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N-Pyridinyl(alkyl)polyhalogenobenzamides Acting as TNF-α Production Inhibitors

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Abstract

A series of *N*-pyridinyl(methyl)fluorobenzamides issued from 2,4-dimethyl-6-aminopyridine and 3-aminomethylpyridine were synthesized and evaluated as inhibitors of TNF-α production. Although less active than the corresponding phthalimides, several pentafluorobenzamides exhibited significant activity at 10 μM. *N*-(4,6-dimethylpyridin-2-yl)pentafluorobenzamide was selected for a preliminary in-vivo assay. Although its inhibitory activity against carrageenan-induced rat paw oedema was moderate, it induced a significant reduction in ear thickness in the PMA-induced mouse ear-swelling test ($49 \pm 6\%$ inhibition after a dose of $0{\cdot}1\ \text{mM kg}^{-1}$, p.o.).

Tumour necrosis factor-α (TNF-α) is produced by immunologically competent cells during the host response to infection, injury or inflammation, and mediates metabolic and biochemical changes in responding cells when present at low concentrations (Tracey & Cerami 1993; Eigler et al 1997). However, overproduction of TNF-α can cause a destructive immune response associated with many inflammatory diseases including rheumatoid arthritis, osteoarthritis, multiple sclerosis, lupus, asthma, inflammatory bowel disease (Badger & Adams 1998), septic shock (Beutler 1992) or brain injury and inflammation following stroke (Trembly & Slikker 1995).

Non-steroidal anti-inflammatory drugs (NSAIDs) act by inhibiting prostaglandin synthesis, but have low efficacy in these type of diseases, at concentrations compatible with their gastrointestinal and renal toxicity. TNF-α production or maturation inhibitors could represent an interesting alternative to NSAIDs.

Neutralizing anti-TNF-α monoclonal antibodies (such as infliximab) have provided the first evidence of the beneficial effect in rheumatoid arthritis and Crohn's disease (Elliot et al 1994); TNF-α-soluble receptors are now undergoing clinical trials for rheumatoid arthritis (Sander et al 1996). However, production costs for biological agents are high and patients require repeated parenteral treatments. Moreover most develop human antibodies which can lead to tachyphylaxis. Thus, there is a need to develop more economic and orally active chemical substances.

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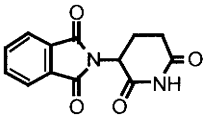
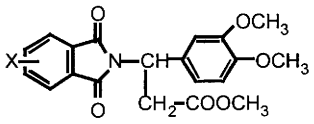
Over the past few years, studies have shown that in addition to its known hypnotic or sedative and teratogenic effects, thalidomide (**1**) (Figure 1) inhibits TNF-α production in lipopolysaccharide-stimulated human monocytes (Sampaio et al 1991). (Poly)fluorine and amino substitution of thalidomide and other *N*-aryl-phthalimides (**2**, **3**) results in analogues with TNF-α inhibition potency approaching 200-times that of thalidomide (Muller et al 1996; Niwayama et al 1996). Formation of the arene oxide metabolite of thalidomide, which is thought to be responsible for its teratogenic effect, could be prevented by tetrafluorine substitution (Niwayama et al 1996).

We recently synthesized and tested a series of *N*-azaaryl(alkyl)phthalimides incorporating amino(alkyl)pyridines (Collin et al 1998). *N*-(4,6-dimethylpyridin-2-yl)tetrafluorophthalimide (**4**) was found to be a potent TNF-α production inhibitor (IC50 = 10 μM). We also studied the TNF-α inhibitory activity of *N*-(4,6-dimethylpyridin-2-yl)heteroarylcarboxamides (Lang et al 1995; Vernhet et al 1997). The furan-2-carboxamide **5** (Robert et al 1995) was found to have greater activity than thalidomide (IC50: 70 and 200 μM approx., respectively).

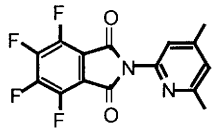
We describe here the synthesis and the evaluation of in-vitro TNF-α production inhibitory activity of



1 (Thalidomide)



2 (X = 4, 5, 6, 7-tetraF)
3 (X = 5–NH_2)



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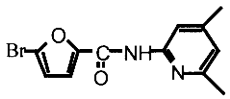

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Figure 1. Chemical structures of phthalimides **1**–**4** and furan-2 carvoxamide **5** inhibiting TNF-α production.

structurally-related compounds, *N*-pyridinyl(alkyl)fluorobenzamides.

Materials and Methods

Chemistry

Melting points were determined on a Tottoli-Büchi apparatus and are uncorrected. Structures were supported by IR, ^{1}H NMR and mass spectra. IR spectra were run with KBr pellets on a Perkin Elmer-Paragon PC 1000 infrared spectrophotometer. ^{1}H NMR spectra were recorded on a Bruker AC 250 spectrometer (250 MHz), using $CDCl_3$ or d_6-$(CH_3)_2SO$ as solvent. Chemical shifts (δ ppm) are reported downfield from tetramethylsilane, the internal standard; coupling constants are in Hz. Mass spectra were recorded on a double beam Varian Mat 112 spectrometer; ionization energy 70 eV. Analytical TLC was performed on precoated silica-gel aluminium plates (0·2 mm, GF254, E Merck). Spots were located by UV illumination. Evaporation (rotating evaporator) was done in-vacuo. Sodium sulphate or phosphorus pentoxide were used as the drying agents. Most of the crude products were passed through short columns of silica gel (silica gel 60, 70–230 mesh, E Merck) with an appropriate mixture of dichloromethane and ethanol.

Commercially available solvents and chemicals were used for syntheses, with the exception of the following polyfunctional amines which were prepared using methods reported in the literature.

Synthesis of intermediary amines

*6-Amino-3-bromo-2,4-lutidine (**6**). Method a.* Monobromination of 6-amino-2,4-lutidine in acetic acid at room temperature led to compound **6** (Mariella & Belcher 1952; Fox & Threlfall 1964). Yield: 78%; mp 143°C (lit. 145–146°C). IR (KBr), ν (cm^{-1}) 3415, 3300, 3160 (ν NH), 840 (ν C–Br). ^{1}H NMR ($CDCl_3$) δ 2·28 (s, 3H, 4-CH_3), 2·50 (s, 3H, 6-CH_3), 4·32 (s, 2H, NH_2), 6·25 (s, 1H, Pyr-3H).

*6-Amino-3,5-dibromo-2,4-lutidine (**7**). Method b.* Dibromination of 6-amino-2,4-lutidine was carried out using the same protocol as for monobromination but adding two equivalents of bromine. Yield: 38%; mp 135°C (lit. 136–137°C). IR (KBr), ν (cm^{-1}) 3130 (ν NH), 670 (ν C–Br). ^{1}H NMR ($CDCl_3$) δ 2·49 (s, 3H, 4-CH_3), 2·53 (s, 3H, 6-CH_3), 4·92 (s, 2H, NH_2).

*6-Amino-3-bromo-5-nitro-2,4-lutidine (**8**). Method c.* This compound was synthesized as described by Graboyes & Day (1957). Nitric acid was added dropwise to a solution of monobrominated amine **6** in sulphuric acid and the mixture was stirred at room temperature. Yield: 61%; mp 169°C (lit. 169–170°C). IR (KBr), ν (cm^{-1}) 3400, 3180 (ν NH_2), 1620 (ν C=N), 1500 (ν_{as} NO_2), 1320 (ν_s NO_2). ^{1}H NMR ($CDCl_3$) δ 2·37 (s, 3H, 4-CH_3), 2·49 (s, 3H, 6-CH_3), 7·06 (s, 2H, NH_2).

*5,6-Diamino-3-bromo-2,4-lutidine (**9**). Method d.* Reduction of compound **8** by $SnCl_2$ in concentrated hydrochloric acid according to Graboyes & Day (1957) gave **9**. Yield: 92%; mp 169°C (lit. 169–170°C). IR (KBr), ν (cm^{-1}) 3400, 3200 (ν NH_2), 1645 (ν C=N). ^{1}H NMR ($CDCl_3$) δ 2·16 (s, 3H, 4-CH_3), 2·30 (s, 3H, 6-CH_3), 4·57 (s, 2H, NH_2), 5·52 (s, 2H, NH_2).

Synthesis of benzamides

*N-(4,6-Dimethylpyridin-2-yl)-2,4-difluorobenzamide (**11**). Method e.* A solution of 2,4-difluorobenzoic acid (0·9 g, 5·7 mmol), 2-amino-4,6-dimethylpyridine (0·69 g, 5·7 mmol) and triethylamine (2·4 mL, 17·1 mmol) in dry 1,2-dichloroethane (60 mL) was cooled in an ice bath. Phenyl dichlorophosphate (0·85 mL, 5·7 mmol) was added dropwise. The mixture was stirred at room temperature for 24 h. The solvent was removed under reduced pressure. The residue was purified by column chromatography using dichloromethane–ethanol (95:5) as eluent. After recrystallization from ethanol, 0·91 g pure product was obtained. Yield: 61%; mp 116°C. IR (KBr), ν (cm^{-1}) 3220 (ν NH), 1700 (ν C=O), 1500 (δ NH), 1290 (combined NH/CN). ^{1}H NMR ($CDCl_3$) δ 2·36 (s, 3H, 4-CH_3), 2·45 (s, 3H, 6-CH_3), 6·80 (s, 1H, Pyr-

5H), 6·95 (m, 1H, Ar-3H), 7·06 (m, 1H, Ar-5H), 8·02 (s, 1H, Pyr-3H), 8·13 (m, 1H, Ar-6H), 9·11 (s, 1H, CONH).

N-*(4,6-Dimethylpyridin-2-yl)-3-fluorobenzamide* ***(10)*** *(Robert et al 1994). Method e.* Yield: 68%; mp 119°C (lit. 115–116°C). IR (KBr), ν (cm^{-1}) 3360 (ν NH), 1675 (ν C = O), 1550 (δ NH), 1280 (combined NH/CN). ^{1}H NMR ($CDCl_3$) δ 2·34 (s, 3H, 4-CH_3), 2·36 (s, 3H, 6-CH_3), 6·76 (s, 1H, Pyr-5H), 6·93 (m, 4H, Ar-H), 8·03 (s, 1H, Pyr-3H), 8·92 (s, 1H, CONH).

N-*(4,6-Dimethylpyridin-2-yl)-2,3,6-trifluorobenzamide* ***(12)****. Method e.* Yield: 69%; mp 182°C. IR (KBr), ν (cm^{-1}) 3440 (ν NH), 1690 (ν C = O), 1620 (δ NH), 1240 (combined NH/CN). ^{1}H NMR ($CDCl_3$) δ 2·37 (s, 3H, 4-CH_3), 2·38 (s, 3H, 6-CH_3), 6·80 (s, 1H, Pyr-5H), 6·93 (m, 1H, Ar-5H), 7·25 (m, 1H, Ar-4H), 8·00 (s, 1H, Pyr-3H), 8·50 (s, 1H, CONH).

N-*(4,6-Dimethylpyridin-2-yl)-2-chloro-4,5-difluorobenzamide* ***(13)****. Method e.* Yield: 72%; mp 123°C. IR (KBr), ν (cm^{-1}) 3205 (ν NH), 1690 (ν C = O), 1565 (δ NH), 1305 (combined NH/CN). ^{1}H NMR ($CDCl_3$) δ 2·37 (s, 3H, 4-CH_3), 2·38 (s, 3H, 6-CH_3), 6·79 (s, 1H, Pyr-5H), 7·29 (dd, 1H, Ar-3H, J = 9·5, 6·8), 7·58 (dd, 1H, Ar-6H, J = 9·5, 8·2), 7·97 (s, 1H, Pyr-3H).

N-*(4,6-Dimethylpyridin-2-yl)pentafluorobenzamide* ***(14)****. Method e.* Yield: 72%; mp 139–140°C. IR (KBr), ν (cm^{-1}) 3205 (ν NH), 1705 (ν C = O), 1495 (δ NH), 1230 (combined NH/CN). EI-MS m/z (%) 316 (M, 15), 269 (100), 195 (57), 167 (45), 117 (17). ^{1}H NMR ($CDCl_3$) δ 2·28 (s, 3H, 4-CH_3), 2·37 (s, 3H, 6-CH_3), 6·78 (s, 1H, Pyr-5H), 7·96 (s, 1H, Pyr-3H), 9·22 (s, 1H, CONH).

Method f. 1,1-Carbonyldiimidazole (1·03 g, 5·6 mmol) was added portionwise to a solution of pentafluorobenzoic acid (1·2 g, 5·6 mmol) in dry tetrahydrofuran (40 mL). The mixture was stirred at room temperature for 1 h. 2-Amino-4,6-dimethylpyridine (0·68 g, 5·6 mmol) was then added. The solution obtained was stirred at room temperature for 24 h. The solvent was removed under reduced pressure. The residue was purified by column chromatography using dichloromethane–ethanol (95:5) as eluent. Recrystallization from ethanol afforded 1·11 g pure compound. Yield: 62%.

N-*(Pyridin-3-ylmethyl))pentafluorobenzamide* ***(15)****. Method e.* Yield: 82%; mp 120–121°C. IR (KBr), ν (cm^{-1}) 3180 (ν NH), 1690 (ν C = O), 1505 (δ NH), 1330 (combined NH/CN). EI-MS m/z (%) 302 (M, 82), 195 (100), 167 (47), 117 (25). ^{1}H NMR ($CDCl_3$) δ 4·62 (d, 2H, CH_2, J = 5·7), 7·18 (m 1H, NH), 7·28 (m, 1H, Pyr-2H), 7·70 (m, 1H, Pyr-4H), 8·45 (m, 2H, Pyr-5H, Pyr-6H).

N-*(5-Bromo-4,6-dimethylpyridin-2-yl)pentafluorobenzamide* ***(16)****. Method e.* Yield: 47%; mp 186°C. IR (KBr), ν (cm^{-1}) 3250 (ν NH), 1705 (ν C = O), 1575 (δ NH), 1270 (combined NH/CN). EI-MS m/z (%) 397 (M + 2, 20), 395 (M, 19), 195 (100), 167 (50), 117 (20). ^{1}H NMR ($CDCl_3$) δ 2·47 (s, 3H, 4-CH3), 2·55 (s, 3H, 6-CH_3), 8·06 (s, 1H, Pyr-3H), 8·43 (s, 1H, CONH).

N-*(3,5-Dibromo-4,6-dimethylpyridin-2-yl)pentafluorobenzamide* ***(17)****. Method e.* Yield: 49%; mp 119°C. IR (KBr), ν (cm^{-1}) 3250 (ν NH), 1705 (ν C = O), 1575 (δ NH), 1270 (combined NH/CN). EI-MS m/z (%) 195 (100), 167 (25), 117 (9). ^{1}H NMR ($CDCl_3$) δ 2·51 (s, 3H, 4-CH_3), 2·68 (s, 3H, 6-CH_3), 6·98 (s, 1H, CONH).

N-*(5-Bromo-3-nitro-4,6-dimethylpyridin-2-yl)-pentafluorobenzamide* ***(18)****. Method e.* Yield: 45%; mp 121°C. IR (KBr), ν (cm^{-1}) 3450 (ν NH), 1665 (ν C = O), 1510 (δ NH), 1525 (ν_{as} NO_2), 1370 (ν_s NO_2), 1225 (combined NH/CN). EI-MS m/z (%) 442 (M + 2, 5), 440 (M, 4), 195 (100), 167 (21), 117 (7). ^{1}H NMR ($CDCl_3$) δ 2·53 (s, 3H, 4-CH_3), 2·62 (s, 3H, 6-CH_3), 9·29 (s,1H, CONH).

N-*(2-Amino-5-bromo-4,6-dimethylpyridin-3-yl) pentafluorobenzamide* ***(19)****. Method e.* Yield: 47%; mp 255°C. IR (KBr), ν (cm^{-1}) 3470 (ν NH), 1675 (ν C = O), 1520 (δ NH), 1330 (combined NH/CN). EI-MS m/z (%) 412 (M + 2, 12), 410 (M, 13), 195 (67), 167 (34), 135 (100), 117 (21). ^{1}H NMR ($CDCl_3$) δ 2·23 (s, 3H, 4-CH_3), 2·45 (s, 3H, 6-CH_3), 5·86 (s, 2H, NH_2), 10·26 (s, 1H, CONH).

Pharmacology

TNF-α inhibitory activity

The effect of the different compounds on the production of TNF-α, by in-vitro activated peritoneal macrophages, was quantified as previously described (Lang et al 1995). Adult male Swiss CF mice, 18–25 g, were obtained from CREJ, France. Briefly, thioglycollate-elicited mouse macrophages were isolated by peritoneal washing with Ca-Mg phosphate-buffered saline (PBS), after cervical dislocation. The cell suspension ($1–2 \times 10^6$ cells/well) was incubated in 24-well culture plate in RPMI 10% foetal calf serum for 2 h at 37°C, 5% CO_2. After three washes with PBS, mouse macrophages were pre-incubated with the different

compounds solubilized in ethanol (maximum final concentration of ethanol: 0·5%) or in dimethylsulphoxide (maximum final concentration: 0·2%) at 10 μM. Macrophages were then stimulated with bacterial lipopolysaccharides or LPS (0·5 μg mL^{-1}) from *Escherichia coli* (Sigma) for 4 h. Supernatants were collected and assessed for TNF-α content using Wehi 164 clone 13 cytotoxic assay (Espevik & Nissen-Meyer 1986).

Acute phorbol ester-induced mouse ear-swelling test

In the acute mouse ear-swelling test (Carlson et al 1985), 2·5 μg phorbol-12- *O*-myristate-13-acetate (PMA) (Sigma) in 10 μL ethanol–water (8:2, v/v) was applied topically in a single dose to the inner and outer surfaces of the right ear of mice. The mice were randomly divided into seven groups, five mice per group: vehicle, 0·1, 0·2 and 0·4 mM kg^{-1} compound **14** and the reference compound, ibuprofen. The appropriate doses of **14** and ibuprofen were orally administered 1 h before PMA application. The left ear of each animal received only vehicle. Ear thickness (mm) as an index of inflammation was measured after 3·5 h, using a Dyer model micrometer gauge (Dyer Co. Inc., Lancaster, PA). The percentage inhibition of the inflammatory reaction was determined by comparison with controls. Results are expressed as mean ± s.e.

Carrageenan-induced rat paw oedema

The inhibitory activity of **14** and ibuprofen on carrageenan-induced rat paw oedema was determined according to the method of Winter et al (1962), with slight modifications (Duflos et al 1998).

Results and Discussion

Chemistry

The synthesis of intermediary amines was carried out as shown in Figure 2. The preparation of benzamides **10**–**19** was carried out as shown in Figure 3. Corresponding substituted benzoic acids were condensed with appropriate amines using the methods which afforded the best results in previous studies: phenyl dichlorophosphate activation of the carboxylic acid in the presence of triethylamine in 1,2-dichloroethane at room temperature (Arrieta et al 1985) or 1,1-carbonyldiimidazole activation in tetrahydrofuran at room temperature (Paul & Anderson 1960). The dichloro phosphate method provided compound **14** in better yield (72%) than the 1,1-carbonyldiimidazole method (62%) and so

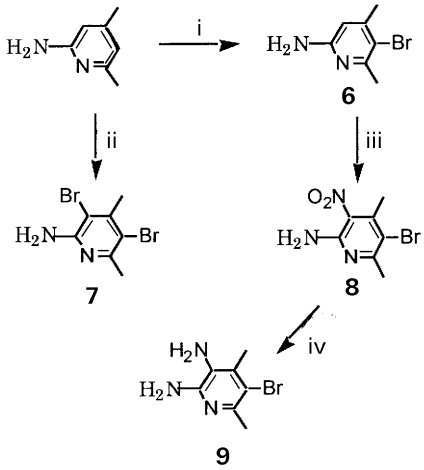


Figure 2. Synthesis of intermediary amines. Reagents: i. Br_2 (1 equiv.), AcOH, r.t., 3 h; ii. Br_2 (2 equiv.) AcOH, r.t., 3 h; iii. conc HNO_3, H_2SO_4, 60°C, 20 min; iv. $SnCl_2$, conc HCl, 80°C, 30 min.

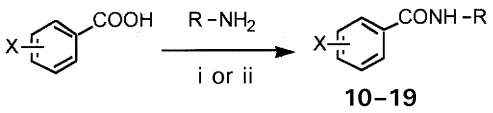


Figure 3. Synthesis of benzamides. Reagents: i. phenyl dichlorophosphate, TEA, 1,2-dichloroethane, r.t., 24 h; ii. 1,1-carbonyldiimidazole, THF, r.t., 24 h.

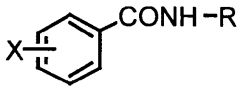
it was adopted for the synthesis of the other benzamides (**10**–**13**, **15**–**19**). The variability in yields obtained (45–82%) by this method could be explained by the difference in nucleophilicity of the starting amines; for instance, β-picolylamine afforded compound **15** in a 82% yield whereas 6-amino-3-bromo-5-nitro-2,4-lutidine gave compound **18** in only 45% yield.

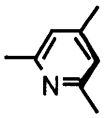
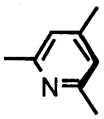
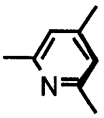
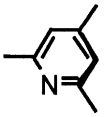
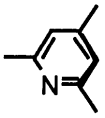
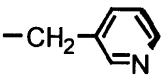
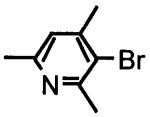
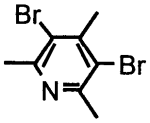
Pharmacology

The effect of the test compounds on the production of TNF-α was quantified in in-vitro LPS-stimulated peritoneal macrophages (Table 1). Although 3-fluorobenzamide **10** was one of the most efficient *N*-lutidinylcarboxamides studied in the rat paw oedema inhibition, it did not exert a significant inhibitory effect on TNF-α production, at least at 10 μM. Even di- and tri-halogenobenzamides **11**–**13** were inefficient at this concentration.

Nevertheless, as previously observed in the phthalimide series (Collin et al 1998), perfluorination of benzamides issued from 6-amino-2,4-lutidine and β-picolylamine allowed emergence of inhibitory activity in compounds **14** and **15** (33% approx.). Pharmacomodulation at the level of the pyridinyl carbons 3 and 5 in the lutidinyl sub-series, was carried out by introducing a bromo, nitro

Table 1. TNF-α inhibitory activity of 10 μM *N*-pyridinyl (alkyl)fluorobenzamides.



Compound	X	R	Inhibition (%)
10	3-F		<10
11	2, 4-diF		<10
12	2, 3, 6-triF		<10
13	2-Cl, 4, 5-diF		<10
14	2, 3, 4, 5, 6-pentaF		33
15	2, 3, 4, 5, 6-pentaF		35
16	2, 3, 4, 5, 6-pentaF		<10
17	2, 3, 4, 5, 6-pentaF		25
19	2, 3, 4, 5, 6-pentaF		40

Inhibition is expressed as the mean of six values obtained in two separate experiments. Individual values differed from the mean by less than 5%. Because of its very low solubility in the assay solvent system, compound **18** could not be tested.

or amino group. Although 5-bromination suppressed activity, emergence of TNF-α inhibition (25%) was observed after 3,5-dibromination. The corresponding 3-nitro compound **18** could not be tested due to insolubility in the solvent system used for the in-vitro assay; trials to increase its aqueous solubility, by maleate salt formation, failed. Reduction of its nitro group, leading to the amino derivative **19**, afforded the most effective compound of the series (40% at 10 μM).

Table 2. Inhibition of phorbol myristate acetate-induced mouse ear oedema after oral administration of compound **14**.

Compound	Inhibition (%)		
	Dose ($mM\,kg^{-1}$)		
	0·1	0·2	0·4
14	49 ± 6	65 ± 9	69 ± 4
Ibuprofen	35 ± 8	56 ± 4	86 ± 6

Of the four active pentafluorobenzamides (**14**, **15**, **17** and **19**), compound **14**, an analogue of the *N*-lutidinyl tetrafluorophthalimide **4**, was selected for preliminary evaluation of in-vivo anti-inflammatory activity. Its effect in the acute PMA-induced mouse ear-swelling test, was measured after oral administration (Table 2). It exerted a significant reduction in ear thickness and its level of activity at a concentration of 0·1 $mM\,kg^{-1}$, compared favourably with ibuprofen.

This benzamide was also tested against carrageenan-induced rat paw oedema; its inhibitory effect after oral administration of 0·2 $mM\,kg^{-1}$ remained moderate (31 ± 14%), and no activity increase was observed at a dose of 0·4 $mM\,kg^{-1}$. At the same doses, percentage inhibition by ibuprofen was 50 ± 1% and 57 ± 2%, respectively.

The multiple PMA-induced model of chronic inflammation is considered to be a relevant model of human psoriasis; we wish to evaluate the most potent polyfluorophthalimides and benzamides in the subchronic mouse ear-swelling test (Alford et al 1992).

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