Inhibition of Leukocyte Functions by the Alkaloid Isaindigotone from *Isatis indigotica* and Some New Synthetic Derivatives

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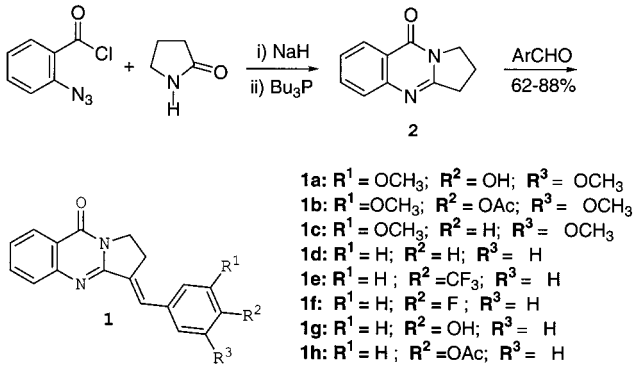
The alkaloid isaindigotone (**1a)** and seven derivatives have been synthesized to study their influence on several leukocyte functions and the generation of inflammatory mediators. Isaindigotone (**1a**) was found to be a scavenger of superoxide generated either by the hypoxanthine/xanthine oxidase system or stimulated human neutrophils. Isaindigotone (**1a**) and its acetylated derivative (**1b**) also inhibited 5-lipoxygenase activity and leukotriene B_4 production in these cells, whereas none of the compounds affected degranulation. In RAW 264.7 macrophages stimulated with lipopolysaccharide, synthetic derivatives exerted higher inhibitory effects on prostaglandin E_2 (PGE_2) and nitric oxide (NO) generation when compared with (**1a**). The presence of an acetoxyl group at C-4′ favors the inhibition of NO and PGE_2 production, whereas the fluoro substituent at C-4′ or the absence of substituents on the aromatic ring of the benzylidene unit improves the inhibition of PGE_2. Thus, this series of compounds can attenuate the production of mediators relevant to the inflammatory response.

Isatis indigotica Fort is a biennial herbaceous plant distributed widely in Chanjiang River valley. The purified extract of its root, named "Ban-Lan-Gen" in Chinese, is popularly used in clinical practice for treatment of influenza, epidemic hepatitis, epidemic encephalitis B, carbuncle, and erysipelas.[1] The root ethanol extract exhibits anti-endotoxic activity in the limulus amebocyte assay and is a source of the new alkaloid isaindigotone (**1a**) isolated from the chloroform/butanol fraction.[2] Recently, we have described a synthetic procedure for the preparation of this natural product,[3] which in preliminary experiments inhibited the leukocyte respiratory burst. We have prepared a series of seven new synthetic derivatives (**1b**−**1h**) to investigate their pharmacological activity on leukocyte functions relevant to inflammatory conditions and to examine structure−activity relationships.

Phagocytic cells produce a wide range of mediators contributing to the inflammatory response. Particularly, activated polymorphonuclear leukocytes release reactive oxygen species, inflammatory leukotrienes (LT) and proteolytic lysosomal enzymes, which can directly induce tissue damage.[4] The rate-limiting enzyme for the synthesis of LTs is 5-lipoxygenase (5-LO), which catalyzes the oxidation of arachidonic acid and the formation of LTA_4, which in turn is converted by LTA_4 hydrolase into the potent phagocyte chemoattractant LTB_4.[5] Conversely, activated macrophages play a key role in inflammatory responses and release a variety of mediators, including eicosanoids[6] and nitric oxide (NO). Prostaglandins (PGs) can be produced by the activity of two enzymes, cyclo-oxygenase-1 (COX-1) and cyclo-oxygenase-2 (COX-2). The first activity is implicated mainly in homeostatic functions, while induction of COX-2 by inflammatory stimuli leads to PG overproduction implicated in the pathophysiology of inflammatory diseases.[7] NO is a potent vasodilator that facilitates leukocyte migration[8] and formation of edema, as well as leukocyte activity and cytokine production.[9] In addition, NO can also react with superoxide anion to form peroxynitrite, a potent oxidizing molecule that contribute to tissue injury during inflammatory responses.[10]

Scheme 1



The aim of this work was to study the effects of isaindigotone (**1a**) and seven new synthetic derivatives on human neutrophil functions such as oxygen-derived species and LTB_4 generation in addition to the production of PGE_2 and NO in mouse macrophages.

Results and Discussion

The strategy employed to prepare 3-arylidenepyrrolo[2,1-*b*]quinazoline-9-ones (**1b**−**1h**) was based on the Claisen−Schmidt condensation between deoxyvasicinone (**2**) (available in two steps and 74% overall yield from *o*-azidobenzoyl chloride and pyrrolidone) and the appropriate aromatic aldehyde (Scheme 1).[3] The reactions were carried out in acetic anhydride as solvent at reflux temperature, and the products were obtained as crystalline solids after chromatographic separation in yields ranging from 62 to 88%. The products were characterized by spectroscopic methods, and the configuration of the new C−C double bond formed was deduced by ^{1}H NMR spectroscopy.

As shown in Table 1, isaindigotone (**1a**) inhibited the generation of oxygen-derived species by human neutrophils stimulated with 12-*O*-tetradecanoyl phorbol 13-acetate

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Table 1. Effect of Isaindigotone (**1a**) and Derivatives on Superoxide Production, Elastase Activity (Measured as *p*-Nitrophenol Release), and LTB_4 Generation in Human Neutrophils[a]

	superoxide (C.L./min/10^6 cells)	*p*-nitrophenol (nmol/mL)	LTB_4 (ng/mL)
basal	398.9 ± 1.1[b]	3.8 ± 0.4[b]	0.4 ± 0.1[b]
control	4736.3 ± 253.3	20.4 ± 0.5	60.7 ± 2.9
1a	570.6 ± 56.9[b]	19.3 ± 0.5	4.2 ± 0.6[b]
1b	2486.9 ± 42.9[b]	18.6 ± 0.6	13.5 ± 1.7[b]
1c	4150.7 ± 66.1	16.8 ± 0.7	58.4 ± 1.1
1d	2960.4 ± 73.6[b]	19.1 ± 0.6	61.6 ± 1.1
1e	4101.1 ± 174.8	19.3 ± 0.4	57.9 ± 1.6
1f	3221.7 ± 111.0[b]	19.7 ± 0.3	58.9 ± 1.3
1g	3269.2 ± 27.0[b]	16.9 ± 1.1	55.9 ± 3.4
1h	2636.4 ± 217.3[b]	19.2 ± 1.4	59.8 ± 3.0
fraxetin	401.1 ± 2.2[b]	N.D.	N.D.
ZM 230,487	N.D.	N.D.	4.5 ± 0.9[b]

[a] Data are mean ± SEM. [b] ($n = 9-12$). $P < 0.01$ with respect to the control group. N.D., not determined. Compounds were assayed at 10 μM. C.L.= chemiluminescence units. Basal = nonstimulated cells. Control = stimulated cells incubated with vehicle. Groups treated with test compounds were stimulated in the same conditions as the control group.

(TPA) in a concentration-dependent manner, with IC_{50} and 95% confidence limits of 41.8 (28.1−50.1) nM, while **1b**−**1h** exerted only a weak inhibitory effect at 10 μM. In addition, isaindogotone (**1a**) exerted a potent scavenging effect on superoxide generated by the system hypoxanthine/xanthine oxidase, with an IC_{50} of 42.2 (26.3−64.5) nM without affecting enzyme activity (data not shown). In this superoxide generating system, synthetic derivatives did not show any influence (data not shown). These results suggest that scavenging activity is related to the presence of the 4′-hydroxyl, 3′,5′-dimethoxyl groups in isaindigotone (**1a**).

We have also assayed the effects of these compounds on LTB_4 synthesis and degranulation of human neutrophils. Isaindigotone (**1a**) and, to a lesser extent, its acetylated derivative (**1b**) significantly inhibited at 10 μM LTB_4 generation in stimulated human neutrophils (Table 1). This effect is due to inhibition of 5-LO activity, since in high-speed supernatants from human neutrophils, both compounds reduced LTB_4 synthesis in a concentration-dependent manner, with IC_{50} values of 0.04 (0.02−0.06) μM and 0.60 (0.20−1.50) μM, respectively. Thus, isaindigotone (**1a**) exhibited a potency similar to that of the reference compound ZM 230,487 (a selective inhibitor of 5-LO), which showed an IC_{50} = 0.09 (0.06−0.10) μM. The free radical scavenging properties of this alkaloid may participate in its inhibitory effects on this enzyme, as redox activity is one of the mechanisms reported for 5-LO inhibition.[11]

In contrast, none of the compounds tested affected degranulation, measured as elastase release, nor caused cellular toxicity at the concentrations used, as determined by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; data not shown).

Incubation of RAW 264.7 macrophages with lipopolysaccharide (LPS) for 20 h induces COX-2[12] and NO synthase (iNOS).[13] As indicated in Table 2, all compounds at 10 μM coincubated with LPS reduced PGE_2 and nitrite accumulation in culture medium, with some differences in activity. Compound **1f** was the most potent, followed by **1b**, **1d**, and **1h**, with IC_{50} values below 1 μM. As expected, nitrite production was strongly inhibited by the selective iNOS inhibitor 1400W, followed by **1h** and **1b** with IC_{50} values in the μM range. It can be deduced from the limited data presented that the presence of an acetoxyl group at C-4′ favors the inhibition of NO and PGE_2 production, whereas the −F group at C-4′ or the absence of substituents on the aromatic ring improves the inhibition of PGE_2.

This is the first report on the biological activity of isaindigotone (**1a**), and we have shown that this alkaloid is a scavenger of superoxide generated either by stimulated human neutrophils or the hypoxanthine/xanthine oxidase system, in addition to its 5-LO inhibitory activity in human neutrophils. We have also synthesized a series of derivatives that show lower inhibitory effects on oxygen-derived species when compared with **1a**, but a better activity to decrease NO and PGE_2 generation by cells relevant to the inflammatory process. Reduction of oxygen- and nitrogen-derived species as well as inhibition of eicosanoid synthesis could contribute to control inflammatory responses. Our results indicate that these compounds serve as valuable leads for the treatment of inflammatory disorders.

Experimental Section

General Experimental Procedures. All reagents were of commercial quality and were obtained from freshly opened containers. Solvents were dried and purified by conventional methods prior to use. Preparative column chromatography: Merck silica gel 60, particle size 0.040−0.063 mm (230−400 mesh, flash). Analytical TLC: silica gel 60 F_{254} plates, Merck, Darmstadt. All melting points were determined on a Kofler hot-plate melting point apparatus and are uncorrected. IR spectra were obtained as Nujol emulsions or films on a Nicolet Impact 400 spectrophotometer. NMR spectra were recorded on a Brucker AC-200 (200 MHz) or a Varian Unity 300 (300 MHz). Mass spectra were recorded on a Hewlett-Packard 5993C spectrometer or a Fisons Autospec 500 VG. Microanalyses were performed on a Perkin-Elmer 240 C instrument.

Table 2. Effect of Isaindigotone (**1a**) and Derivatives on Nitrite and PGE_2 Accumulation in 20 h Lipopolysaccharide-Stimulated RAW 264.7 Macrophages

	[a]nitrite (ng/mL)	[b]IC_{50}	[a]PGE_2 (ng/mL)	[b]IC_{50}
basal	11.4 ± 2.8[c]		3.7 ± 0.4[c]	
control	613.3 ± 41.5		25.1 ± 1.9	
1a	459.3 ± 15.8[c]	>10 μM	20.3 ± 0.4[c]	>10 μM
1b	143.4 ± 10.3[c]	4.2 (1.9−8.9) μM	6.7 ± 0.5[c]	0.2 (0.1−0.4) μM
1c	437.2 ± 10.7[c]	>10 μM	14.8 ± 0.9[c]	>10 μM
1d	337.1 ± 21.8[c]	>10 μM	4.8 ± 0.3[c]	0.2 (0.4−0.3) μM
1e	448.0 ± 9.2[c]	>10 μM	15.6 ± 0.8[c]	>10 μM
1f	438.0 ± 15.0[c]	>10 μM	5.7 ± 0.4[c]	80 (30−160) nM
1g	429.8 ± 23.8[c]	>10 μM	15.4 ± 0.7[c]	>10 μM
1h	151.2 ± 15.6[c]	1.8 (1.5−2.1) μM	9.9 ± 0.5[c]	0.7 (0.3−1.1) μM
1400W	11.6 ± 0.2[c]	0.3 (0.2−0.5) μM	N. D.	>10 μM
NS 398	N.D.	>10 μM	3.7 ± 0.0[c]	2.1 (1.0−3.5) nM

[a] Compounds were assayed at 10 μM. Data show mean ± SEM ($n = 9-12$). [b] Values represent the concentration required to produce 50% inhibition of the response, along with the 95% confidence limits. [c] $P < 0.01$ with respect to the control group. N.D., not determined. Basal = not stimulated cells. Control = stimulated cells incubated with vehicle. Groups treated with test compounds were stimulated in the same conditions as the control group.

General Procedure for the Preparation of 3-Arylidene-1,2-dihydropyrrolo[2,1-*b*]quinazoline-9-ones (1). A mixture of deoxyvasicinone (**2**) (200 mg, 1.07 mmol), the appropriate aromatic aldehyde (8.56 mmol), and Ac_2O (10 mL) was heated at reflux temperature for 24 h. After cooling, the solvent was removed under reduced pressure, and the crude product was purified by column chromatography on silica gel using EtOAc/hexane (2:1) as eluent to give **1**.

Isaindigotone (1a). It was prepared in 64% yield,[3] and it shows spectroscopic data identical to those reported for the natural product.[2]

3-(4′-Acetoxy-3′,5′-dimethoxy)benzylidene-1,2-dihydropyrrolo[2,1-*b*]quinazoline-9-one (1b): yield 63%; mp 276−280 °C; IR (Nujol) ν_{max} 1767, 1680, 1594, 1133, 775, 688 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$/TMS) δ 2.35 (3H, s), 3.14 (2H, t, J = 7.0 Hz), 3.83 (6H, s), 4.20 (2H, t, J = 7.0 Hz), 6.72 (2H, s), 7.32−7.37 (1H, m), 7.54−7.65 (3H, m), 8.18 (1H, d, J = 8.1 Hz); ^{13}C NMR (50 MHz, $CDCl_3$/TMS) δ 20.3 (CH_3), 25.1 (CH_2), 43.9, (CH_2), 56.0 (CH_3), 106.3 (CH), 120.7 (q), 126.0 (CH), 126.1 (CH), 127.0 (CH), 129.0 (q), 130.0 (CH), 131.4 (q), 133.5 (q), 134.0 (CH), 149.4 (q), 152.1 (CH), 155.1 (q), 160.9 (C═O), 168.4 (C═O); EIMS (70 eV), *m*/*z* 392 [M$^+$] (12), 350 (100), 335 (15), 319 (11), 275 (8); *anal*. C, 67.20%; H 5.29%; N 7.32%, calcd for $C_{22}H_{20}N_2O_5$, C 67.34%; H 5.14%; N 7.14%.

3-(3′,5′-Dimethoxy)benzylidene-1,2-dihydropyrrolo-[2,1-*b*]quinazoline-9-one (1c): yield 67%; mp 191−192 °C; IR (Nujol) ν_{max} 1679, 1583, 1128, 773 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$/TMS) δ 3.19 (2H, t, J = 7.2 Hz), 3.78 (6H, s), 4.19 (2H, t, J = 7.2 Hz), 6.43 (1 H, t, J = 2.1 Hz), 6.63−6.64 (2H, m), 7.33−7.42 (1H, m), 7.69−7.70 (3H, m), 8.22 (1 H, d, J = 7.8 Hz); ^{13}C NMR (50 MHz, $CDCl_3$/TMS) δ 25.4 (CH_2), 43.9 (CH_2), 55.3 (CH_3), 100.9 (CH), 107.8 (CH), 120.7 (q), 126.0 (CH), 126.2 (CH), 127.1 (CH), 130.4 (CH), 132.0 (q), 134.0 (CH), 137.0 (CH), 149.5 (q), 155.3 (q), 160.8 (q), 161.0 (C═O); EIMS (70 eV), *m*/*z* 334 [M$^+$] (85), 333 (100), 319 (90), 303 (55), 275 (12), 163 (14); *anal*. C 71.60%; H 5.31%; N 8.29%, calcd for $C_{20}H_{18}N_2O_3$, C 71.84%; H 5.43%; N 8.38%.

3-Benzylidene-1,2-dihydropyrrolo[2,1-*b*]quinazoline-9-one (1d): yield 68%; mp 175−176 °C; IR (Nujol) ν_{max} 1680, 1590, 1345, 769, 688 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$/TMS) δ 3.26 (2H, t, J = 6.8 Hz), 4.26 (2H, t, J = 6.8 Hz), 7.35−7.48 (4H, m), 7.53 (2H, d, J = 8.0 Hz), 7.71−7.74 (2H, m), 7.83 (1H, t, J = 2.0 Hz), 8.27 (1H, d, J = 8.0 Hz); ^{13}C NMR (50 MHz, $CDCl_3$/TMS): δ 25.4 (CH_2), 43.9 (CH_2), 120.8 (q), 126.0 (CH), 126.3 (CH), 127.2 (CH), 128.7 (CH), 128.9 (CH), 129.7 (CH), 130.6 (CH), 131.5 (q), 134.1 (CH), 135.4 (q), 149.6 (q), 155.4 (q), 161.1 (C═O); EIMS (70 eV), *m*/*z* 274 [M$^+$] (100), 245 (13), 228 (19), 185 (18), 128 (23), 115 (51); *anal*. C 78.60%; H 4.98%; N 10.30%, calcd for $C_{18}H_{12}N_3O$, C 78.81%; H 5.14%; N 10.21%.

3-(4′-Trifluoromethyl)benzylidenepyrrolo[2,1-*b*]quinazoline-9-one (1e): yield 62%; mp 194−195 °C; IR (Nujol) ν_{max} 1679, 1616, 1594, 1078, 828, 769 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$/TMS) δ 3.22 (2H, t, J = 6.9 Hz), 4.23 (2H, t, J = 6.9 Hz), 7.32−7.41 (4H, m), 7.64−7.68 (2H, m), 7.77 (1H, t, J = 2.7 Hz), 8.18 (1H, d, J = 7.8 Hz); ^{13}C NMR (50 MHz, $CDCl_3$/TMS) δ 25.5 (CH_2), 43.9 (CH_2), 120.9 (q), 123.4 (q, $^1J_{CF}$ = 271 Hz), 125.7 (q, $^3J_{CF}$ = 4 Hz), 126.4 (CH), 127.3 (CH), 128.8 (CH), 129.7 (CH), 131.0 (q), 131.7 (q), 134.3 (CH), 135.5 (q, $^2J_{CF}$ = 32.2 Hz), 149.4 (q), 154.9 (q), 161.0 (C═O); EIMS (70 eV), *m*/*z* 342 [M$^+$] (24), 341 (100), 273 (23), 183 (19), 119 (33), 91 (35); *anal*. C 66.50%; H 3.77%; N 8.32%, calcd for $C_{19}H_{13}F_3N_2O$, C 66.67%; H 3.83%; N 8.18%.

3-(4′-Fluoro)benzylidenepyrrolo[2,1-*b*]quinazoline-9-one (1f): yield 73%; mp 201−203 °C; IR (Nujol) ν_{max} 1673, 1589, 1158, 834, 772 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$/TMS) δ 3.19 (2H, t, J = 6.6 Hz), 4.23 (2H, td, J = 6.6 Hz, J = 3.0 Hz), 7.07−7.13 (2H, m), 7.36−7.41 (1H, m), 7.42−7.52 (2H, m), 7.75 (1H, t, J = 3.0 Hz), 8.22 (1H, d, J = 8.2 Hz); ^{13}C NMR (50 MHz, $CDCl_3$/TMS) δ 25.3 (CH_2), 43.9 (CH_2), 115.9 (CH $^2J_{CF}$ = 20.6 Hz), 120.8 (q), 126.1 (CH), 126.3 (CH) 127.2 (CH) 129.3 (CH), 131.0 (q), 131.5 (CH $^3J_{CF}$ = 8.4 Hz), 131.7 (q), 134.1 (CH), 149.6 (q), 155.3 (q), 161.4 (C═O), 162.7 (q, $^1J_{CF}$ = 249.7 Hz); EIMS (70 eV), *m*/*z* 292 [M$^+$] (15), 291 (100), 261 (27), 146 (47), 132 (57), 118 (47); *anal*. C 73.79%; H 4.60%; N 9.45%, calcd for $C_{18}H_{13}FN_2O$, C 73.96%, H 4.48%, N, 9.58%.

3-(4′-Hydroxy)benzylidene-1,2-dihydropyrrolo[2,1-*b*]-quinazoline-9-one (1 g): yield 88%; mp 272−275 °C (dec); IR (Nujol) ν_{max} 3335, 1650, 1576, 842, 774 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$/TMS) δ 3.08 (2H, t, J = 6.6 Hz), 4.05 (2H, t, J = 6.6 Hz), 6.77 (2H, d, J = 8.4 Hz), 7.32 (1H, td, J = 8.1 Hz, J = 1.2 Hz), 7.40 (2H, d, J = 8.7 Hz), 7.53−7.58 (2H, m), 7.66 (1H, td, J = 8.1 Hz, J = 1.2 Hz), 7.99 (1H, dd, J = 8.1 Hz, J = 1.2 Hz), 9.89 (1H, s); ^{13}C NMR (50 MHz, $CDCl_3$/TMS) δ 24.9 (CH_2), 46.3 (CH_2), 115.8 (CH), 120.3 (q), 125.6 (CH), 125.7 (CH), 126.5 (q), 126.8 (CH), 128.7 (q), 129.3 (CH), 131.7 (CH), 134.0 (CH), 149.5 (q), 156.1 (q), 159.4 (q), 160.2 (C═O); EIMS (70 eV), *m*/*z* 290 [M$^+$] (73), 289 (100), 261 (12), 146 (16), 119 (20); *anal*. C 74.22%; H 4.71%; N 9.55%, calcd for $C_{18}H_{14}N_2O_2$, C 74.47%; H 4.86%; N 9.65%.

3-(4′-Acetoxy)benzylidene-1,2-dihydropyrrolo[2,1-*b*]-quinazoline-9-one (1h): yield 88%; mp 156−158 °C (dec); IR (Nujol) ν_{max} 1746, 1676, 1589, 1230, 926, 776 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$/TMS) δ 2.32 (3H, s), 3.25 (2H, t, J = 6.6 Hz), 4.27 (2H, t, J = 6.6 Hz), 7.17 (2H, d, J = 8.7 Hz), 7.40−7.42 (1H, m), 7.56 (2H, d, J = 8.7 Hz), 7.72−7.73 (2H, m,), 7.8 (1H, t, J = 2.4 Hz), 8.26−8.29 (1H, dd, J = 8.1 Hz, J = 1.2 Hz); ^{13}C NMR (50 MHz, $CDCl_3$/TMS) δ 21.1 (CH_2), 25.3 (CH_2), 43.9 (CH_2), 120.8 (q), 122.0 (CH), 126.1 (CH), 126.3 (CH), 127.2 (CH), 129.5 (CH), 130.8 (CH), 131.5 (q), 133.1 (q), 134.1 (CH), 149.5 (q), 150.8 (q), 155.3 (q), 161.1 (C═O), 169.1 (C═O); EIMS (70 eV), *m*/*z* 332 [M$^+$] (54), 289 (100), 261 (18), 146 (16), 119 (18); *anal*. C 72.33%, H 4.80%, N 8.67%, calcd for $C_{20}H_{16}N_2O_3$, C 72.28%, H 4.85%, N 8.43%.

Human Neutrophil Functions and Scavenging Activity. Human neutrophils were obtained as previously described.[14] Cells (2.5 × 10^6 cells/mL) were incubated with luminol (40 μM) and stimulated with TPA (1 μM) for 7 min. Superoxide anions were also generated by the hypoxanthine/xanthine oxidase system.[15] To measure degranulation, neutrophils were stimulated with cytochalasin B (10 μM) and *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (10 nM) for 10 min. Elastase activity in supernatants was estimated by *p*-nitrophenol release.[16] LTB_4 was determined by radioimmunoassay after stimulation of neutrophils (5 × 10^6/mL) with calcium ionophore A23187 (1 μM) for 10 min at 37 °C. The reduction of MTT was used to determine possible cytotoxic effects of compounds at the concentrations used. High-speed (100,000*g*) supernatants from sonicated human neutrophils were obtained as previously described.[17] Aliquots (50 μg of protein/tube) were incubated with 5 μM arachidonic acid at 37 °C for 5 min, in the presence of test compounds or vehicle. The LTB_4 levels in supernatants were measured by radioimmunoassay as above.

Production of PGE_2 and Nitrite by Stimulated Macrophages. RAW 264.7 mouse macrophages (2 × 10^6/ mL) in DMEM supplemented with 10% foetal bovine serum were stimulated with *Escherichia coli* LPS (1 μg/mL) for 20 h in the presence of test compounds or vehicle (methanol, 1%, v/v). PGE_2 levels were assayed in culture supernatants by radioimmunoassay,[18] and nitrite concentration, as a reflection of NO released, was assayed fluorometrically by the method of Misko.[19] Aliquots of culture supernant (10 μL) were incubated with 2,3-diaminonaphthalene (0.05 mg/mL in 0.62 M HCl) for 10 min in the dark. The reaction was terminated with 5 μL of 2.8 N NaOH, and formation of 2,3-diaminonaphthotriazole was measured with excitation at 365 nm and emission at 450 nm. The amount of nitrite was obtained by extrapolation from a standard curve with sodium nitrite.

Statistical Analysis. Results are presented as mean ± SEM. Statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons. Inhibitory concentration 50% (IC_{50}) and 95% confidence limits were obtained by linear regression analysis.

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