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Original article

N-Benzyl-2-chloroindole-3-carboxylic acids as potential anti-inflammatory agents. Synthesis and screening for the effects on human neutrophil functions and on COX1/COX2 activity

Aldo Andreani [a,*], Massimiliano Granaiola [a], Alberto Leoni [a], Alessandra Locatelli [a], Rita Morigi [a], Mirella Rambaldi [a], Aldo Roda [a], Massimo Guardigli [a], Serena Traniello [b], Susanna Spisani [b]

[a] *Dipartimento di Scienze Farmaceutiche, Università di Bologna, Via Belmeloro 6, 40126 Bologna, Italy*
[b] *Dipartimento di Biochimica e Biologia Molecolare, Università di Ferrara, Via L.Borsari 46, 44100 Ferrara, Italy*

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Abstract

The synthesis of *N*-benzyl-2-chloroindole-3-carboxylic acids related to indomethacin is reported. These compounds were tested on in vitro human neutrophil activation. Some of them, more soluble in water, were tested to define the influence on prostaglandin biosynthesis via inhibition of cyclooxygenases (COX1 and COX2) by a chemiluminescent method suitable for fast screening. Several derivatives showed inhibitory effects and in some cases were more active than the parent compound.

Keywords: Indole; Inflammation; Neutrophil; COX1/COX2

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) show in vitro effects on neutrophil functions [1–3]. Some NSAIDs inhibit neutrophil activation by inflammatory stimuli, such as C5a, LTB_4, or formyl-methionine-leucine-phenylalanine (FMLP), by interfering with early events of signal transduction [4–7]. Indomethacin inhibits neutrophil chemotaxis and leukotriene B4 generation [8]. Moreover indomethacin, salicylic acid and acetylsalicylic acid were found to inhibit superoxide generation by human neutrophils exposed to phorbol 12-myristate 13-acetate (PMA) in a whole-cell system [9] while prednisolone and NSAIDs inhibited elastase release from neutrophils exposed to FMLP [10,11]. For a recent review on neutrophils in the inflammatory process see Ref. [12]. The trend of measuring the effects on neutrophil functions for the evaluation of new potential anti-inflammatory agents is confirmed by a recent paper [13].

In pursuing our search on the anti-inflammatory activity of heterocyclic compounds [14–18], we planned the synthesis of 2-chloro-1-(4-chlorobenzyl)-5-methoxyindole-3-carboxylic acid **3c** (Scheme 1) which has a steric hindrance analogous to indomethacin with a shorter acidic chain (the approach of chain shortening has been applied for example in the case of diclofenac and its fenamic analog). The substituents at the indole ring are for example the methoxy group (see indomethacin) and the acethyl group (see acetylsalicylic acid where it is well known that the acetyl group binds a serine residue in COX).

Indomethacin was first reported by Shen et al. [19]. The paper stimulated the search in this field and a lot of derivatives have been prepared, including rigid analogs [20] and benzyl derivatives [21,22].

The encouraging effects shown by compound **3c** on neutrophil functions prompted us to prepare a small series of analogues in order to study the effect of different substituents at the position 4–6 of the indole and at the position 4 of the benzyl ring. The present work investigates the in vitro effects of new compounds and indomethacin on human neutrophil activation induced by different stimuli. Chemotaxis, super-

* Corresponding author. Tel.: +39-51-2099714; fax: +39-51-2099734.
E-mail address: aldo.andreani@unibo.it (A. Andreani).

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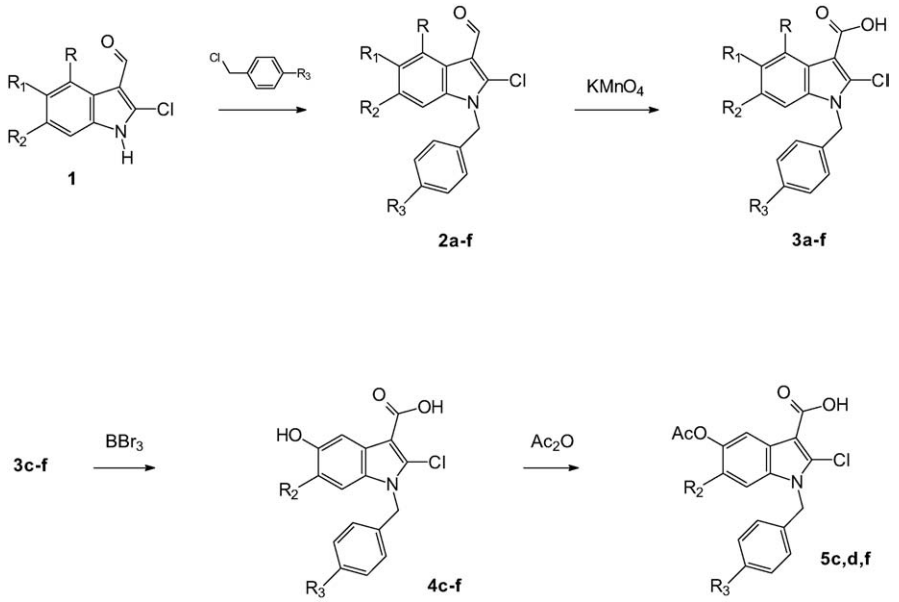

Scheme 1. (R–R_3 see Table 1).

oxide anion (O_2^-) generation and lysozyme degranulation were evaluated after cell pulse with different concentrations of the drugs.

In order to better define the biological profile of the newly synthesized compounds we also evaluated the inhibitory activity in vitro on two COX isoenzymes, COX1 and COX2. Among the numerous assays developed for this purpose over the years, we decided to measure the inhibition using a chemiluminescent (CL) method which allows to measure the rate of the peroxidase reaction, i.e., the second step of the PGH synthase-catalyzed reaction, in which the intermediate product prostaglandin G_2 is converted into the final product prostaglandin H_2 [23], by using luminol (a well-known CL substrate) as the reducing cosubstrate [24]. It has been previously demonstrated that the cyclooxygenation reaction is the rate limiting step for the generation of the CL signal, thus the easily detectable CL emission produced by the oxidation of luminol could be regarded as a real-time index of cyclooxygenase acitivity [24]. This method allowed us to determine the IC_{50} value of the newly synthesized compounds in a very short time and to consume small amounts of samples and reagents.

2. Chemistry

The starting 2-chloroaldehydes **1** (Scheme 1) have been previously reported in the literature [25,26]. *N*-Alkylation was conducted in dimethylformamide in the presence of sodium hydride [27]. The oxidation of the resulting *N*-benzyl-2-chloroindole-3-carboxaldehydes **2a–f** (Table 1) to the corresponding carboxylic acids **3a–f** was performed with potassium permanganate in a mixture of acetone–water. The acids bearing a methoxy group at the 5 position **(3c–f)** were treated with boron tribromide in methylene chloride in order to obtain the corresponding phenols **4c–f**. Aim of the subsequent step was the synthesis of the corresponding 5-acetyloxy derivatives but under the planned experimental conditions we were able to obtain only the expected compounds **5c,d,f**. Under the same experimental conditions compound **4e** led to complex reaction mixtures in which little, if any, of the desired product was present. The spectroscopic data of the new compounds (see Table 2) are in agreement with the assigned structures and with the data reported on a previous papers [28]. The ^{1}H-NMR spectra of compounds **2–5** show the methylene of the benzyl group in the range 5.32–5.67 ppm; in all compounds **4**, the indole 5-hydroxy group is between 9.10 and 9.25 ppm i.e. clearly separated from the OH of the 4-hydroxyphenyl group present in two of them (9.40–9.44 ppm).

3. Biological results

One of the targets of the present research was to determine whether the tested compounds **3–5** affect some important neutrophil functions. We analyzed the in vitro effect (see Refs. [17,18]) of the new compounds on a wide range of neutrophil functionality triggered by different stimuli acting either through specific cell surface receptors (e.g. FMLP) or by post-receptor signaling (e.g. casein and PMA). It is generally accepted that receptor and nonreceptor agonists utilize different transduction mechanisms to activate chemotaxis and free radical production [29,30]. In order to assess possible cytotoxic effects of the tested compounds, the cell viability was measured. Release of the cytoplasmic enzyme lactate dehydrogenase (LDH) was used as an indicator of cell viability. In none of the above described experiments the percentage of total LDH release was >3% (data not shown). In the first step of our study, we tested whether compounds

Table 1
Compounds **2–5**

Compound	R	R_1	R_2	R_3	Formula	MW	m.p. (°C) or literature
2a	H	H	H	Cl	$C_{16}H_{11}Cl_2NO$	304.2	[27]
2b	Cl	H	H	Cl	$C_{16}H_{10}Cl_3NO$	338.6	180–184
2c	H	OCH_3	H	Cl	$C_{17}H_{13}Cl_2NO_2$	334.2	[16]
2d	H	OCH_3	CH_3	Cl	$C_{18}H_{15}Cl_2NO_2$	348.2	175–180 dec
2e	H	OCH_3	H	OCH_3	$C_{18}H_{16}ClNO_3$	329.8	134–136
2f	H	OCH_3	CH_3	OCH_3	$C_{19}H_{18}ClNO_3$	343.8	130–133
3a	H	H	H	Cl	$C_{16}H_{11}Cl_2NO_2$	320.2	[27]
3b	Cl	H	H	Cl	$C_{16}H_{10}Cl_3NO_2$	354.6	160–163 dec
3c	H	OCH_3	H	Cl	$C_{17}H_{13}Cl_2NO_3$	350.2	215–218 dec
3d	H	OCH_3	CH_3	Cl	$C_{18}H_{15}Cl_2NO_3$	364.2	240–241 dec
3e	H	OCH_3	H	OCH_3	$C_{18}H_{16}ClNO_4$	345.8	211–214 dec
3f	H	OCH_3	CH_3	OCH_3	$C_{19}H_{18}ClNO_4$	359.8	229–231 dec
4c	–	–	H	Cl	$C_{16}H_{11}Cl_2NO_3$	336.2	237–239
4d	–	–	CH_3	Cl	$C_{17}H_{13}Cl_2NO_3$	350.2	210–212 dec
4e	–	–	H	OH	$C_{16}H_{12}ClNO_4$	317.7	200–205 dec
4f	–	–	CH_3	OH	$C_{17}H_{14}ClNO_4$	331.7	203–206 dec
5c	–	–	H	Cl	$C_{18}H_{13}Cl_2NO_4$	378.2	223–225
5d	–	–	CH_3	Cl	$C_{19}H_{15}Cl_2NO_4$	392.2	261–264 dec
5f	–	–	CH_3	OAc	$C_{21}H_{18}ClNO_6$	415.8	233–236

3–5 and indomethacin were chemotaxins for human neutrophils, secretagogue agents or were able per se to trigger superoxide anion production. None of the molecules was able to mediate these activities. In the second step, we tested a possible influence of the compounds on neutrophil functions. In order to study the initial event of inflammation, we analyzed the influence of the prepared compounds and indomethacin on random locomotion in vitro. All compounds showed slight effects: however, these were not statistically significant ($P > 0.05$) at the concentrations tested (10^{-11}, 10^{-9}, 10^{-7} and 10^{-5} M) (data not shown). Conversely, when the same compounds were tested for their ability to influence directed migration, inhibition of FMLP-induced chemotaxis (Table 3) was observed in the presence of compounds **3a,d–f, 4d–f** and **5f**. Most of the compounds were able to affect FMLP-induced respiratory burst. The inhibition became statistically significant ($P < 0.05$) at a drug concentration of 10^{-9} M, reaching the maximal inhibition at the highest concentrations ($P < 0.01$). Compounds **4c,d,f, 5c,d** reached about 60% inhibition at 10^{-5} M. Only few compounds (**3f, 4e,f** and **5f**) elicited a PMA-induced response (inhibition = 30% at 10^{-5} M). Only compounds **3e,f** were active in PMA-stimulated lysozyme release (inhibition = 35% at 10^{-5} M). None of the tested derivatives affect either casein-activated chemotaxis or FMLP-induced lysozyme secretion (data not shown). No activity by any of the molecules was found at 10^{-11} M (data not shown). Ineffective derivatives are not presented in the Table 3.

The second target of the biological activity tests was the study of COX1 and COX2 inhibition. The performance of the CL method was determined by measuring the IC_{50} values of known cyclooxygenase inhibitors (indomethacin, ibuprofen and rofecoxib). The method proved to be reliable in term of reproducibility and the obtained data for known inhibitors (Table 4) were in reasonable agreement with those reported in the literature, in particular as concerned the COX1/COX2 selectivity and the relative potency of these compounds [31,32]. Cyclooxygenase inhibitory activity was measured only for compounds **3a,b, 4c–f, 5c,d,f** which showed a good solubility in DMSO/water 1:10 (v/v). The IC_{50} values found for these compounds were in the range 10^{-4} to 10^{-6} M (Table 4). Most of the compounds were more active than ibuprofen and some of them (**4d** and **4f**) showed an inhibitory effect close to that of indomethacin. As indomethacin, both the derivatives were approximately equally effective on both isoenzymes. The IC_{50} values obtained for compounds **4d** and **4f** in COX2 inhibition experiments ($IC_{50} = 10^{-6}$ M) were an order of magnitude higher than that of the COX2 selective inhibitor rofecoxib ($IC_{50} = 10^{-7}$ M).

4. Conclusion

According to our results, compounds **3–5** are not toxic (measurement of cell viability, for experimental see Refs. [17,18]). In particular, chemotaxis and O_2^- production triggered by the optimal active concentration of the agonist FMLP, seems more sensible to these derivatives. Some of them display interesting properties as inhibitors of human neutrophil functions and retain much of the inhibitory power of indomethacin. In some cases they are even more active (see **3d, 4c,d,f** and **5c,d** in FMLP-triggered O_2^- generation) and are able to inhibit neutrophil activity at concentrations as low as 10^{-9} M and for this reason may be considered as potential anti-inflammatory agents. They could block recruitment of neutrophils into inflammatory lesions and control neutrophil degranulation, showing a protective effect against the direct injury to the target tissue derived from proteolityc

Table 2
IR and ^{1}H-NMR of compounds **2–5**

Compound	IR [a]: ν_{max}, cm^{-1}	^{1}H-NMR [b]: δ (ppm); J (Hz) in DMSO-d$_6$; ph, phenyl; ind, indole
2b	1650, 1035, 770, 725	5.65 (2H, s, CH_2), 7.15 (2H, d, ph, J = 8.4), 7.31 (1H, t, ind, J = 8.1), 7.39 (3H: 2H, d, ph, J = 8.4 + 1H, d, ind, J = 8.1), 7.70 (1H, d, ind, J = 8.1), 10.67 (1H, s, CHO)
2d	1640, 1500, 1035, 715	2.23 (3H, s, CH_3), 3.84 (3H, s, OCH_3), 5.57 (2H, s, CH_2), 7.16 (2H, d, ph, J = 6.6), 7.42 (2H, d, ph, J = 6.6), 7.49 (1H, s, ind), 7.59 (1H, s, ind), 10.00 (1H, s, CHO)
2e	1645, 1510, 1245, 1140	3.70 (3H, s, OCH_3), 3.79 (3H, s, OCH_3), 5.49 (2H, s, CH_2), 6.89 (2H, d, ph, J = 8.7), 6.94 (1H, dd, ind6, J = 9.1, J = 2.8), 7.16 (2H, d, ph, J = 8.7), 7.59 (1H, d, ind7, J = 9.1), 7.61 (1H, d, ind4, J = 2.8), 10.00 (1H, s, CHO)
2f	1650, 1510, 1250, 1030	2.20 (3H, s, CH_3), 3.67 (3H, s, OCH_3), 3.80 (3H, s, OCH_3), 5.43 (2H, s, CH_2), 6.86 (2H, d, ph, J = 8.4), 7.11 (2H, d, ph, J = 8.4), 7.48 (1H, s, ind), 7.54 (1H, s, ind), 9.96 (1H, s, CHO)
3b	1670, 1230, 1165, 760	5.59 (2H, s, CH_2), 7.13 (2H, d, ph, J = 8.5), 7.26(2H, d, ind, J = 4.6), 7.41 (2H, d, ph, J = 8.5), 7.62 (1H, t, ind, J = 4.6)
3c	1680, 1520, 1280, 1225	3.78 (3H, s, OCH_3), 5.56 (2H, s, CH_2), 6.90 (1H, dd, ind6, J = 9.0, J = 2.5), 7.11 (2H, d, ph, J = 8.5), 7.39 (2H, d, ph, J = 8.5), 7.51 (1H, d, ind7, J = 9.0), 7.55 (1H, d, ind4, J = 2.5)
3d	1665, 1505, 1210, 1180	2.22 (3H, s, CH_3), 3.82 (3H, s, OCH_3), 5.53 (2H, s, CH_2), 7.09 (2H, d, ph, J = 8.5), 7.40 (3H: 2H, d, ph, J = 8.5 + 1H, s, ind), 7.51 (1H, s, ind)
3e	1650, 1610, 1500, 1170	3.66 (3H, s, OCH_3), 3.75 (3H, s, OCH_3), 5.43 (2H, s, CH_2), 6.84 (2H, d, ph, J = 8.8), 6.86 (1H, dd, ind6, J = 9.1, J = 2.7) 7.06 (2H, d, ph, J = 8.8), 7.49 (1H, d, ind7, J = 9.1), 7.50 (1H, d, ind4, J = 2.7)
3f	1680, 1510, 1210, 1175	2.22 (3H, s, CH_3), 3.69 (3H, s, OCH_3), 3.81 (3H, s, OCH_3), 5.43 (2H, s, CH_2), 6.87 (2H, d, ph, J = 8.6), 7.06 (2H, d, ph, J = 8.6), 7.42 (1H, s, ind), 7.49 (1H, s, ind)
4c	3150, 1660, 1510, 1165	5.51 (2H, s, CH_2), 6.73 (1H, dd, ind6, J = 8.8, J = 2.4), 7.12 (2H, d, ph, J = 8.5), 7.38 (1H, d, ind, J = 8.8), 7.40 (2H, d, ph, J = 8.5), 7.48 (1H, d, ind, J = 2.4), 9.20 (1H, s, OH)
4d	3450, 1650, 1520, 1225	2.21 (3H, s, CH_3), 5.51 (2H, s, CH_2), 7.12 (2H, d, ph, J = 8.5), 7.32 (1H, s, ind), 7.43 (2H, d, ph, J = 8.5), 7.52 (1H, s, ind), 9.25 (1H, s, OH)
4e	3230, 1675, 1515, 1235	5.33 (2H, s, CH_2), 6.67 (2H, d, ph, J = 7.9), 6.70 (1H, dd, ind6, J = 8.8, J = 1.6), 6.97 (2H, d, ph, J = 7.9), 7.36 (1H, d, ind7, J = 8.8), 7.41 (1H, d, ind4, J = 1.6), 9.10 (1H, s, OH-5), 9.40 (1H, s, OH-*para*)
4f	3300, 1660, 1500, 1250,	2.18 (3H, s, CH_3), 5.32 (2H, s, CH_2), 6.69 (2H, d, ph, J = 8.5), 6.97 (2H, d, ph, J = 8.5), 7.28 (1H, s, ind), 7.46 (1H, s, ind), 9.16 (1H, s, OH-5), 9.44 (1H, s, OH-*para*)
5c	1730, 1650, 1490, 1200	2.29 (3H, s, CH_3), 5.61 (2H, s, CH_2), 7.04 (1H, dd, ind6, J = 8.8, J = 2.2), 7.15 (2H, d, ph, J = 8.4), 7.41 (2H, d, ph, J = 8.4), 7.64 (1H, d, ind7, J = 8.8), 7.76 (1H, d, ind4, J = 2.2)
5d	1740, 1650, 1500, 1200	2.19 (3H, s, CH_3), 2.33 (3H, s, CH_3), 5.58 (2H, s, CH_2), 7.15 (2H, d, ph, J = 7.7), 7.42 (2H, d, ph, J = 7.7), 7.56 (1H, s, ind), 7.69 (1H, s, ind)
5f	1740, 1680, 1640, 1185	2.16 (3H, s, CH_3), 2.22 (3H, s, CH_3), 2.30 (3H, s, CH_3), 5.54 (2H, s, CH_2), 7.07 (2H, d, ph, J = 8.7), 7.14 (2H, d, ph, J = 8.7), 7.54 (1H, s, ind), 7.65 (1H, s, ind)

[a] The COOH group is broad (3400–2300 cm^{-1}).
[b] The COOH group is a singlet in the range 12.5–13 ppm.

enzyme release. In some cases (see for example **5d** in Table 3) the pharmacological response was peculiar and difficult to explain.

We demonstrated that, thanks to the use of 96-well microtiter plates and the high detectability and fast kinetics of the CL signal, the CL COX activity assay facilitated the rapid screening of the biological activity of newly synthesized compounds and the identification of the most promising molecules with a reduced consumption of reagents.

5. Experimental

5.1. Chemistry

The melting points are uncorrected. Analyses (C, H, N) were within ±0.4% of the theoretical values. TLC was performed on Bakerflex plates (Silica gel IB2-F): the eluent was a mixture of petroleum ether/acetone in various proportions. The same eluent was used for column chromatography with Kieselgel 60 (Merck) as the stationary phase. The IR spectra were recorded in nujol on a Nicolet Avatar 320 ESP. The ^{1}H-NMR spectra were recorded in $(CD_3)_2SO$ on a Varian Gemini (300 MHz); the chemical shift (referenced to solvent signal) is expressed in δ (ppm) and J in Hz (see Table 2).

5.1.1. General procedure for the synthesis of the N-benzyl-2-chloroindole-3-carboxaldehydes **2a–f**

The appropriate 2-chloroindolaldehyde **1** (10 mmol) was dissolved in 20 ml of DMF and the stirred solution was treated with small portions of NaH (15 mmol). The reaction mixture was stirred at room temperature for 10 min, treated with the appropriate benzyl chloride (60 mmol) and maintained at 90 °C for 1–3 h according to a TLC test. The reaction mixture was then poured into ice and acidified with 2 N HCl. The crude *N*-benzyl-2-chloroindole-3-carboxaldehydes **2a–f** thus obtained was collected by filtration and crystallized from ethanol with a yield of 80–90%.

5.1.2. General procedure for the synthesis of the N-benzyl-2-chloroindole-3-carboxylic acids **3a–f**

The appropriate *N*-benzyl-2-chloroindole-3-carboxaldehyde (**2a–f**, 5 mmol) was dissolved in 100 ml of acetone and treated with a solution of $KMnO_4$ (15 mmol) in water

Table 3
Inhibitory activity (%) of compounds **3–5** and indomethacin on human neutrophil functions

Compound	Concentration (M)	FMLP-induced chemotaxis [a]	FMLP-triggered O_2^- generation [a]
3a	10^{-7}	n.s. [b]	31
	10^{-5}	52	33
3b	10^{-9}	n.s.	32
	10^{-7}	n.s.	35
	10^{-5}	n.s.	35
3c	10^{-9}	n.s.	30
	10^{-7}	n.s.	31
	10^{-5}	n.s.	31
3d	10^{-9}	25	41
	10^{-7}	n.s.	44
	10^{-5}	n.s.	51
3e	10^{-9}	28	n.s.
	10^{-7}	29	29
	10^{-5}	31	31
3f	10^{-9}	25	n.s.
	10^{-7}	33	n.s.
	10^{-5}	35	n.s.
4c	10^{-9}	n.s.	44
	10^{-7}	n.s.	53
	10^{-5}	n.s.	55
4d	10^{-9}	25	51
	10^{-7}	44	58
	10^{-5}	35	62
4e	10^{-9}	29	n.s.
	10^{-7}	33	n.s.
	10^{-5}	35	28
4f	10^{-9}	26	41
	10^{-7}	28	44
	10^{-5}	30	49
5c	10^{-9}	n.s.	61
	10^{-7}	n.s.	60
	10^{-5}	n.s.	59
5d	10^{-9}	n.s.	58
	10^{-7}	n.s.	53
	10^{-5}	n.s.	52
5f	10^{-9}	25	28
	10^{-7}	27	32
	10^{-5}	33	47
Indomethacin	10^{-9}	49	22
	10^{-7}	52	22
	10^{-5}	59	36

[a] Cells were incubated for 10 min before the assay with the compounds at the concentration of 10^{-11} (n.s.), 10^{-9}, 10^{-7}, 10^{-5} M. Each value is the average of the inhibitory activity from 8–10 separate experiments carried out in triplicate. SEM are within 10%. Statistical significance is reported in the text.
[b] n.s.=not significant.

(40 ml). The reaction mixture was stirred at room temperature for 5–16 h (according to a TLC test), discolored with 10% H_2O_2 and filtered. Acetone was evaporated under reduced pressure and the resulting solution, filtered again if necessary, was acidified with 2 N HCl. The precipitate was collected by filtration and crystallized from ethanol with a yield of 60–70%.

*5.1.3. General procedure for the synthesis of the 5-hydroxy derivatives **4c–f***

The appropriate 5-methoxy derivative (**3c–f**, 3 mmol) was dissolved in 40 ml of anhydrous CH_2Cl_2 and treated under nitrogen at 0–5 °C with BBr_3 (7 mmol). The mixture was stirred overnight at room temperature, treated with 33% NaOH (pH 9) and stirred for additional 10 min. The aqueous phase was separated and acidified with 2 N HCl at 0 °C: the precipitate thus obtained was collected by filtration with a yield of 50–60% and was not subjected to further purification.

*5.1.4. General procedure for the synthesis of the 5-acetyl derivatives **5c,d,f***

The appropriate 5-hydroxy derivative (**4c–f**, 2 mmol) was dissolved in 15 ml of acetic anhydride and two drops of 98% H_2SO_4. The mixture was stirred at 50–60 °C for 15 min and water (30 ml) was added. The aqueous phase was extracted

Table 4
COX1 and COX2 inhibition IC_{50} values measured for compounds **3a,b, 4c–f, 5c,d,f** and three reference drugs, using the CL method [a]

Compound	IC_{50}: μmol/l ± SEM	
	COX1 inhibition	COX2 inhibition
Ibuprofen	100 ± 30	350 ± 200
Indomethacin	0.4 ± 0.1	0.20 ± 0.06
Rofecoxib	60 ± 12	0.08 ± 0.02
3a	95 ± 15	230 ± 120
3b	70 ± 15	12 ± 6
4c	4.8 ± 0.4	1.7 ± 0.3
4d	0.48 ± 0.06	0.65 ± 0.15
4e	3.2 ± 0.6	30 ± 6
4f	0.6 ± 0.1	1.2 ± 0.5
5c	7.5 ± 3.0	4.6 ± 2.6
5d	3.3 ± 0.3	22 ± 10
5f	1.2 ± 0.2	1.7 ± 0.7

[a] IC_{50} values were calculated from inhibition graphs containing data collected in three independent experiments.

with CH_2Cl_2 (3 × 20 ml) and the crude product thus obtained (**5c,d,f**) was purified by column chromatography with a yield of 20–25%. Under the above experimental conditions, compound **4e** led to complex reaction mixtures.

5.2. Effects on human neutrophil functions

The detailed procedures have been described in a previous papers [17]. Cells were purified employing the standard techniques of dextran sedimentation of heparinized blood, followed by centrifugation on Ficoll–Paque and hypotonic lysis of contaminating red cells. Random locomotion and chemotaxis were evaluated with a 48-well microchemotaxis chamber, by estimating the distance in micrometers which the leading front of the cell migrated. The chemotactic factors used were FMLP (10^{-8} M in KRPG-A) or casein (2 mg/ml in KRPG-A). The actual control of chemotaxis was 95 ± 4 μm or 83 ± 2 μm SEM, from 15 separate experiments induced, respectively, by FMLP or casein. The superoxide anion production was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome modified for microplate-based assays. The stimulants employed were PMA (100 ng/ml in KRPG) or FMLP (10^{-6} M in KRPG). Neutrophils were preincubated with cytochalasin B 5 μg/ml for 5 min prior to activation by FMLP. The actual control of O_2^- generation was, 35 ± 2 nmoles or 30±2 nmoles/1 × 10^6 cells/5 min from 15 separate experiments induced, respectively, by PMA or FMLP. The release of lysozyme was quantified nephelometrically by the lysis rate of cell-wall suspension of *Micrococcus lysodeikticus*. The enzyme was expressed as a net percentage of the total enzyme content released by 0.1% Triton X-100. The total enzyme activity was 85 ±1 μg/1 × 10^7 cells/min. Spontaneous release was less than 10%. The degranulating agents used were PMA (100 ng/ml) or FMLP (10^{-6} M), in KRPG. Cells were preincubated with cytochalasin B 5 μg/ml for 15 min prior to activation by FMLP. Assays were currently done in triplicate for each experimental condition. The actual control of lysozyme release was 45 ± 4% or 52 ± 5%/3 × 10^6 cells/min from 15 separate experiments induced, respectively, by PMA or FMLP. Stock solutions in dimethylsulfoxide (DMSO) were diluted before use in Krebs–Ringer-phosphate buffer: 10^{-2} M for the tested compounds and FMLP; 1 mg/ml for PMA. DMSO used did not interfere with any of the biological assays performed. stock solution. The nonparametric Wilcoxon test was used in the statistical evaluation of differences between groups (level of significance $P \leq 0.05$).

5.3. COX1/COX2 inhibition

Ovine COX1 and COX2 were purchased from Cayman Chemical, Ann Arbor, MI. Porcine hematin, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione sodium salt) and arachidonic acid, as well as the reference inhibitor ibuprofen, were from Sigma Chemical Co. (St. Louis, MO). Rofecoxib was obtained from Merck & Co., Inc. (Rahway, NJ). Chemiluminescence measurements were performed in 96-well white polystyrene microtiter plates (White Microstrip Breakable, Labsystems Oy, Helsinki, Finland) using a microtiter plate luminometer (Luminoskan Ascent, Labsystems Oy) equipped with an on-board incubator and a reagent dispenser.

5.3.1. Evaluation of cyclooxygenase inhibition

The CL cyclooxygenase activity was measured following the procedure reported in the literature [24], with slight modifications. Briefly, cyclooxygenases (COX1 or COX2, 50 U/ml in 0.1 M phosphate buffer, pH 6.5, with 1 mg/ml gelatine), hematin (6 μM in 0.1 M phosphate buffer, pH 6.5) and luminol (500 μM in 0.1 M TRIS buffer, pH 6.5) were added to the microtiter plate wells to obtain a reaction mixture containing 1.0 U of cyclooxygenase, 350 μM luminol and 1 μM hematin in a 150 μl final volume. Then, 10 μl of scalar dilutions of the inhibitors in DMSO/water 1:10 (v/v) were added, and the solution was incubated in the microtiter plate luminometer at 35 °C for 30 min. The enzymatic reaction was started by adding to each well 200 μl of a 35 μM arachidonic acid solution in water (corresponding to a final arachidonic acid concentration of 20 μM), and the CL emission was measured at 1-s intervals up to 60 s after initiation of the reaction with substrate. The cyclooxygenase activity was evaluated by interpolating the integrated CL signal (which is proportional to the amount of product formed) on a calibration curve obtained by measuring in the same analytical session samples containing different amounts of cyclooxygenases. The inhibition graph for each inhibitor was constructed from data collected in three independent experiments. Data were analyzed by using the software package Prism 3.0 (Graph Pad, San Diego, CA) and the results were expressed as IC_{50} ± SEM (mol/l).

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