-Chloro-5-nitro-N-butylbenzamide (**8**)*

To a solution of 2-chloro-5-nitrobenzoic acid (**7**) (20 g, 99.0 mmol) in anhydrous DMF (150 mL) was added HOBt (14.7 g, 109 mmol) and DCC (22.5 g, 109 mmol) in the above order at 0 °C. The resulting reaction mixture was stirred for 30 min and then *n*-butylamine (10.8 mL, 109 mmol) was added. The reaction mixture was stirred at 0 °C for 2 h and overnight at r.t. *N*,*N*′-Dicyclohexylurea (DCU) was filtered off and the DMF was evaporated in vacuum. The residue was dissolved in CH_2Cl_2, washed consecutively with brine, 1 N NaOH, brine, 1 N HCl and brine. The organic phase was dried over magnesium sulphate, filtered, concentrated in vacuum and the residue was purified by chromatography on a silica gel column (elution with diethyl ether–petroleum ether (7:3), v/v). Recrystallisation by diethyl ether–ethanol afforded 19.6 g (77%) of pure **8** as a yellow solid, m.p. 134 °C. ^{1}H-NMR ($CDCl_3$, δ, ppm): 8.46 (d, 1H, $J = 2.9$ Hz, ArH); 8.19 (dd, 1H, $J = 2.9$ Hz, ArH); 7.59 (d, 1H, $J = 7.9$ Hz, ArH); 6.23 (br, 1H, CONH); 3.48 (q, 2H, $J = 6.9$ Hz, CH_2NH); 1.89 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.44 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 0.98 (t, 3H, $J = 6.9$ Hz, CH_2CH_3). Anal. ($C_{11}H_{13}ClN_2O_3$): C, H, Cl, N.

*6.1.8. 2-Cyclohexyloxy-5-nitro-N-butylbenzamide (**9a**)*

A solution of sodium hydride (60% in mineral oil, 3.35 g, 84.0 mmol) in dry dioxane (150 mL) was added to cyclohexanol (7.91 g, 79.0 mmol) in an ice-water bath under nitrogen atmosphere. After the evolution of hydrogen had ceased the mixture was stirred at 70 °C for 2 h. Then intermediate **8** (19.6 g, 76.4 mmol) in anhydrous dioxane (100 mL) was added dropwise and the resulting reaction mixture was stirred at 70 °C for 4 h and overnight at ambient temperature. The solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate and washed with brine. The organic phase was dried over magnesium sulphate, filtered, concentrated in vacuum and the residue purified by chromatography on a silica gel column (elution with diethyl ether–hexane (6:4), v/v) to give **9a** (9.4 g, 75%), m.p. 64–65 °C. ^{1}H-NMR ($CDCl_3$, δ, ppm): 9.09 (d, 1H, $J = 2.9$ Hz, ArH); 8.44 (d, 1H, $J = 7.9$ Hz, ArH); 7.88 (br, 1H, CONH); 7.05 (dd, 1H, $J = 2.9$ Hz, ArH); 4.62 (m, 1H, cyclohexyl); 3.49 (q, 2H, $J = 6.9$ Hz, CH_2NH); 2.10–1.85 (m, 8H, cyclohexyl); 1.67 (q, 2H, $J = 6.9$ Hz, CH_2CH_2NH); 1.58 (m, 2H, cyclohexyl); 1.48 (m, 2H, $J = 7.9$ Hz, CH_2CH_3); 0.98 (t, 3H, $J = 6.9$ Hz, CH_2CH_3). Anal. ($C_{11}H_{23}N_2O_4$): C, H, N.

Using the procedure described for preparing **9a** the following compounds were prepared: 2-phenyloxy-5-nitro-*N*-butylbenzamide (**9b**), 2-*p*-fluorophenyloxy-5-nitro-*N*-butylbenzamide (**9c**), 2-*p*-chlorophenyloxy-5-nitro-*N*-butylbenzamide (**9d**), and 2-*p*-isopropylphenyloxy-5-nitro-*N*-butylbenzamide (**9e**) (yields ranging between 70 and 80%). Similar ^{1}H-NMR data occur in intermediates **9b**–**e** and were consistent with the proposed structures.

*6.1.9. 2-Cyclohexyloxy-5-amino-N-butylbenzamide (**10a**)*

To a suspension of Pd/C (0.15 g) in 15 mL of water was added a suspension (20 mL) of $NaBH_4$ (1.0 g, 26.4 mmol) in water. The resulting reaction mixture was stirred at r.t. under nitrogen atmosphere for 10 min. Then, **9a** (2.89 g, 8.1 mmol) in 100 mL of methanol was added dropwise and the mixture was stirred for 30 min. The reaction mixture was filtered through a Celite bed. The solution was acidified with 1 N HCl to remove the excess of $NaBH_4$. The acidified solution was adjusted to alkaline pH with 2 N NaOH solution and the resulting suspension was extracted with diethyl ether. The organic phase was dried over magnesium sulphate, filtered, and evaporated to dryness to yield intermediate **10a** as a white solid (2.7 g, 87%). ^{1}H-NMR data were reported as hydrochloride salts.

Using the procedure described for preparing **10a** the following compounds were prepared: 2-phenyloxy-5-amino-*N*-butylbenzamide (**10b**), 2-*p*-fluorophenyloxy-5-amino-*N*-butylbenzamide (**10c**), 2-*p*-chlorophenyloxy-5-amino-*N*-butylbenzamide (**10d**), and 2-*p*-isopropylphenyloxy-5-amino-*N*-butylbenzamide (**10e**) (yields ranging between 65 and 70%). Similar ^{1}H-NMR data occur in intermediates **10b**–**e** and were consistent with the proposed structures.

*6.1.10. 5-Amino-N-butyl-2-cyclohexyloxy-benzamide hydrochloride (**11a**)*

Free base **10a** (2.5 g, 8.6 mmol) was dissolved in ethanol (50 mL) and treated with an excess of diethyl ether saturated with dry, gaseous HCl. Recrystallisation

from diethyl ether–ethanol (8:2, v/v) provides **11a** (2.5 g, 89%) as a white solid.

Using the procedure described for preparing **11a** the following compounds were prepared: 5-amino-*N*-butyl-2-phenoxy-benzamide hydrochloride (**11b**), 5-amino-*N*-butyl-2-(4-fluorophenyloxy) benzamide hydrochloride (**11c**), 5-amino-*N*-butyl-2-(4-chlorophenyloxy) benzamide hydrochloride (**11d**), and 5-amino-*N*-butyl-2-(4-isopropylphenyloxy) benzamide hydrochloride (**11e**).

*6.1.11. 5-Acetamide-N-butyl-2-cyclohexyloxy-benzamide (**12a**)*

To a solution of amine **10a** (2.71 g, 7.0 mmol) in anhydrous DMF (70 mL) was added acetic anhydride (0.75 mL, 8.0 mmol). The resulting reaction mixture was stirred at r.t. for 30 min and then the solvent removed in vacuum. The crude residue was dissolved in CH_2Cl_2, washed with 0.5 N NaOH and brine. The organic layer was dried over magnesium sulphate, filtered, and concentrated in vacuum. The crude residue was purified by chromatography on a silica gel column (elution with diethyl ether–hexane (5.5:4.5), v/v). Recrystallisation from diethyl ether–ethanol provides 1.6 g (71%) of **12a** as a white solid.

Using the procedure described for preparing **12a** the following compounds were prepared: 5-acetamide-*N*-butyl-2-phenoxy-benzamide (**12b**), 5-acetamide-*N*-butyl-2-(4-fluorophenyloxy) benzamide (**12c**), 5-acetamide-*N*-butyl-2-(4-chlorophenyloxy) benzamide (**12d**), and 5-acetamide-*N*-butyl-2-(4-isopropylphenyloxy) benzamide (**12e**).

*6.1.12. 5-Methanesulfonamide-N-butyl-2-cyclohexyloxy-benzamide (**13a**)*

To a solution of **10a** (2.71 g, 7.0 mmol) and pyridine (0.6 mL) in toluene (60 mL) was added dropwise methanesulfonyl chloride (0.9 mL, 7.6 mmol) in 10 mL of toluene. The resulting reaction mixture was stirred at 70 °C for 5 h. After cooling at ambient temperature the solvent was removed in vacuum. The crude residue was dissolved in CH_2Cl_2, washed with brine and the organic phase was dried over magnesium sulphate and concentrated in vacuum. The obtained residue was purified by chromatography on a silica gel column (elution with diethyl ether–hexane (5.5:4.5), v/v). Recrystallisation from diethyl ether–ethanol affords 1.52 g (59%) of pure **13a** as a white solid.

Using the procedure described for preparing **13a** the following compounds were prepared: 5-methanesulfonamide-*N*-butyl-2-phenoxy-benzamide (**13b**), 5-methanesulfonamide-*N*-butyl-2-(4-fluorophenyloxy)benzamide (**13c**), 5-methanesulfonamide-*N*-butyl-2-(4-chlorophenyloxy)benzamide (**13d**), and 5-methanesulfonamide-*N*-butyl-2-(4-isopropylphenyloxy)benzamide (**13e**).

6.1.13. Determination of pK_a values: general procedure

The pK_a values of compounds **5b**, **11a** and **11b** have been evaluated by reading the pH at the midpoint (50% neutralisation) of the plot versus millilitres of titrant. The titrations have been performed potentiometrically on the hydrochlorides of compounds **5b**, **11a** and **11b** with NaOH standard solution in water–ethanol (2:1, v/v); pK_a values were 4.20, 3.80 and 3.15, respectively.

6.2. Enzyme assay

6.2.1. Pure ovine COX-1 and COX-2 enzyme assay

Purified ovine COX-1 and COX-2 were obtained from Cayman Chemicals (Ann Arbor, MI). Pure enzymes and co-factors adrenaline (5 mM) and hematin (1 μm) were dissolved in 50 mM Tris-buffer (pH 7.5). Hematin was first dissolved as a concentrated stock of 100 mM in 1 M NaOH before being further diluted in Tris-buffer. Enzymatic reactions were carried in individual wells of 96-well plates with a final reaction volume of 200 μL. Drugs were diluted in Tris-buffer to obtain different concentrations (10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} M) and were added to the plate, followed by co-factors and 10 U mL^{-1} of the enzyme (180 μL). The plates were incubated at 37 °C for 30 min before addition of AA (10 μM). The reaction was stopped after a further 15 min by the addition of 10 μL 1 M HCl, then the pH 7.5 was balanced with 10 μL 1 M NaOH. By measuring the formation of PGE_2, a non-enzymatic metabolite of PGH_2, the activity of the enzymes and the inhibitory effects of different compounds were assessed. The content of PGE_2 in the incubates was measured by using radioimmunoassay. Data are presented as a percentage of control PGE_2 production. The inhibition graphs for each COX inhibitor were plotted as a mean ± S.E.M. and were constructed from data collected on at least four experimental days. Data were analysed by using a computerised software package PRISM 3.0 (Graphpad software, USA).

6.2.2. Carrageenin footpad edema

Male Wistar rats (120–140 g) were injected in the right hind paw with 0.1 mL of a 1% solution of λ-carrageenin in saline. Compounds were suspended in carboxymethylcellulose 0.5% and administered orally 1 h

prior to carrageenin injection. Paw volume was measured by using a hydroplethysmometer (Ugo Basile, Italy) immediately before carrageenin injection and at hourly intervals for 5 h. Control animals received an equal volume of vehicle. Results were analysed by ANOVA followed by Dunnett's test.

6.2.3. Acute gastric damage

Rats (120–140 g) were deprived of food, but not water, for 18 h and were then given Parsalmide or the compounds to be examined orally at the dose of 100 mg/kg. A further group was treated with an equal volume of the vehicle. Each group was made up of six rats. The rats were anaesthetised with sodium pentobarbital and the stomach was excised and opened by an incision along the greater curvature. The extent of macroscopic damage was determined by an observer unaware of the treatments that the rats received, as described previously [17]. The method involved measuring the length of the lesions in millimetres and then summing the length of all lesions observed in each stomach. Data were analysed by ANOVA for non-parametric measure Kruskall–Wallis test followed by Dunn's post-test analysis.

6.2.4. Indomethacin-induced gastric damage

Rats (120–140 g) were deprived of food, but not water, for 18 h and then given Parsalmide or the compounds to be analysed 1 h prior to the administration of indomethacin at the dose of 20 mg/kg. After 3 h animals were sacrificed and the stomach was excised and the extent of macroscopic damage determined by an observer unaware of the treatments as described in the acute gastric damage section. Data were analysed by ANOVA for non-parametric measure Kruskall–Wallis test followed by Dunn's post-test analysis.

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