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6-Dimethylamino 1H-Pyrazolo[3,4-*d*]pyrimidine Derivatives as New Inhibitors of Inflammatory Mediators in Intact Cells

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Abstract—The synthesis of 6-dimethylamino 1H-pyrazolo[3,4-*d*]pyrimidines substituted at positions 1 and 4, and their effects on murine macrophage and human neutrophil functions are described. Several compounds and especially **4b**–**6b** are potent inhibitors of PGE_2 generation in murine macrophages. This action is related to a direct effect on COX-2 activity without affecting the enzyme expression. Some of these compounds also inhibited COX-1 and COX-2 in human monocytes and **4b** showed selectivity for COX-2 inhibition.

Introduction

Neutrophils are the first line of host cellular defence but also contribute to propagation and maintenance of acute and chronic inflammation using several mechanisms. Activated neutrophils release oxygen species, inflammatory leukotrienes (LT) and some proteolytic lysosomal enzymes, such as elastase, which can directly induce tissue damage.[1]

Macrophages play an important role in immune and inflammatory responses leading to the production of a number of mediators including cytokines, nitrogen species and prostanoids.[2] Prostaglandins (PGs) regulate a great number of physiological processes and are synthesized by two cyclooxygenase (COX) isoforms. COX-1 synthesizes different prostanoids involved in homeostatic functions, whereas COX-2 whose expression is restricted under basal conditions, is upregulated by inflammatory stimuli resulting in increased PGE_2 production,[3] which has been suggested to play an important role in the pathophysiology of inflammation and arthritis.[4,5]

In a recent paper,[6] we reported that certain isoxazolopyrimidines are potent inhibitors of nitrite and PGE_2 production in endotoxin-stimulated macrophages, which can be related to a reduced expression of iNOS and COX-2 in some cases. These products were also found active on nitrite levels by oral administration, in the mouse air pouch model of inflammation.

In the search for new drugs active on NO production in different inflammatory and NO-related pathologies, we reasoned that the modulation of the isoxazolpyrimidine system by substitution of the isoxazole by a pyrazole ring, should be investigated. After some preliminary screening, those expectations were found to be correct and we describe in this study, the synthesis of a series of 6-dimethylamino 1H-pyrazolo[3,4-*d*]pyrimidine derivatives (Scheme 1), and their effects on murine macrophage and human leukocyte functions.

Chemistry

For the preparation of the title compounds, we used a synthetic procedure based on the use of β-enaminonitriles[7] and phosgeniminium chloride that allows the introduction of two substituents R_1 and R_2 at positions 1 and 4, respectively, of the heterocycle.[8] Thus, reaction of *ortho*-aminonitriles **1a** (5-amino-4-cyano-1-phenylpyrazole) and **1b** (5-amino-1-*tert*-butyl-4-cyanopyrazole) with phosgeniminium salt followed by treatment

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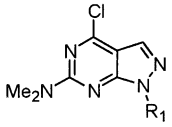
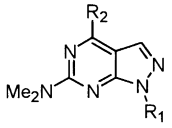
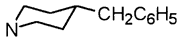
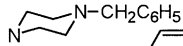
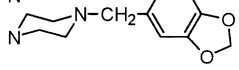


2a $R_1 = C_6H_5$
2b $R_1 = C(CH_3)_3$



3a-9a $R_1 = C_6H_5$
3b-9b $R_1 = C(CH_3)_3$
3c-9c $R_1 = H$

Compound	R_2	Compound	R_2
3	OCH_3	**7**	
4	$NHCH_2C_6H_5$	**8**	
5	$NHCH_2C_5H_4N$	**9**	
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Scheme 1.

with hydrogen chloride afforded the 4-chloro-pyrazolo[3,4-*d*]pyrimidines **2a** (4-chloro-6-dimethylamino-1-phenyl-1H-pyrazolo[3,4-*d*]pyrimidine) and **2b** (1-*tert*-butyl-4-chloro-6-dimethylamino-1H-pyrazolo[3,4-*d*]pyrimidine), that were converted into pyrazolopyrimidines **3a–9a** ($R_1 = Ph$), and **3b–9b** ($R_1 = tert$-butyl), by nucleophilic halide displacement with primary and secondary amines and methoxide ion (R_2 substituent). The pyrazolo[3,4-*d*]pyrimidines **3c–9c** ($R_1 = H$), were prepared by cleavage of the *tert*-butyl group of **3b–9b** with formic acid. All compounds gave satisfactory elemental analyses and spectral data (IR, ^1H NMR, ^{13}C NMR, and MS) that are consistent with the structures proposed. Some pyrazolo[3,4-*d*]pyrimidines mentioned in this paper have been recently reported as histamine release inhibitors in rat peritoneal mast cells.[9]

Results and Discussion

The 6-dimethylamino 1H-pyrazolo[3,4-*d*]pyrimidines were tested in several biological systems using human leukocytes and mouse peritoneal macrophages, but first, we checked that the 6-dimethylamino 1H-pyrazolo[3,4-*d*]pyrimidines investigated were not cytotoxic for either cells, as was demonstrated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (data not shown). Only the chloro substituted compound **2a** (4-chloro-6-dimethylamino-1-phenyl-1H-pyrazolo[3,4-*d*]pyrimidine) at 10 μM reduced cell viability by 25% in human neutrophils.

To assay if these compounds were able to inhibit some neutrophil functions, we tested them on chemiluminescence induced by 12-*O*-tetradecanoylphorbol 13 acetate (TPA), and found that none of these 6-dimethylamino 1H-pyrazolo[3,4-*d*]pyrimidines affected superoxide generation by human neutrophils (Table 1). In these cells, the chloro substituted **2b** (1-*tert*-butyl-4-chloro-6-dimethylamino-1H-pyrazolo[3,4-*d*]pyrimidine) was found to be a strong inhibitor of elastase release induced by cytochalasin B plus *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) with an IC_{50} value of 1.30 (0.91–1.62) μM, without affecting elastase activity. Human neutrophil degranulation was also inhibited to a lesser extent by compounds **5a**, **3b**, **4b**, **5b**, **6b**, **9b**, **3c**, **4c** and **5c** (Table 1).

Table 1. Biological effects of compounds on human neutrophils

	Chemiluminescence (Units/min/10^6 cells)	Elastase release (nmol *p*-nitrophenol/mL)	LTB_4 (ng/mL)
Basal	162.6±2.8	4.8±0.7	0.2±0.02
Control	2281.2±128.5	24.0±0.8	51.2±2.9
2a	2056.8±75.6	24.0±0.4	31.8±3.7**
3a	2243.6±47.8	23.6±0.8	51.6±0.9
4a	2317.2±210.0	23.3±1.8	48.0±2.2
5a	2010.4±158.8	18.0±14*	45.8±3.1
2b	2181.2±50.0	10.9±0.3**	29.7±2.5**
3b	2255.2±62.4	19.2±0.6**	47.6±3.9
4b	2230.4±123.9	19.4±1.0**	44.2±3.6
5b	2148.4±49.9	11.3±1.3**	37.2±3.2**
6b	2294.4±115.6	16.4±1.4*	33.8±2.4**
7b	2080.8±107.0	21.0±1.5	51.1±2.6
8b	2034.0±84.0	24.0±1.6	20.3±2.2**
9b	2374.4±50.0	18.2±1.0**	18.7±1.9**
3c	2120.4±125.2	18.6±0.6**	42.8±1.8
4c	2280.0±55.2	16.4±1.1**	37.7±1.1
5c	2003.2±55.8	19.0±0.8**	45.2±4.2
6c	2112.4±58.0	21.0±0.8	49.8±4.4

Compounds were assayed at 10 μM, except **2a** at 1 μM. Data shown are mean±SEM ($n = 6$-12). **$p < 0.01$; *$p < 0.05$.

After those results, we decided to investigate if our compounds were able to inhibit LTB_4 synthesis. Table 1 shows that in fact compounds **2a**, **2b**, **5b**, **6b**, **8b** and **9b** are good inhibitors of LTB_4 synthesis in stimulated human neutrophils. We calculated the IC_{50} for the most effective compounds **8b** and **9b**, with values of 2.74 (1.67–5.27) μM and 2.19 (1.77–5.62) μM, respectively.

It is well-known that stimulation of macrophages with bacterial lipopolysaccharide (LPS) causes expression of iNOS and COX-2 with the consequent generation of large quantities of NO and PGs.[10]

We determined the influence of this series of 6-dimethylamino 1H-pyrazolo[3,4-*d*]pyrimidines on the accumulation of nitrite and PGE_2, as an index of expression/activity. When the compounds were incubated with LPS for 20 h as inhibitors of NO formation, four compounds gave significant results although only one of them **2a**, reduced nitrite levels by 60% at 10 μM. In contrast, most of them were effective on the accumulation of PGE_2, with IC_{50} values in the micromolar and submicromolar range, as shown in Table 2.

This could be due to inhibition of either COX-2 induction or activity. To test this last possibility in intact cells, we first induced COX-2 by LPS stimulation for 20 h and then the cells were washed and incubated with test compounds for 2 h. Our results showed that the selective COX-2 activity inhibitor NS-398 potently inhibited PGE_2 generation both in the presence and absence of LPS. Most of the compounds tested exerted similar effects on both experimental systems, which suggests that they inhibited COX-2 activity only.

Table 2. Effects of compounds on mouse peritoneal macrophages stimulated with LPS

	20 h[a]	20 h[a]		20 h[b]	
	NO_2^- (ng/mL)	PGE_2 (ng/mL)	IC_{50} μM	PGE_2 (ng/mL)	IC_{50} μM
Basal	97.2±4.7	0.8±0.1		0.5±0.0	
Control	890.8±36.0	7.5±0.3		71±0.3	
2a	367.6±33.6**	0.7±0.6**	1.7 (1.3–2.3)	3.1±0.2**	5.0 (4.1–5.9)
3a	775.2±16.5*	1.2±0.1**	0.3 (0.3–0.4)	2.6±0.1**	3.9 (2.8–4.1)
4a	838.2±28.5	2.2±0.4**	0.8 (0.6–1.3)	3.3±0.3**	ND
5a	722.0±28.7*	1.8±0.2**	1.9 (1.4–2.7)	3.4±0.4**	ND
2b	889.9±63.1	2.8±0.3**	3.1 (2.2–4.5)	2.6±0.1**	ND
3b	899.3±32.4	1.2±0.2**	1.1 (0.8–1.3)	2.4±0.2**	3.3 (2.7–3.8)
4b	878.6±18.2	0.6±0.1**	23.5 (13.0–37.8) nM	2.0±0.2**	0.1 (0.1–0.2)
5b	772.0±21.5	0.7±0.0**	0.3 (0.2–0.4)	1.7±0.1**	1.1 (1.0–1.2)
6b	845.5±34.9	0.8±0.1**	0.4 (0.3–0.5)	1.8±0.2**	1.4 (1.0–1.8)
7b	921.2±41.0	7.5±0.4	ND	ND	ND
8b	892.3±23.0	7.8±0.4	ND	ND	ND
9b	874.8±21.6	7.1±0.4	ND	ND	ND
3c	799.6±31.8	7.4±0.3	ND	ND	ND
4c	758.0±27.6*	4.0±0.2**	ND	ND	ND
5c	882.1±16.7	4.1±0.2**	ND	4.9±0.2**	ND
6c	873.0±52.0	1.5±1.5**	2.1 (1.7–2.6)	3.2±0.3**	ND
NS-398	ND	0.7±0.1**	3.1 (1.3–5.4) nM	1.2±0.2**	14.0 (2.0–43.6) nM
1400W	113.0±4.5**	ND	ND	ND	ND

[a]Cells were estimulated with LPS for 20 h in either the presence or absence (control) of test compounds.
[b]After 20 h stimulation with LPS, cells were washed and incubated for 2 h with test compounds and arachidonic acid. Data shown are mean±SEM (n=6–12) of results obtained at 10 μM and IC_{50} values (with 95% confidence limits) for the most effective compounds. **p <0.01; *p <0.05. ND, not determined.

In order to confirm that the inhibition observed in cultured cells is dependent on a direct action on enzymatic activity, we studied the effect of the active pyrazolo[3,4-*d*]-pyrimidines on COX-2 expression by Western blot assay. For comparison, we used NS-398, a COX-2 inhibitor, and dexamethasone, an inhibitor of protein expression, which are very effective in reducing eicosanoid levels.

As expected, dexamethasone potently inhibited the expression of this enzyme, whereas when the cells were treated with NS-398 or pyrazolo[3,4-*d*]pyrimidines, protein expression was unaffected. Only for **3b** a weak inhibition of protein expression was observed (Fig. 1). Our results thus indicate that the inhibitory effects on PGE_2 production produced by the compounds of Scheme 1 are not due to a reduction in COX-2 expression.

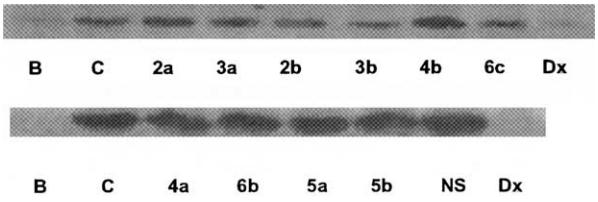

Figure 1. Effect of compounds (10 μM), NS-398 (10 μM) or dexamethasone (1 μM) on COX-2 protien expression in 20 h LPS-stimulated murine peritoneal macrophages.

To evaluate the structure–activity relationship in murine macrophages, the effect on activity of the different substituents in position 1 and 4 of the skeleton was studied. Comparative analysis of the data shown in Table 2 indicates some trends: The 6-dimethylamino 1H-pyrazolo[3,4-*d*]pyrimidines with a phenyl substituent at

Table 3. Inhibition of COX-1 and COX-2 activity in human monocytes at 1 μM

Compd	COX-1		COX-2	
	% Inhibition 1 μM	IC_{50} nM	% Inhibition 1 μM	IC_{50} nM
2a	31.8±2.4*	ND	61.5±4.1**	ND
3a	14.2±3.8	ND	61.3±3.3**	ND
4a	0.0±0.0	ND	39.9±5.1**	ND
5a	0.0±0.0	ND	31.9±4.6*	ND
2b	12.5±4.1	ND	34.7±6.7*	ND
3b	12.5±2.4	ND	60.5±4.7**	ND
4b	72.5±3.1**	59.6 (51.1–68.1)	97.8±1.1**	0.9 (0.3–2.0)
5b	55.7±4.0**	753.8 (527.5–1000.0)	74.9±1.3**	88.2 (56.2–136.4)
6b	2.6±2.0	ND	61.7±5.0**	ND
6c	13.4±5.2	ND	64.6±3.9**	ND
Ind	90.5±3.2**	6.2 (1.0–23.4)	88.5±5.0**	50.3 (20.4–100.0)
NS-398	87.0±5.0**	66.1 (15.6–197.7)	96.6±3.0**	0.5 (0.2–2.2)

Data show mean±SEM (n=6–12). *p <0.05 **p <0.01 compared with control values (8.8±0.5 ng/mL PGE_2 in COX-1 and 25.2±1.5 ng/mL PGE_2 in COX-2 activity). ND, not determined.

position 1 (**2a–5a**), were found to be good inhibitors of PGE_2 production with a reduction at 10 μM, of over 90% of the control level in most cases. Compounds **2b–6b** with *tert*-butyl substituent at that position, inhibited PGE_2 production also, showing a greater effect those compounds with an open-chain amine at C-4 (**4b** and **5b**). In fact a piperazine substituent at C-4 is associated with a total loss of activity (**8b** and **9b**). The absence of substituent at position 1 is related with a reduction of this effect (**3c–6c**). Compounds having a piperidine group at position 4 (**6b** and **6c**) are active on PGE_2 synthesis, but it seems that the presence of a substituent in this ring abolishes the activity (**7b**).

We considered of interest to study the effects of the most active compounds on the COX-1 and COX-2 activities in intact human cells. As we show in Table 3 all tested compounds were more effective as COX-2 inhibitors in human monocytes. We calculated the IC_{50} values against COX-1 and COX-2 for the strongest inhibitors, **4b** and **5b**, and we found that **4b** showed selectivity for COX-2 inhibition.

Experimental

General

All melting points were measured by using a Büchi 510 instrument and are given uncorrected. Ir spectra (potassium bromide) were recorded on a Perkin-Elmer 383 spectrophotometer. ^1H and ^{13}C NMR (200 and 50 MHz) were measured on a Bruker AC200F spectrometer. Chemical shifts are given on δ and using tetramethylsilane as internal standard. Mass spectra were obtained on a VG4 spectrometer. Microanalyses for C, H and N were performed by the Elemental Analyses General Service of the University of La Coruña. [5,6,8,11,12,14,15(*n*)-^3H]PGE_2 and [5,6,8,9,11,12,14,15(*n*)-^3H]leukotriene B_4 were from Amersham Iberica (Madrid, Spain). Antibody against leukotriene B_4 was provided by Zeneca-Pharmaceuticals, Macclesfield, Cheshire, UK. NS398 and 1400W were purchased from Cayman Chemicals (SPI-Bio, Massy, France). The rest of the reagents were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Compounds **2a–b**, **3a–c**, **4a–c**, **6a–c**, and **9a–c**, are known compounds and were prepared according to the published procedure.[9] Their spectroscopic and physical properties were coincident with those reported.

Preparation of 6-dimethylamino-1*H*-pyrazolo[3,4-*d*]pyrimidines 5a–b, 7a–b and 8a–b

General procedure. A solution of **2a** or **2b** (0.5 mmol) and the appropriate amine (2 mmol) in THF (5 mL) was refluxed until the starting material had disappeared (TLC) (2–15 h). The solid was filtered and recrystallized or was purified by flash chromatography as indicated.

4-(3′-Aminomethylpyridino)-6-dimethylamino-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidine (5a). Purified by flash chromatography using CH_2Cl_2 as eluent. Yield 97%; mp 196–198 °C. IR (cm^{-1}): ν = 1600, 1500, 1390, 1350, 960. ^1H NMR ($CDCl_3$): δ 3.22 (s, 6H, $N(CH_3)_2$); 4.80 (d, 2H, J = 5.90 Hz, $NHCH_2$); 5.76.79 (m, 1H, NH); 7.19.74 (m, 5H, C_6H_5); 7.80 (s, 1H, H-3); 8.52 (dd, 1H, J = 1.50, 4.90 Hz, Py); 8.63 (d, 1H, J = 1.96 Hz, Py). ^{13}C NMR ($CDCl_3$): δ 37.3; 42.1; 95.9; 120.3; 123.5; 125.1; 128.8; 132.0; 134.7; 135.4; 140.0; 148.7; 149.2; 156.3; 156.5; 161.7. MS (EI, *m*/*z*, %): 345 (M^+, 7), 69 (61); 111 (21); 97 (55); 85 (78); 83 (70); 71 (100); 69 (61). Anal. calcd $C_{19}H_{19}N_7$: C, 66.07; H, 5.54; N, 28.39. Found: C, 66.07; H, 5.61; N, 28.52.

4-(3′-Aminomethylpyridino)-6-dimethylamino-1-*t*-buthyl-1*H*-pyrazolo[3,4-*d*]pyrimidine (5b). Purified by flash chromatography using CH_2Cl_2–MeOH (50:1) as eluent. Yield 97%; mp 183–185 °C. IR: (cm^{-1}): ν = 1620; 1580; 1520, 1390. ^1H NMR ($CDCl_3$): δ 1.74 (s, 9H, $C(CH_3)_3$); 3.16 (s, 6H, $N(CH_3)_2$); 4.75 (d, 2H, J = 5.90, $NHCH_2$); 5.56 (s ancho, 1H, NH); 7.18.27 (m, 1H, Py); 7.56 (s, 1H, H-3); 7.67.72 (Py); 7.67.72 (m, 1H, Py); 8.48 (dd, 1H, J = 1.47, 4.88 Hz, Py); 8.60 (d, 1H, J = 1.95 Hz, Py). ^{13}C NMR ($CDCl_3$): δ 28.7; 37.2; 42.1; 58.9; 96.0; 123.5; 128.4; 135.0; 135.4; 148.5; 149.2; 156.0; 156.4; 160.5. MS (EI, *m*/*z*, %): 325 (M^+, 33); 269 (23); 97 (63); 92 (68); 85 (81); 83 (84); 71 (100). Anal. calcd $C_{17}H_{23}N_7$: C, 62.74; H, 7.12; N, 30.14. Found: C, 62.66; H, 7.15; N, 30.21.

4-(4′-Benzylpiperidino)-6-dimethylamino-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidine (7a). Purified by flash chromatography using CH_2Cl_2 as eluent. Yield 97%; mp 150–152 °C. IR (cm^{-1}): ν = 1595, 1495, 1390, 960, 780, 750. ^1H NMR ($CDCl_3$): δ 1.30–142 (m, 2H, C_5H_9N), 1.79.91 (m, 3H, C_5H_9N); 2.59 (d, 2H, J = 6.80 Hz, CH_2Ph); 2.98.12 (m, 2H) (C_5H_9N); 3.22 (s, 6H, $N(CH_3)_2$); 4.67.73 (m, 2H, C_5H_9N); 7.15.51 (m, 8H, C_6H_5); 7.89 (s, 1H, H-3); 8.29.34 (m, 2H, C_6H_5). ^{13}C NMR ($CDCl_3$): δ 31.8; 37.1; 38.3; 43.0; 45.8; 95.7; 120.7; 125.0; 126.0; 128.3; 128.7; 134.5; 140.0; 156.9; 161.2. MS (EI, *m*/*z*, %): 412 (M^+, 66); 111 (33); 97 (69); 85 (77); 71 (100). Anal. calcd $C_{25}H_{18}N_6$: C, 72.79; H, 6.84; N, 20.37. Found: C, 72.77; H, 7.25; N, 20.57.

4-(4′-Benzylpiperidino)-6-dimethylamino-1-*t*-butyl-*1H*-pyrazolo[3,4-*d*]pyrimidine (7b). Purified by flash chromatography using CH_2Cl_2–hexanes (7:3) as eluent. Yield 96%; mp 117–119 °C. IR (cm^{-1}): ν = 1620; 1545; 1405; 935. ^1H NMR ($CDCl_3$): δ 1.26.40 (m, 2H, C_5H_9N); 1.76 (s, 9H,$C(CH_3)_3$); 1.80–192 (m, 3H, C_5H_9N); 2.57 (d, 2H, J = 6.8 Hz, CH_2Ph); 2.92.07 (m, 2H, C_5H_9N); 3.18 (s, 6H, $N(CH_3)_2$), 4.46.71 (m, 2H, C_5H_9N); 7.14.35 (m, 5H, C_6H_5); 7.65 (s, 1H, H-3). ^{13}C NMR ($CDCl_3$), δ 28.6; 31.9; 37.1; 38.4; 43.1; 45.8; 58.8; 96.0: 125.9; 128.3; 129.1; 130.9; 140.1; 157.0; 160.1. MS (EI, *m*/*z*, %): 392 (M^+, 100), 336 (38); 245 (22); 162 (31); 91 (67); 71 (38). Anal. calcd $C_{23}H_{32}N_6$: C, 70.37; H, 8.22; N, 21.41. Found: C, 70.25; H, 816; N, 21.40.

4-(4′-Benzylpiperazino)-6-dimethylamino-1-phenyl-1*H*-pyrazolo[3.4-*d*]pyrimidine (8a). Purified by flash chromatography using CH_2Cl_2–AcOEt (4:1) as eluent. Yield 98%; mp: 160–162 °C. IR (cm^{-1}): ν = 1560, 1500; 1395; 965. ^1H NMR ($CDCl_3$): δ 2.58 (m, 4H, $C_4H_8N_2$); 3.22 (s, 6H, $N(CH_3)_2$); 3.59 (s, 2H, CH_2Ph); 3.92.30 (m, 4H,

$C_4H_8N_2$); 7.19.50 (m, 8H, C_6H_5); 7.88 (s, 1H, H-3); 8.28.32 (m, 2H, C_6H_5). ^{13}C NMR ($CDCl_3$): δ 37.1; 45.2; 58.2; 63.0; 95.7; 120.7; 125.0; 127.3; 128.3; 128.7; 129.2; 134.4; 137.7; 139.9; 157.1; 157.4. MS (EI, *m*/*z*, %): 413 (M^+, 21); 267 (100); 254 (40); 91 (79); 71 (36). Anal. calcd $C_{24}H_{27}N_7$: C, 69.71; H, 6.58; N, 23.71. Found: C, 69.51; H, 6.62; N, 23.70).

4-(4′-Benzylpiperazino)-6-dimethylamino-1-*t*-butyl-1*H*-pyrazolo[3.4-*d*]pyrimidine (8b). Purified by flash chromatography using CH_2Cl_2–AcOEt (9:1) as eluent. Yield: 98%. IR (cm^{-1}): ν = 1570; 1385; 990; 740. ^1H NMR ($CDCl_3$): δ 1.75 (s, 9H, $C(CH_3)_3$); 2.53.59 (m, 4H, $C_4H_8N_2$); 3.17 (s, 6H, $N(CH_3)_2$); 3.56 (s, 2H, CH_2Ph); 3.87.92 (m, 4H, $C_4H_8N_2$); 7.27.38 (m, 5H, C_6H_5), 7.63 (s, 1H, H-3). ^{13}C NMR ($CDCl_3$): δ 28.7; 37.1; 45.1; 52.8; 58.9; 63.1; 96.0: 127.2; 128.3; 129.2; 130.7; 137.7; 157.2; 160.0. MS (EI, *m*/*z*, %): 393 (M^+, 25); 247 (100); 234 (72); 191 (75); 162 (26); 97 (37); 91 (81), 83 (43); 71 (47). Anal. calcd $C_{22}H_{31}N_7$: C, 67.15; H, 7.94; N, 24.91. Found: C, 67.09; H, 7.66; N, 24.70.

Preparation of 6-dimethylamino-1*H*-pyrazolo[3,4-*d*] pyrimidines 3c–9c

General procedure. A solution of **3b–9b** (6 mmol) in formic acid (2.5 mL) was heated at 90 °C until the starting material had disappeared (TLC). The solvent was removed under reduced pressure and the residue was recrystallized or purified by flash chromatography as indicated to yield **3c–9c**.

4-(3′-Aminomethylpyridino)-6-dimethylamino-1*H*-pyrazolo [3,4-*d*]pyrimidine (5c). Purified by flash chromatography using CH_2Cl_2/MeOH (17:3) as eluent. Yield 84%; mp 266–268 °C. IR (cm^{-1}): ν = 2360; 1600; 1520; 1350. ^1H NMR ($CDCl_3$). δ 3.06 (s, 6H, $N(CH_3)_3$; 4.64 (d, 2H, *J* = 5.80 Hz, $NHCH_2$; 7.29.36 (m, 1H, Py); 7.27.75 (m, 1H, Py); 7.80 (s, 1H, H-3); 8.32.44 (m, 2H, Py); 12.53 (s, 1H, NH). EM (EI, *m*/*z*, %): 269 (M^+, 22); 149 (37); 105 (56); 85 (50); 71 (100). Anal. calcd $C_{13}H_{15}N_7$: C, 57.97; H, 5.61; N, 36.41. Found: C, 58.03; H, 5.54; N, 36,63.

4-(4′-Benzylpiperidino-6-dimethylamino-1*H*-pyrazolo[3.4-*d*] pyrimidine (7c). Purified by flash chromatography using AcOEt as eluent. Yield 93%; mp 191–193 °C. IR (cm^{-1}): ν = 1610; 1555; 1400, 940. ^1H NMR ($CDCl_3$). δ 1.23.40 (m, 2H, C_5H_9N); 1.78.90 (m, 3H, C_5H_9N); 2.59 (d, 2H, *J* = 6.80 Hz, CH_2Ph); 2.91.14 (m, 2H, C_5H_9N); 3.26 (s, 6H, $N(CH_3)_2$); 4.67.73 (m, 2H, C_5H_9N); 7.14.35 (m, 5H, C_6H_5); 7.30 (s, 1H, NH); 7.77 (s, 1H, H-3). ^{13}C NMR ($CDCl_3$): δ 31.9; 37.7; 38.3; 43.0; 45.8; 94.4; 126.0; 128.3; 129.1; 134.0; 135.4; 140.0; 156.6; 158.8; 160.8. MS (EI, *m*/*z*, %): 336 (M^+, 100), 245 (44); 97 (67); 85 (71); 71 (90). Anal. calcd $C_{19}H_{24}N_6$: C, 67.82; H, 7.19; N, 24.99. Found: C, 67.89; H, 7.27; N: 24.87.

4-(4′-Benzylpiperazino)-6-dimethylamino-1H-pyrazolo [3,4-*d*]pyrimidine (8c). Purified by flash chromatography using CH2Cl2/MeOH (95.5:0.5) as eluent. Yield 95%; mp 197–199 °C. IR (cm^{-1}): ν = 1610: 1560; 1505; 1400; 940. ^1H NMR ($CDCl_3$): 2.56.62 (m, 4H, $C_4H_8N_2$); 3.25 (s, 6H, $N(CH_3)_2$); 3.58.58 (s, 2H, CH_2Ph); 3.91.96 (m, 4H, $C_4H_8N_2$); 7.23.46 (m, 5H, C_6H_5); 7.38 (s, 1H, NH); 7.78 (s, 1H, H-3). MS (EI, *m*/*z*, %): 337 (M^+, 22); 191 (100); 91 (52); 71 (38). Anal. calcd $C_{18}H_{23}N_7$: C, 59.83; H, 6.87; N, 29.06. Found: C, 60.18; H, 6.50; N, 28.99.

Preparation of human neutrophils. Venous blood leukocytes from healthy volunteers, were obtained and purified as previously described.[11] Viability was greater than 95% according to trypan blue exclusion test. The mitochondrial-dependent reduction of MTT to formazan was used to assess the possible cytotoxic effects of test compounds.[12]

Chemiluminescence. Neutrophils (2.5×10^6 cells/mL) were incubated with luminol (40 μM) and stimulated with TPA (1 μM). Chemiluminescence was recorded with a Microbeta trilux counter (Wallac, Turku, Finland).

Elastase release by human neutrophils. Neutrophils (2.5×10^6 cells/mL) were preincubated with test compound or vehicle for 5 min and then stimulated with cytochalasin B (10 μM) and fMLP (10 nM) for 10 min. Elastase release was measured in supernatants.[13] Possible direct inhibitory effects on elastase activity were also assessed.[14]

LTB_4 release. Neutrophils (5×10^6 cells/mL) were preincubated with test compound or vehicle for 5 min and then stimulated with calcium ionophore A23187 (1 μM) for 10 min at 37 °C. Leukotriene B_4 levels in supernatants were measured by radioimmunoassay.[15]

Mouse macrophages culture. Highly purified peritoneal macrophages were harvested by peritoneal lavage 4 days after ip injection of 1 mL of 10% thioglycollate broth and were resuspended in culture medium (120 mM NaCl, 4.7 mM KCl, 1.2 mM Ca. Cl_2–$7H_2O$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES, 1 mM arginine and 10 mM glucose) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and incubated at a concentration of 2×10^6 cells/mL at 37 °C for 2 h. The adherent cells were incubated with *Escherichia coli* LPS (10 μg/mL) at 37 °C for 20 h in the presence of test compounds or vehicle. Nitrite and PGE_2 levels were assayed in culture supernatants by a fluorometric method[16] and by radioimmunoassay, respectively. Cytotoxicity was evaluated as above.

COX-2 activity in intact cells. Murine peritoneal macrophages incubated with LPS (10 μg/mL) at 37 °C for 20 h were washed and Hank's buffer supplemented with arachidonic acid (10 μM) was added for a 2 h incubation with test compound to determine their effects on COX-2 activity. Supernatants were collected for the measurement of PGE_2 accumulation for the last 2 h by radioimmunoassay as above.

Western blot analysis. Inducible COX-2 protein expression was studied from LPS-stimulated peritoneal macrophages. Equal amounts of protein were separated by

12.5% sodium dodecyl sulphate-polycrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes for 90 min at 125 mA. Membranes were blocked in phosphate buffer saline (0.002 M, pH 7.0)-Tween-20 (0.1%) containing 3% w/v unfatted milk. For inducible COX-2, membranes were incubated with specific anti-COX-2 polyclonal antiserum (1/1000) and then they were incubated with peroxidase-conjugated goat anti-rabbit Immunoglobulin G (1/20,000). The immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL) Amersham Iberica (Madrid, Spain).

Preparation of human monocytes. The mononuclear cell interphase was obtained from human leukocytes by Ficoll-Paque density gradient centrifugation. Cells were resuspended in RPMI 1640 supplemented with 10% fetal bovine serum. 10^7 cells/mL were incubated in Petri dishes and some plates contained aspirin (300 μM). Monocytes, purified by discarding non-adherent cells after 2-h incubation, were resuspended at a concentration of 2×10^6/mL. Cell viability was greater than 95% by the tripan blue-exclusion test.

COX-1/COX-2 activity in intact human monocytes. To assess the effects of the compounds on COX-2 activity, aspirin-treated monocytes were incubated with LPS (1 μg/mL) for 24 h to induce COX. Cultured medium was changed and supplemented with AA (10 μM) for a 2 h incubation with test compounds or vehicle. In pararel experiments, aspirin-untreated monocytes were incubated for 2 h with AA (10 μM) and test compounds or vehicle, to assess the effects of compounds on COX-1 activity. After the incubation period, supernatants were collected for the measurement of PGE_2 levels by radioimmunoassay.

Statistical analysis. The results are presented as mean ± SEM; *n* represents the number of experiments. Inhibitory concentration 50% (IC_{50}) values were calculated from at least four significant concentrations ($n = 6$). The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons.

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