JPP Journal of Pharmacy and Pharmacology

JPP 2001, 53: 1379–1385

Received March 27, 2001
Accepted July 3, 2001
ISSN 0022-3573

Effect of some hexahydroimidazo[1,2-*c*]pyrimidines in inflammatory responses involving leucocytes and macrophages

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Abstract

We have studied the effects of some hexahydroimidazo[1,2-*c*]pyrimidine derivatives (HIPs) on leucocyte functions in-vitro and we have assayed the anti-inflammatory activity of these compounds in two models of inflammation. All HIPs inhibited the human neutrophil degranulation process and superoxide generation at concentrations in the μM range. In mouse peritoneal macrophages stimulated with lipopolysaccharide, HIP-4 and HIP-5 inhibited nitrite production without affecting prostaglandin E_2 (PGE_2) accumulation. HIP-4 was also active in the zymosan-injected mouse air pouch model (at 100 nmol/pouch), with significant reductions in leucocyte migration and PGE_2 and leukotriene B_4 levels in the air pouch exudate. To confirm the anti-inflammatory effects of this compound, we tested HIP-4 orally (10–40 mg kg^{-1}) on carrageenan mouse-paw oedema where it exerted a dose-dependent inhibition of paw swelling with significant reductions of myeloperoxidase and elastase activity and PGE_2 levels in paw homogenates. This study demonstrates that some HIPs inhibit leucocyte functions and one of these derivatives (HIP-4) shows anti-inflammatory activity when administered by the oral route, which can be related to inhibition of leucocyte migration.

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Funding and acknowledgements: This work was supported by grant 1FD97-0373-C02-01, CICYT-FEDER. A. Vidal thanks Generalitat Valenciana for a scholarship. We wish to thank Dr S. J. Foster (Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK) for the kind gift of antibody against LTB_4.

Introduction

Neutrophils adhere to endothelium and emigrate to ingest and kill invading pathogens, a process which is dependent upon their ability to produce reactive oxygen species and to secrete lytic proteins. Neutrophils' oxidative bursts generate large quantities of powerful tissue-damaging species such as superoxide, hydrogen peroxide and hypochlorous acid (produced by the myeloperoxidase-catalysed oxidation of Cl^- by H_2O_2) (Smith 1994; Babior 2000). There is evidence that reactive oxygen species are implicated in tissue damage in different pathological conditions, including rheumatoid arthritis, atherosclerosis, pancreatitis, reperfusion injury, etc. (Poch et al 1999; Babior 2000).

Macrophages also play an important role in acute and chronic inflammation and can synthesize and secrete a large number of biologically active substances (cytokines, chemotactic factors, lipid mediators, oxidants, etc.) (Laskin & Pendino 1995; MacMicking et al 1997). Interactions between macrophages and neutrophil-secreted mediators can perpetuate chronic inflammation (Lefkowitz et al 1999).

Nitric oxide (NO) is a radical produced in a reaction catalysed by the enzyme nitric oxide synthase (NOS) and possesses a wide range of biological activities. The inducible isoform of the enzyme (iNOS) is expressed in many inflammatory cells

after stimulation with endotoxins, such as bacterial lipopolysaccharide (LPS) or cytokines, leading to increased NO generation during inflammatory reactions (Knowles & Moncada 1994). Superoxide and NO interact to form the potent oxidant peroxynitrite that may contribute to the cytotoxic actions of macrophages (Xia & Zweier 1997).

Hexahydroimidazo[1,2-*c*]pyrimidine derivatives (HIPs) (Figure 1) can be precursor molecules of imidazo[1,2-*a*]pyrimidines and a new synthetic procedure for both has been described (Acero-Alarcon et al 1999). Since we have recently shown that the latter class of compounds is a potential template for anti-inflammatory drugs (Vidal et al 2001), this work was aimed at assessing the influence of some HIPs on leucocyte functions and models of inflammation. We have used in-vitro assays in human neutrophils and mouse peritoneal macrophages, as well as the zymosan-injected mouse air pouch and the carrageenan-induced mouse-paw oedema. This is the first report on the anti-inflammatory activity of this class of compounds.

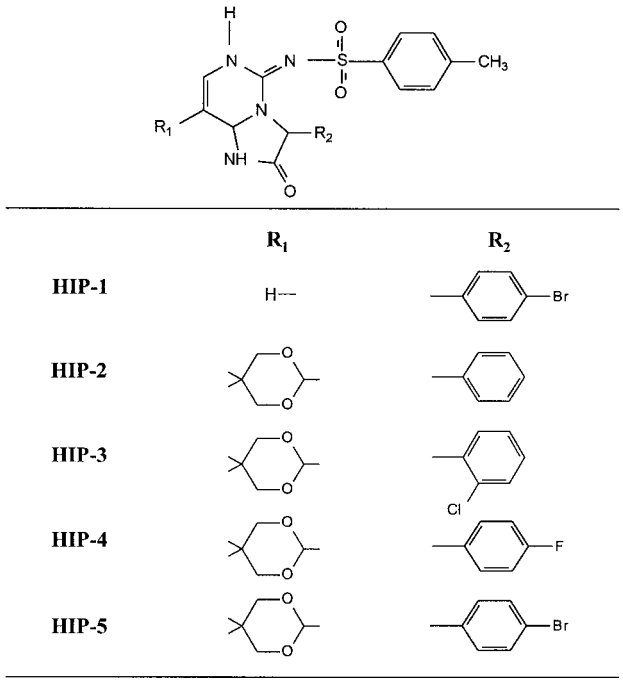

Figure 1 Chemical structure of hexahydroimidazo[1,2-*c*]pyrimidine derivatives (HIPs).

Materials and Methods

Materials

HIPs were obtained as described by Acero-Alarcon et al (1999). Bis-(benzyloxycarbonyl-Ala-Ala)-rhodamine 110 was from Molecular Probes Europe BV (Leiden, The Netherlands) and 1400W from Cayman Chem. (Ann Arbor, MI). Other reagents were purchased from Sigma Chem. (St Louis, MO).

Animals

Female Swiss mice, 25–30 g (Harlan, Barcelona, Spain), were housed according to the guidelines of the institutional care and use committee.

Elastase and myeloperoxidase release by human neutrophils

Human neutrophils were isolated from peripheral blood taken from healthy volunteers. Contaminating red cells were removed by hypotonic lysis. Neutrophils were isolated by Ficoll–Hypaque sedimentation and resuspended in phosphate-buffered saline (PBS) containing 1.26 mM Ca^{2+} and 0.9 mM Mg^{2+} (Escrig et al 1997). Viability was greater than 95% by the trypan blue exclusion test. Neutrophils (2.5×10^6 mL^{-1}) were pre-incubated with test compound or vehicle for 5 min and then stimulated with cytochalasin B (10 μM) and either *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 10 nM) or platelet activating factor (PAF, 0.5 μM) for 10 min. Elastase activity was estimated in supernatants by the fluorescence increase due to hydrolysis of rhodamine 110 bispeptide substrate. Myeloperoxidase activity was determined spectrophotometrically by the tetramethylbenzidine oxidation assay, as previously described (De Young et al 1989). Percentage inhibition of degranulation was calculated with respect to elastase/myeloperoxidase activity in control stimulated groups. Possible direct inhibitory effects on elastase or myeloperoxidase activity were assessed by incubating test compounds with supernatants from cytochalasin B + FMLP-stimulated neutrophils. The mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan was used to assess the possible cytotoxic effects of test compounds (Gross & Levi 1992).

Superoxide generation by human neutrophils

Neutrophils (2.5×10^6 mL^{-1}) were mixed with luminol (40 μM) and stimulated with 12-*O*-tetradecanoyl phorbol 13-acetate (TPA, 1 μM). In another set of experiments, superoxide anions were also generated by the

hypoxanthine–xanthine oxidase system. The chemiluminescence was recorded in a Microbeta Trilux counter (Wallac, Turku, Finland) after 7 min (Escrig et al 1997).

Synthesis and release of LTB_4 by human neutrophils

A suspension of human neutrophils (5×10^6 mL^{-1}) was pre-incubated with test compounds or vehicle and then stimulated with 1 μM A23187. The leukotriene B_4 (LTB_4) levels in supernatants were measured by radioimmunoassay (RIA) (Moroney et al 1988).

Nitrite and PGE_2 production by mouse peritoneal macrophages

Peritoneal macrophages were obtained from female Swiss mice weighing 25–30 g. Cells were harvested by peritoneal lavage 4 days after intraperitoneal injection of 1 mL of 10% thioglycolate broth. Cells were resuspended and incubated at 37°C for 2 h. Adherent cells were stimulated with *Escherichia coli* LPS (1μg mL^{-1}) for 18 h in the presence of test compounds or vehicle. Nitrite, as an index of NO production, was assayed fluorimetrically (Misko et al 1993). Prostaglandin E_2 (PGE_2) levels were measured by RIA (Moroney et al 1988). In another set of experiments, macrophages were stimulated with LPS for 18 h to induce iNOS and then test compounds were added to fresh medium supplemented with L-arginine (0.5 mM), followed by incubation for a further 2-h period. Nitrite levels were determined in supernatants as above.

Zymosan-injected air pouch model

The zymosan-injected air pouch model was performed in mice as previously described (Edwards et al 1981). Sterile saline, 1% w/v zymosan + vehicle or 1% w/v zymosan + test drug was injected in a volume of 1 mL into the 6-day-old air pouches. Four hours after administration, the mice were killed by cervical dislocation and the exudate in the pouch was collected with 1 mL of saline. Leucocytes present in exudates were counted using a Coulter counter. After centrifugation of exudates at 1200 *g* at 4°C for 10 min, the supernatants were used to measure LTB_4 and PGE_2 levels by RIA as above.

Carrageenan paw oedema

Swelling was induced following a modification of the technique of Sugishita et al (1981). Mice were fasted for 12 h with free access to water. Drugs or vehicle (ethanol–Tween 80–distilled water, 5:5:90 v/v/v) were administered orally (0.5 mL) 1 h before the injection of carrageenan (0.05 mL; 3% w/v in saline) into the subplantar area of the right hind paws of groups of six mice. The volume of injected and contralateral paws were measured at 1, 3 and 5 h after induction of oedema by using a plethysmometer (Ugo Basile, Comerio, Italy). The volume of oedema was expressed for each mouse as the difference between the carrageenan-injected and contralateral paws. After death (5 h after induction of oedema), paws were amputated above the ankle and homogenized in 2.5 mL saline. After centrifugation at 10000 *g* at 4°C for 15 min, supernatants were used for the determination of elastase, myeloperoxidase and PGE_2 as described above.

Statistical analysis

The results are presented as mean ± s.e.m.; n represents the number of experiments. Inhibitory concentration 50% (IC50) values were calculated by linear regression analysis using Graph Pad Prism II. The level of statistical significance was determined by analysis of variance followed by Dunnett's *t*-test for multiple comparisons.

Results

Effects of HIPs on neutrophil function

Stimulation of human neutrophils induced the degranulation process releasing enzymes such as elastase and myeloperoxidase. All HIPs at 10 μM inhibited elastase and myeloperoxidase release from neutrophils stimulated with cytochalasin B + FMLP or cytochalasin B + PAF (Table 1). HIP-4 and HIP-5 were the most active compounds on elastase released by cytochalasin B + FMLP, with IC50 values of 3.9 and 3.0 μM, respectively. None of these derivatives showed a direct inhibition on elastase or myeloperoxidase activity. In addition, generation of LTB_4 by calcium ionophore A23187 in human neutrophils was not modified by HIPs (data not shown).

Generation of superoxide was reduced by all compounds (Table 1). The observed effect was not due to direct scavenging of superoxide, since none of these derivatives was able to affect superoxide generated by the enzymatic system hypoxanthine–xanthine oxidase (data not shown). No cytotoxic effects on neutrophils were observed at the concentrations used in our study,

Table 1 Percentage inhibition, by HIPs, of human neutrophil degranulation and superoxide generation.

	CytB + FMLP		CytB + PAF		TPA
	Elastase	Myeloperoxidase	Elastase	Myeloperoxidase	Superoxide
HIP-1	65.5 ± 1.7**	74.3 ± 1.8**	39.5 ± 7.3**	61.8 ± 9.6**	30.9 ± 1.6**
HIP-2	52.9 ± 6.7**	85.3 ± 1.7**	47.9 ± 5.1**	59.7 ± 9.3**	43.6 ± 7.1**
HIP-3	45.4 ± 9.1**	70.7 ± 4.7**	46.2 ± 5.5**	74.7 ± 8.6**	38.7 ± 3.6**
HIP-4	83.9 ± 5.0**	60.6 ± 3.1**	49.8 ± 3.9**	72.8 ± 6.6**	53.3 ± 2.1**
HIP-5	85.3 ± 4.3**	60.7 ± 2.0**	46.6 ± 4.1**	81.9 ± 3.6**	53.8 ± 2.3**

Data represent mean ± s.e.m., n = 6–10; $^{**}P < 0.01$ with respect to the control group. HIPs were assayed at 10 μM. Elastase and myeloperoxidase release was induced by cytochalasin B (CytB) + FMLP or cytochalasin B + PAF and superoxide generation by TPA. Values of elastase (fluorescence units min^{-1} mL^{-1}: unstimulated cells, 498 ± 78; CytB + FMLP-treated cells, 4045 ± 183 and CytB + PAF-treated cells, 3403 ± 214. Values of myeloperoxidase (OD min^{-1} mL^{-1}): unstimulated cells, 0.55 ± 0.08; CytB + FMLP-treated cells, 6.20 ± 0.30 and CytB + PAF-treated cells, 4.66 ± 0.15.

as assessed by mitochondrial reduction of MTT after 30 min challenge (data not shown).

Effects of HIPs on nitrite and PGE_2 production in mouse peritoneal macrophages

HIPs (10 μM) were included in the 18-h period during which mouse peritoneal macrophages were stimulated with LPS. Levels of nitrite as an index of iNOS activity and PGE_2 as an index of cyclooxygenase-2 activity were determined in culture medium. HIP-4, HIP-5 and 1400W inhibited nitrite accumulation in a concentration-dependent manner, whereas HIP-3 caused a minor reduction of nitrite levels and the rest of the compounds were inactive at 10 μM (Table 2). PGE_2 levels were not modified by HIPs (data not shown).

Table 2 Effect of HIPs on nitrite production by LPS-stimulated mouse peritoneal macrophages.

	18 h Incubation[a]		2 h Incubation[b]
	Nitrite (μM)	IC50 (μM)	Nitrite (μM)
Control	8.0 ± 0.5	–	4.5 ± 0.3
HIP-1	8.1 ± 0.2	N.D.	N.D.
HIP-2	7.4 ± 0.2	N.D.	N.D.
HIP-3	5.7 ± 0.5**	N.D.	3.1 ± 0.1**
HIP-4	4.2 ± 0.4**	8.9 (8.1–9.7)	2.4 ± 0.2**
HIP-5	3.5 ± 0.3**	5.3 (3.6–6.9)	2.5 ± 0.2**
1400W	0.8 ± 0.3**	2.4 (1.3–3.2)	1.6 ± 0.3**

[a]Cells were co-incubated with LPS and test compound for 18 h. [b]Cells were stimulated with LPS for 18 h. After washing the cells, test compounds were added and incubated for 2 h in the presence of L-arginine (0.5 mM). Nitrite levels were determined in supernatants. Data represent mean ± s.e.m., n = 6–10; $^{**}P < 0.01$ with respect to the LPS control group. Compounds were assayed at 10 μM. IC50 values were determined for the HIPs that reached 50% inhibition. N.D. not determined.

To clarify whether the reduction of nitrite accumulation caused by HIP-3, HIP-4 and HIP-5 is related either to an interference with enzyme induction or to a direct action on iNOS activity, these compounds were added at 10 μM after the induction of the enzyme with LPS in macrophages, and incubated in fresh medium supplemented with L-arginine (0.5 mM) for 2 h. Under these conditions, HIP-3, HIP-4 and HIP-5 showed an inhibitory effect on nitrite levels similar to that observed in the induction assay (Table 2). Our results suggest that these HIPs inhibit nitrite levels after 18 h co-incubation with LPS by a direct action on iNOS activity.

Effects of HIPs on zymosan-injected mouse air pouch

Four hours after induction of inflammation with zymosan, a great increase in leucocyte migration was observed in the zymosan-injected control group of mice, in comparison with the saline-injected group (Table 3). HIP-4, administered intrapouch, reduced cell accumulation in exudates dose dependently with an IC50 value of 54.0 (47–58) nmol. The inflammatory response also generated high levels of PGE_2 and LTB_4 in the air pouch exudate and HIP-4 at the highest dose (100 nmol/pouch) caused a significant decrease in PGE_2 and LTB_4 levels (Table 3). The rest of the compounds were inactive in this assay.

Table 3 Effect of HIPs on 4-h zymosan-injected air pouch in mice.

	Cell migration (cells $\times 10^6$ mL^{-1})	PGE_2 (ng mL^{-1})	LTB_4 (ng mL^{-1})
Saline	3.7 ± 1.0**	0.2 ± 0.0**	1.6 ± 0.7**
Zymosan	31.5 ± 1.8	29.1 ± 1.2	65.9 ± 8.3
HIP-1	26.0 ± 1.8	21.4 ± 1.8	N.D.
HIP-2	28.3 ± 5.3	35.8 ± 2.6	N.D.
HIP-3	27.6 ± 3.9	26.8 ± 8.3	N.D.
HIP-4	10.5 ± 1.5**	19.3 ± 0.5**	26.9 ± 3.7**
HIP-5	26.8 ± 1.3	25.2 ± 2.6	28.9 ± 4.1
Indometacin	25.2 ± 2.6	0.2 ± 0.0**	55.3 ± 4.2

Data represent mean ± s.e.m., n = 6; $^{**}P < 0.01$ with respect to the zymosan control group. Compounds were administered at 100 nmol/pouch.

Effects of HIP-4 on carrageenan mouse-paw oedema

To further study the anti-inflammatory activity of HIP-4, this compound was administered orally (10–40 mg kg^{-1}) to mice, 1 h before carrageenan injection. HIP-4, at a dose of 20 and 40 mg kg^{-1}, significantly reduced paw oedema at three points (1, 3 and 5 h) after carrageenan. Indometacin at 10 mg kg^{-1} was also effective at all points considered (Table 4). Elastase and myeloperoxidase activity in paw homogenates was determined as an index of leucocyte migration. Indometacin (10 mg kg^{-1}) and HIP-4 (40 mg kg^{-1}) inhibited both types of activity significantly and also reduced PGE_2 levels in paws homogenates (Table 5).

Discussion

Tissue damage during the inflammatory response has been linked to leucocyte infiltration and production of reactive oxygen species, such as superoxide, hydrogen peroxide and hydroxyl radicals, as well as to the release of proteolytic enzymes (Smith 1994). All the HIPs tested significantly inhibited superoxide generation by human neutrophils, with HIP-4 and HIP-5 showing the highest effects, and they also inhibited degranulation, with small differences depending on the stimulus used and the enzyme assayed. These results demonstrate that HIPs

Table 4 Effect of compounds on carrageenan-induced mouse-paw oedema.

	Oedema (μL)		
	1 h	3 h	5 h
Control	128.3 ± 9.7	160.0 ± 8.8	176.4 ± 13.9
HIP-4 10 mg kg^{-1}	94.0 ± 5.1*	112.5 ± 4.8*	134.0 ± 12.9
HIP-4 20 mg kg^{-1}	76.9 ± 8.8**	95.0 ± 19.4**	125.0 ± 8.7*
HIP-4 40 mg kg^{-1}	60.0 ± 10.0**	62.5 ± 11.1**	98.0 ± 5.8**
Indometacin 10 mg kg^{-1}	57.5 ± 8.5**	60.0 ± 8.2**	110.0 ± 5.5**

Data represent mean ± s.e.m., n = 6; $^{*}P < 0.05$, $^{**}P < 0.01$ with respect to the carrageenan control group. Compounds were administered orally 1 h before carrageenan injection.

Table 5 Effect of HIP-4 and indometacin on mouse-paw oedema and some parameters measured in homogenates from carrageenan-inflamed paws.

	% Oedema inhibition	% Elastase inhibition	% Myeloperoxidase inhibition	% PGE_2 inhibition
HIP-4 40 mg kg^{-1}	44.4 ± 3.6**	37.2 ± 9.9*	40.1 ± 7.3**	55.9 ± 8.3**
Indometacin 10 mg kg^{-1}	37.6 ± 3.4**	35.4 ± 5.3**	52.1 ± 5.3**	98.4 ± 0.1**

Data represent mean ± s.e.m., n = 6; $^{*}P < 0.5$, $^{**}P < 0.01$ with respect to the control group (PGE_2 control group = 98.3 ± 8.0 ng mL^{-1}). HIP-4 and indometacin were administered orally 1 h before carrageenan injection. Mice were killed 5 h after carrageenan injection and inflamed paws were amputated and homogenized.

possess inhibitory activity on human neutrophil functions in-vitro since these compounds inhibited the degranulation process that contributes to tissue damage and were also effective in suppressing the TPA-stimulated oxidative burst.

Three of these compounds, and mainly HIP-4 and HIP-5, also decreased NO generation by LPS-stimulated mouse macrophages, probably due to inhibition of iNOS activity. These active derivatives may inhibit the production of superoxide and NO thus preventing the formation of strong oxidants with an important role in tissue injury during inflammatory responses.

Nevertheless, only HIP-4 exhibited in-vivo anti-inflammatory activity in the two models used, the zymosan air pouch and the carrageenan paw oedema in mice. In both assays HIP-4 inhibited leucocyte migration, and at the higher dose used it also reduced eicosanoid levels. This last effect could be dependent on inhibition of cell migration, since this compound did not affect the production of these lipid mediators in the in-vitro systems we have used. Although a recent report showed that NOS inhibitors can exert pro-inflammatory or anti-inflammatory effects depending on the route of administration in the carrageenan-induced pleuresy model (Paul-Clark et al 2001), in the experimental model used in this study (zymosan air pouch), we have previously observed that aminoguanidine (200 mg kg^{-1}) administered intraperitoneally 30 min before zymosan injection did not modify either the cell number or eicosanoids level in exudates (Posadas et al 2000). Thus, the anti-inflammatory effect of HIP-4 in the zymosan-injected air pouch model is probably related to the reduction of infiltration and activation of neutrophils.

The presence of different substituents on the benzene ring (R2) did not exert significant influences on degranulation, although either -F or -Br slightly improved the inhibitory activity on superoxide generation. In contrast, the introduction of 5,5-dimethyl-1,3-dioxane at R1 and either a -Br or -F substituent at C-4 of the benzene ring (R2) resulted in higher inhibition of NO production by mouse macrophages, in addition to anti-inflammatory activity in the latter case.

Conclusion

Our data indicate that HIPs can exert in-vitro inhibitory effects on human neutrophil functions similar to those of imidazo[1,2-*a*]pyrimidines (Vidal et al 2001). They also decrease iNOS activity in mouse peritoneal macrophages. In addition, HIP-4 is active after oral administration to mice in carrageenan oedema as well as after intrapouch administration in the zymosan air pouch, exhibiting a greater anti-inflammatory activity than that of imidazo[1,2-*a*]pyrimidines. Thus, HIP is a new class of compounds of interest for the design of anti-inflammatory drugs.

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