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1-Methyl and 1-(2-hydroxyalkyl)-5-(3-alkyl/cycloalkyl/phenyl/naphthylureido)-1*H*-pyrazole-4-carboxylic acid ethyl esters as potent human neutrophil chemotaxis inhibitors

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fMLP-OMe
N-Formyl-methionyl-leucyl-phenylalanine methyl ester
Zymosan-induced peritonitis

ABSTRACT

In this paper we report the synthesis and the chemotaxis inhibitory activity of a number of 1*H*-pyrazole-4-carboxylic acid ethyl esters **2** functionalized in N1 with a methyl group or different hydroxyalkyl chains and in position 5 with a series of 3-substituted urea groups. These compounds were designed as development of previous pyrazole-urea derivatives that resulted potent IL8-induced neutrophil chemotaxis inhibitors in vitro. Most of the new compounds revealed a potent inhibition of both IL8- and fMLP-OMe-stimulated neutrophil chemotaxis. The most active compounds in the fMLP-OMe induced chemotaxis test showed IC_{50} in the range 0.19 nM–2 μM; but we observed a very strong inhibition in the IL8-induced chemotaxis test, having the most active compounds IC_{50} at pM concentrations. In vivo compounds **2e** and **2f**, although to a lesser extent, at 50 mg/kg os decreased granulocyte infiltration in zymosan-induced peritonitis in mice.

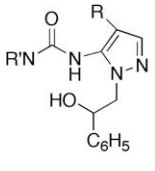
1. Introduction

Neutrophils are the main cells infiltrated in the course of acute inflammation. Their activation consists in subsequent events: margination, diapedesis and migration (chemotaxis) to site of inflammation where they take part in the phagocytosis of foreign particles and reactive free radicals production.[1,2] Chemotaxis is a very complex multi-step process induced by various chemoattractans as *N*-formyl-methionyl-leucyl-phenylalanine (fMLP),[3,4] platelet activating factor (PAF),[5] leukotriene B4 (LTB4),[6] C5a anaphylotoxin,[7] and different chemokines such as the CC chemokine MIP 1β (macrophage inhibition factor), or the CXC chemokine interleukine 8 (IL8, also named CXCL8).[8]

When the control of cellular recruitment breaks down, both acute and chronic autoimmune inflammatory disorders (e.g., asthma, rheumatoid arthritis, multiple sclerosis) will ensue.

In the last years the search for new small molecules able to interfere with numerous and different mechanisms involved in neutrophil upregulation greatly engaged many medicinal chemistry researchers.

In this context, we recently reported many pyrazoles (compounds **1**, Fig. 1) which resulted active as IL8-induced chemotaxis inhibitors in vitro.[9] These compounds, obtained by functionalization with a urea moiety of the 5-amino-1-(2-hydroxy-2-phenylethyl)-1*H*-pyrazole-4-carboxylic acid ethyl ester, blocked the IL8-



1

R = H, CN, $COOC_2H_5$
NR' = $NHCH(CH_3)_2$, cycloalkylamines
NHC_6H_5, $NHC_6H_4F(o,m,p)$,
$NHC_6H_4Cl(m,p)$, $NHC_6H_4Br(m,p)$,
$NHC_6H_4CH_3(m,p)$, $NHC_6H_4OCH_3(o,m)$
$NHCH_2C_6H_5$, $NHCH_2C_6H_4F(o,m,p)$,
$NHCH_2C_6H_4Cl(o,m,p)$, $NHCH_2C_6H_4Br(o,m,p)$

Figure 1. Chemical structure of compounds **1**.

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induced neutrophil chemotaxis, while they did not block fMLP-mediated chemotaxis. The most active compounds were 3-benzyl-, 3-(4-benzylpiperazinyl)-, 3-phenyl- and 3-isopropyl-ureido derivatives, which showed an IC_{50} of 10, 14, 45 and 55 nM, respectively. They were inactive in the binding assays on CXCR1 and CXCR2 (IL8 receptors), whereas they inhibited the phosphorylation of PTKs (Protein Tyrosine Kinases) in the 50–70 kDa region. Moreover, in the presence of the same derivatives, we observed a complete block of F-actin rise and pseudopod formation. The results of a preliminary in vivo study of these compounds and some SAR (structure–activity relationship) considerations suggested a relation between anti-chemotaxis activity and molecular lipophilicity.

To acquire further insight into the SAR of the pyrazolyl-ureas we synthesized a new series of 4-carboxyethyl-pyrazole derivatives (compounds **2a**–**u**, Fig. 2) substituted in N1 with more or less lipophilic chains, keeping constant the urea moieties in position 5 of the pyrazole scaffold which have given the best results in a previous series. Among compounds **1** the 3-fluorophenyl derivative showed a good anti-inflammatory activity in vivo, therefore we prepared now also some naphthyl-ureido derivatives as fluorophenyl bioisosteres and we tested some of the most active compounds for their anti-inflammatory activity in intraperitoneal zymosan-induced peritonitis in mice.

2. Chemistry

The condensation of ethyl (ethoxymethylene)cyanoacetate with commercial hydrazines (namely, methylhydrazine **3a**) or with hydrazines **3b–e**, in its turn obtained from proper oxyranes with hydrate hydrazine, gives the intermediates 5-amino-1-methyl- or 5-amino-1-(2-hydroxyalkyl)-1*H*-pyrazole-4-carboxylic acid ethyl esters **4a–e.** Title compounds (**2a–u**) were then synthesized starting from compounds **4a–e** following Method A or Method B (Scheme 1).

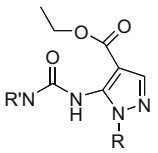
In the Method A the pyrazoles **4a–d** were preliminarily treated with phosgene in anhydrous THF and, later, with suitable amines yielding compounds **2b–d**, **i–k**, **p–u**. In the Method B compounds **4b–e** reacted with a little excess of proper isocyanate in anhydrous toluene, at reflux for 6 h, to give derivatives **2a**, **2e–h** and **2l–o**. IR and ^1H NMR spectral data confirmed the urea structure and excluded the involvement of the hydroxy group.

3. Biological results

Release of the cytoplasmic enzyme LDH was used as an indicator of cell viability. In none of the experiments described below (stimulated, unstimulated or compounds-treated neutrophils) was the percentage of total LDH release >3% (data not shown).

All compounds were tested in in vitro chemotaxis assays, in human neutrophils stimulated by 10 nM fMLP-OMe and 1 nM IL8 following the methods already reported.[10] Molecules were added to neutrophils 10 min before the incubation step for chemotaxis. The data of antagonism (percentage of activity) were obtained by comparing the chemotactic index (C.I.), in the absence and in the presence of derivatives.

In Figures 3 and 4 we reported the dose–response curves of the most active compounds toward the fMLP-OMe and IL8-induced chemotaxis, respectively. The potency of the novel antagonists was calculated through IC_{50} values, which represent the concentration able to inhibit the 50% of the fMLP-OMe- or IL8-induced chemotaxis. The experimental data represented by the percentage of the chemotaxis reduction were analysed through the computer



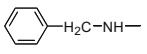
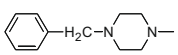
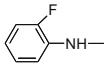
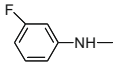
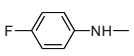
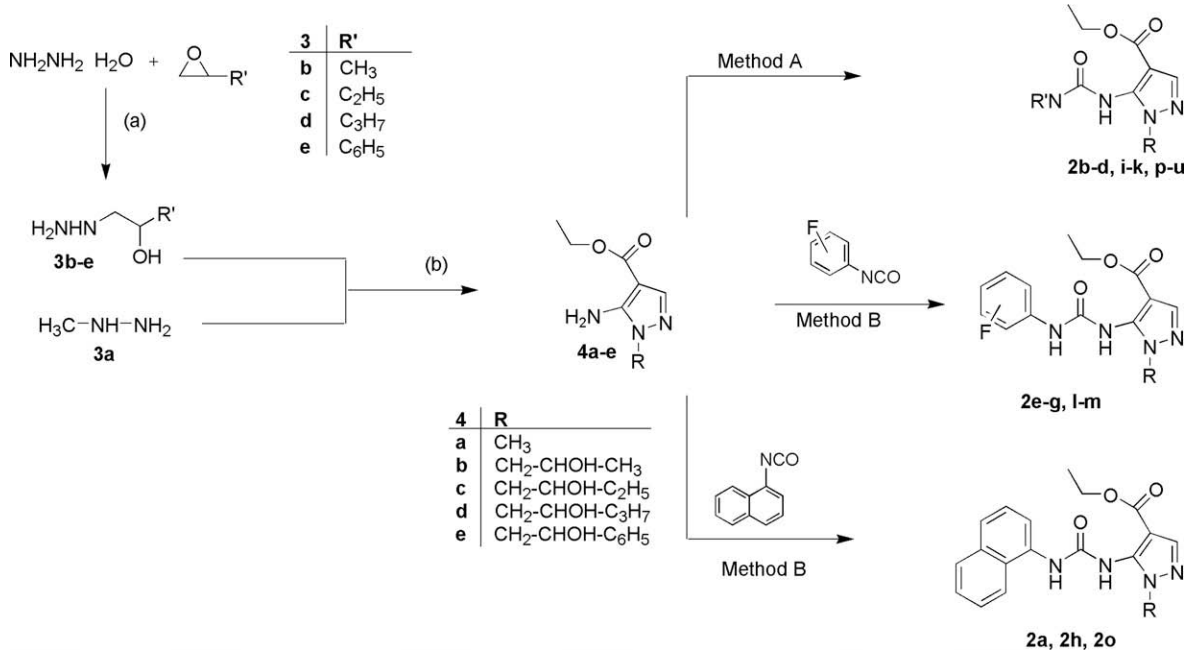
R =	R =	R =	R =	R =	NR’
	2b	**2i**	**2p**	**2s**	
	2c	**2j**	**2q**	**2t**	
	2d	**2k**	**2r**	**2u**	
	2e	**2l**			
	2f	**2m**			
	2g	**2n**			
2a	**2h**	**2o**			

Figure 2. Chemical structure of compounds **2a**–**u.**



Scheme 1. Reagents and conditions: (a) 15 min, 70–100 °C; (b) (ethoxymethylene)cyanoacetate, anhydrous toluene or absolute ethanol, reflux, 8–12 h. Method A: (1) anhydrous THF cooled at 0 °C, anhydrous CH_3COONa, phosgene (20% in toluene), reflux, 4 h; (2) anhydrous THF, amines added at 0 °C, rt overnight. Method B: isocyanate, anhydrous toluene, reflux, 6 h.

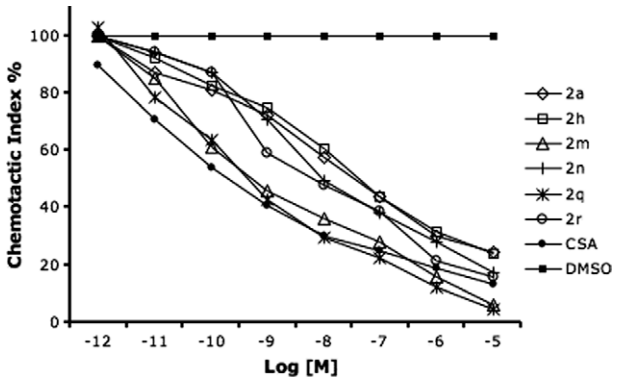


Figure 3. Dose responses curve of compounds **2a**, **2h**, **2m**, **2n**, **2q**, **2r** in neutrophil fMLP-OMe-stimulated chemotaxis. Data are expressed as a percentage of the C.I. (Chemotactic Index). SEs are within 10% of the mean value.

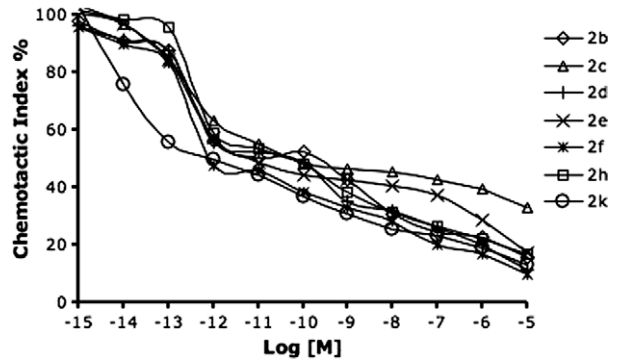


Figure 4. Dose responses curve of compounds **2b**, **2c**, **2d**, **2e**, **2f**, **2h**, **2k** in neutrophil IL8-stimulated chemotaxis. Data are expressed as a percentage of the C.I. (Chemotactic Index). SEs are within 10% of the mean value.

analysis by using the non-linear regression curve fitting computer program Graph Pad Prism (Graph Pad, San Diego, CA, USA) (Table 1). In addition, IC_{50} values were compared with positive (cyclosporine A, CSA) and negative (DMSO, blank) controls.

The most active compounds toward chemotaxis (**2c**, **2d**, **2e**, **2f**, **2h**, **2k, 2m** and **2q)** were selected to evaluate their anti-inflammatory effect in a mice model of zymosan-induced peritonitis. The study showed that compounds **2e** and **2f** (50 mg/kg os) were able to reduce granulocyte infiltration in the peritoneal exudates of 34 ± 5% ($P < 0.05$), and 21 ± 4%, respectively. The reference drug Dexamethasone (3 mg/kg os) reduced infiltration of 35 ± 5% ($P < 0.05$). Data for active compounds are reported in Table 2.

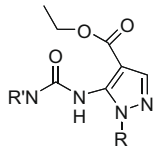
4. Discussion

All tested compounds inhibited the fMLP-OMe- and the IL8-induced chemotaxis at very low concentrations. With respect to the previous compounds (**1**) we remark a notable increase of activity for all the new pyrazolyl-ureas, particularly for those having a hydroxypropyl chain in N1 (see compounds **2b–h**). In fact, the most active compounds in the series **1** had IC_{50} ranging from 10 to 33 nM toward IL8-induced chemotaxis, while in this new series the most active compounds (**2b–h** and **2k**) showed IC_{50} inferior to 1 pM concentration; anyway, all the other compounds were very active, their IC_{50} ranging from 1.6 pM (compound **2l**) to 9.6 nM (compound **2o**).

In addition, while compounds **1** were completely inactive in the fMLP-OMe-induced chemotaxis, most part of compounds **2** showed relevant inhibitory activity, the IC_{50} ranging from 0.19 nM (compound **2m**) to 2 μM (compound **2k**); only few compounds (**2d,f,g,i,j**) showed low inhibition percent at 10 μM concentration, thereafter their IC_{50} values were not calculated.

We can also remark that the inhibitory activity toward the IL8-induced chemotaxis is more than thousand times greater than exhibited toward fMLP-OMe-induced cell recruitment. Interestingly, some of the most active compounds in the IL8-induced chemotaxis test (**2d–g, 2i–k**) were the less active in the fMLP-OMe-induced challenge. This evidence prompted us to hypothesize that compounds **2** differently affect chemotaxis activated by fMLP-OMe or IL8.

Table 1
Potency expressed as IC_{50} values of the novel compounds **2a–u** in the chemotaxis inhibition of human neutrophils



Compound	R	NR′	Inhibition of fMLP-OMe-induced neutrophil migration IC_{50} (nM)[a]	Inhibition of IL8-induced neutrophil migration IC_{50} (pM)[a]
2a	CH_2–CHOH–C_6H_5	α-Naphthylamino	7.8 ± 0.52	7.60 ± 1.0
2b	CH_2–CHOH–CH_3	Isopropylamino	90 ± 9.3	0.82 ± 0.07
2c	CH_2–CHOH–CH_3	Benzylamino	15 ± 1.1	0.54 ± 0.06
2d	CH_2–CHOH–CH_3	*N*-Benzylpiperazino	>10 μM (63%)	0.99 ± 0.08
2e	CH_2–CHOH–CH_3	*o*-F-anilino	100 ± 10.3	0.55 ± 0.01
2f	CH_2–CHOH–CH_3	*m*-F-anilino	>10 μM (60%)	0.70 ± 0.01
2g	CH_2–CHOH–CH_3	*p*-F-anilino	>10 μM (55%)	200 ± 60
2h	CH_2–CHOH–CH_3	α-Naphthylamino	9.8 ± 0.90	0.97 ± 0.06
2i	CH_2–CHOH–C_2H_5	Isopropylamino	>10 μM (52%)	4.2 ± 0.7
2j	CH_2–CHOH–C_2H_5	Benzylamino	>10 μM (54%)	7.4 ± 0.2
2k	CH_2–CHOH–C_2H_5	*N*-Benzylpiperazino	2000 ± 200	0.08 ± 0.003
2l	CH_2–CHOH–C_2H_5	*o*-F-anilino	120 ± 12	1.6 ± 0.2
2m	CH_2–CHOH–C_2H_5	*m*-F-anilino	0.19 ±0.01	360 ± 60
2n	CH_2–CHOH–C_2H_5	*p*-F-anilino	3.2 ± 0.40	1,900 ± 130
2o	CH_2–CHOH–C_2H_5	α-Naphthylamino	12 ± 1.2	9,600 ± 1,000
2p	CH_2–CHOH–C_3H_7	Isopropylamino	20 ± 2.2	1,000 ± 50
2q	CH_2–CHOH–C_3H_7	Benzylamino	0.43 ± 0.05	3.2 ± 0.1
2r	CH_2–CHOH–C_3H_7	*N*-Benzylpiperazino	1.1 ± 0.10	90 ± 4
2s	CH_3	Isopropylamino	77 ± 7.7	1.8 ± 0.5
2t	CH_3	Benzylamino	18 ± 1.6	1300 ± 110
2u	CH_3	*N*-Benzylpiperazino	150 ± 14.2	54.6 ± 0.5
CSA			0.047 ± 0.006	
DMSO			>10,000	

[a] The values are the means ± SEM of six independent experiments. IC_{50} values obtained from functional experiments are the concentration of tested compounds that inhibits the 50% of the maximal effect; the percentage in the parentheses represents the % of the reduction in neutrophil migration by the novel compounds at 10 μM concentration.

Table 2
In vivo anti-inflammatory activity of compounds **2e** and **2f** on zymosan-induced peritonitis in mice

Treatments	Protein conc. (μg/mL)	Cells (millions/cavity)	Granulocytes (millions/cavity)	Relative composition	
				Granulo cytes (%)	Mono/lympho cytes (%)
Saline	0.63 ± 0.07	5.5 ± 0.8	1.8 ± 0.3	33 ± 3	67 ± 3
Zymosan	2.65 ± 0.18	31.6 ± 2.3	27.2 ± 2.3	86 ± 1	14 ± 1
Zymosan + Dexamethasone 3 mg/kg os	1.75 ± 0.13**	21.8 ± 1.8**	17.6 ± 1.6**	81 ± 2	20 ± 2
Zymosan + **2e** 50 mg/kg os	2.62 ± 0.27	24.3 ± 2.1*	18.0 ± 2.2**	74 ± 4**	26 ± 4**
Zymosan + **2f** 50 mg/kg os	2.68 ± 0.39	28.1 ± 3.1	21.6 ± 2.2	77 ± 2**	23 ± 3**

*$p < 0.05$ **$p < 0.01$ by student's *t*-test compared with zymosan treated mice. All the data of zymosan group are significantly different ($p < 0.001$) when compared with saline group. Animals received the compounds under study or dexamethasone 1 h before intraperitoneal injection of 1 mg zymosan. Peritoneal exudates were collected 4 h later and analyzed. Data for inactive compounds (**2c**, **2d**, **2h**, **2k**, **2m**, **2q**) are not reported.

As concerns the structure–activity relationship we can underline that the presence of hydroxypropylchain in N1 gave the most active compounds in IL8-induced chemotaxis test (**2c,d,e,f,h**): its very strong activity seems independent of the urea moiety substituents. In this sub-group compound **2g** (NR′ = *p*-F-anilino) is the sole exception having an IC_{50} of 200 pM.

The increase of chain length and lipophilicity causes, in general, a proportional decrease of chemotaxis inhibition. Compound **2k** (hydroxybutyl chain in N1 and NR′ = *N*-benzylpiperazino) is an exception being the most active with an IC_{50} of 0.08 pM. Also the presence of a simple methyl group in N1 (see compounds **2s–u**) is detrimental for the action. No clear correlation has been found from the urea substituents and activity, except for the fluoroanilino derivatives which were always active in the order *ortho* > *meta* > *para* (see compounds **2e–g** and **2l–n**). Moreover, it is worthy to note that the introduction of a α-naphthyl-ureido group gave active compounds, also in the presence of hydroxyphenylethyl chain (incidentally, the chain present in the previous compounds **1**) in N1 (compound **2a**).

In the fMLP-OMe-induced chemotaxis compounds **2m** (hydroxybutyl chain, NR′ = *m*-F-anilino) and **2q** (hydroxypentyl chain, NR′ = benzylamino) were the most active agents with IC_{50} of 0.19 and 0.43 nM, respectively. The other results pointed out only the detrimental effect of hydroxypropyl chain in N1, while it is difficult to correlate the activity with the urea substituents.

To study the in vivo anti-inflammatory effect of the most attractive compounds we chose the zymosan-induced peritonitis test, an experimental model of inflammation characterized by an early phase (4 h) with a considerable infiltration of granulocytes at the inflammation site and a late phase with mononuclear and lymphocyte number increase during progression of the process (24 h). The

analysis of mice peritoneal exudates taken 4 h after zymosan injection revealed a significant increase of protein concentration, cells and granulocyte infiltration in comparison with saline-treated animals. Dexamethasone (3 mg/kg os) decreases the cell number, and in particular granulocyte infiltration, due to the inhibition of chemokine production whereas it is known that NSAIDs like indomethacin (10 mg/kg os) are completely inactive because of the poor participation of arachidonic acid metabolites in this process.[11] (Table 2)

In the previous study on group **1** compounds, the 3-fluoroanilino derivative evidenced a good anti-inflammatory activity, causing a 33% inhibition of granulocyte recruitment at 100 mg/kg os. In the present work, most of the tested compounds (**2c**, **2d**, **2h**, **2k**, **2m**, **2q**) did not exhibit anti-inflammatory activity leaving unmodified protein content, cells and granulocytes present in the peritoneal cavity lavage of zymosan-treated mice. Also compounds **2e** and **2f** (*ortho*- and *meta*-fluoroanilino derivatives) (50 mg/kg os) did not modify pleural fluid protein content at variance with dexamethasone, but they reduced relative and absolute granulocyte infiltration. In particular compound **2e** showed a higher potency than compound **2f**.

5. Conclusions

The structural modification of previously reported pyrazolyl-ureas **1** gave a new series of 1-methyl- and 1-(2-hydroxyalkyl)-5-(3-alkyl/cycloalkyl/phenyl/naphthylureido)-1*H*-pyrazole-4-carboxylic acid ethyl esters **2a**–**u** that resulted potent inhibitors of human neutrophil chemotaxis both fMLP-OMe- and IL8-induced. Particularly, compounds having a hydroxypropyl chain in N1 showed a very strong inhibitory activity toward IL8. Among them, the 5-[3-(2-fluorophenyl)ureido]- and 5-[3-(3-fluorophenyl)ureido]-derivatives (**2e** and to a lesser extent **2f**) reduced granulocyte migration in mice peritoneal exudates caused by intraperitoneal zymosan application.

The SAR study evidenced a good correlation between chemotaxis inhibitory activity and length and lipophilicity of N1 chain, while the in vitro activity not strictly depends on the nature of urea group. Only the 2-fluoroanilino and 3-fluoroanilino derivatives showed anti-inflammatory activity in vivo, in the mouse model of zymosan-induced peritonitis, confirming the results of the previous compounds **1**.

Members of the CXC and FPR (formyl peptide) receptor families all induce multiple neutrophil functions, including cell migration, granule secretion and superoxide anion generation. As all belong to the pertussis toxin-sensitive GPCR superfamily, it has been generally accepted that they possess many signalling similarities. However, we have previously reported that neutrophils chemotaxis, activated by fMLP and pure chemoattractants, is almost insensitive to any variation of Ca^{++} concentration and this could be related to its poor efficacy in enhancing cAMP level.[12,13] Moreover, more recently, it has been demonstrated that the chemotactic response is associated with specific PKC β_1 isoform translocation and p38 mitogen-activated protein kinase phosphorylation by two independent pathways.[14] Heit and co-workers[15] proposed a model in which the end target chemoattractants (such as fMLP) functions primarily by stimulating p38 MAPK, whereas intermediary chemoattractants (such as IL8) primarily function via the PI3 K/Akt (protein kinase B) pathway, and a hierarchy between the two pathways exists. The same signalling hierarchy exists with regard to NADPH-oxidase activation by formylated peptide and IL8.[16]

Results of the in vitro screening suggested a possible interference of our compounds in different mechanisms of neutrophil recruitment. Therefore, a more complete understanding of intracellular signalling triggered by different chemotaxines, is of great importance in models of inflammation and these considerations represent the rational base and stimulate further studies for the design of new anti-inflammatory agents.

6. Experimental protocols

6.1. Chemistry

6.1.1. General

Chiminord and Aldrich Chemical, Milan, Italy purchased all chemicals. Solvents were reagent grade. THF was dried by filtration on neutral Al_2O_3 column and stored on molecular sieves (5 Å 1/1600 pellets). Toluene was stored in the presence of sodium. Unless otherwise stated, all commercial reagents were used without further purification. Organic solutions were dried over anhydrous magnesium sulphate.

Aluminium backed silica gel plates (Merck DC-Alufolien Kieselgel 60 F254) were used in thin-layer chromatography (TLC) for routine monitoring the course of reactions. Detection of spots was made by UV light. Merck silica gel, 230–400 mesh, was used for chromatography.

Melting points are not 'corrected' and were measured with a Büchi 540 instrument. IR spectra were recorded with a Perkin–Elmer 398 spectrophotometer. ^{1}H NMR spectra were recorded on a Varian Gemini 200 (200 MHz) instrument; chemical shifts are reported as δ (ppm) relative to tetramethylsilane (TMS) as internal standard; signals were characterized as s (singlet), d (doublet), t (triplet), q (quartet), sept (septet) m (multiplet), br s (broad signal); *J* in Hertz. Elemental analyses, indicated by the symbols of the elements were within ±0.4% of the theoretical values and were determined with an Elemental Analyzer EA 1110 (Fison-Instruments, Milan, Italy).

6.1.2. Syntheses of 1-hydrazino-alkan-2-ols 3b–e

1-Hydrazinopropan-2-ol **3b** and 1-hydrazinobutan-2-ol **3c** were synthesized following the method already reported.[17] 2-Hydrazino-2-phenylethanol **3e** was synthesized following the method already reported.[18]

6.1.2.1. Synthesis of 1-hydrazinopentan-2-ol 3d. To hydrazine hydrate (30 mL, 0.6 mol) heated at 60–70 °C, 1,2-epoxypentane (14.64 g, 0.17 mol) was added drop wise and the mixture was heated at 90–95 °C for 1 h. Then, the excess of hydrazine hydrate was removed under reduced pressure and the crude was distilled bulb to bulb to give a viscous oil that becomes wax solid.

Yield 82%, bp 120 °C/0.6 mmHg.

6.1.3. General procedure for 5-amino-1-(2-hydroxyalkyl)-1*H*-pyrazole-4-carboxylic acid ethyl esters 4b,c,d

To a solution of the proper 1-hydrazino-alcohol (**3b, 3c**, **3d)** (20 mmol) in anhydrous toluene (for **3b**) or in absolute ethanol (for **3c** and **3d**) (20 mL), ethyl(ethoxymethylene)cyanoacetate (3.38 g, 20 mmol) was added and the mixture was heated at 70–80 °C for 8 h. After cooling, the toluene was washed with H_2O (20 mL), dried ($MgSO_4$) and concentrated under reduced pressure (compound **4b**). Whereas, the ethanol solutions were concentrated under reduced pressure and cooled to room temperature to give yellow solids (compounds **4c** and **4d**). The crudes were purified by flash-chromatography using a mixture of CH_2Cl_2/CH_3OH (8:2) as eluent and then were crystallized by adding diethyl ether. The solids obtained were recrystallized from absolute ethanol.

6.1.3.1. 5-Amino-1-(2-hydroxypropyl)-1*H*-pyrazole-4-carboxylic acid ethyl ester 4b. Yield 61%, mp 66–68 °C. ^{1}H NMR ($CDCl_3$): δ 1.26 (d, *J* = 6.4, 3H, CH_3CH), 1.35 (t, *J* = 7.0, 3H, CH_3CH_2), 3.13 (br s,

1H, OH, disappears with D_2O), 3.78–4.42 (m, 5H, CH_2O + CH_2N + CH), 5.44 (br s, 2H, NH_2, disappears with D_2O), 7.61 (s, 1H, H_3). IR (KBr): cm^{-1} 3426, 3390, 3313 (NH_2 + OH), 1697 (CO).

Anal. Calcd for ($C_9H_{15}N_3O_3$): C, 50.69; H, 7.09; N, 19.71. Found: C, 50.79; H, 7.06; N, 19.88.

6.1.3.2. 5-Amino-1-(2-hydroxybutyl)-1*H*-pyrazole-4-carboxylic acid ethyl ester 4c. Yield 61%, mp 90–91 °C. ^1H NMR (DMSO-d_6): δ 0.88 (t, J = 7.4, 3H, CH_3), 1.23 (t, J = 7.0, 3H, CH_3CH_2O), 1.24–1.43 (m, 2H, CH_2CH), 3.62–3.77 (m, 1H, CH), 3.85 (d, J = 5.6, 2H, CH_2N), 4.16 (q, J = 7.4, 2H, CH_2O), 4.98 (d, J = 5.2, 1H, OH, disappears with D_2O), 6.09 (br s, 2H, NH_2, disappears with D_2O), 7.45 (s, 1H, H_3). IR (KBr): cm^{-1} 3433, 3343 (NH_2), 3209 (OH), 1689 (CO).

Anal. Calcd for ($C_{10}H_{17}N_3O_3$): C, 52.85; H: 7.54; N, 18.49. Found: C, 52.42; H, 7.60; N, 18.66.

6.1.3.3. 5-Amino-1-(2-hydroxypentyl)-1*H*-pyrazole-4-carboxylic acid ethyl ester 4d. Yield 72%, mp 84–86 °C. ^1H NMR ($CDCl_3$): δ 0.95 (t, J = 7.0, 3H, CH_3), 1.37 (t, J = 7.0, 3H, CH_3CH_2O), 1.38–1.57 (m, 4H, $2CH_2$), 3.19 (br s, 1H, OH, disappears with D_2O), 3.75–3.91 (m, 1H, CH), 3.86–4.08 (m, 2H, CH_2N), 4.25 (q, J = 7.0, 2H, CH_2O), 5.46 (br s, 2H, NH_2, disappears with D_2O), 7.59 (s, 1H, H_3). IR (KBr): cm^{-1} 3405, 3291, 3215 (NH_2 + OH), 1690 (CO).

Anal. Calcd for ($C_{11}H_{19}N_3O_3$): C, 54.76; H, 7.94; N, 17.41. Found: C, 54.74; H, 7.92; N, 17.44.

6.1.3.4. 5-Amino-1-methyl-1*H*-pyrazole-4-carboxylic acid ethyl ester 4a. Compound **4a** was prepared by condensation of 1-methyl-hydrazine **3a** with ethyl (ethoxymethylene)cyanoacetate in anhydrous toluene, as previously reported.[19]

6.1.3.5. 5-Amino-1-(2-hydroxy-2-phenylethyl)-1*H*-pyrazole-4-carboxylic acid ethyl ester 4e. Compound **4e** was prepared by condensation of 2-hydrazino-1-phenylethanol **3e** with ethyl (ethoxymethylene)cyanoacetate in anhydrous toluene, as previously reported.[20]

6.1.4. Synthesis of 1-(2-hydroxy-2-phenylethyl)-5-[3-(1-naphthyl)ureido]-1*H*-pyrazole-4-carboxylic acid ethyl ester 2a (Method A)

To a suspension of 5-amino-1-(2-hydroxy-2-phenylethyl)-1*H*-pyrazole-4-carboxylic acid ethyl ester **4e** (1.38 g, 5 mmol) in anhydrous toluene (10 mL), α-naphthylisocyanate (1.02 g, 6 mmol) was added and the reaction mixture was refluxed for 6 h. After cooling, the solvent was evaporated under reduced pressure and the crude was solved in CH_2Cl_2 (10 mL), washed with H_2O (2 × 10 mL) and with 1 M HCl (10 mL), dried ($MgSO_4$) and concentrated under reduced pressure. The crude crystallized as a white solid by adding a mixture of diethyl ether/ petroleum ether (bp 40–60 °C) (1:1).

Yield 52%, mp 181–183 °C. ^1H NMR ($CDCl_3$): δ 1.26 (t, J = 7.2, 3H, CH_3), 1.61 (br s, 2H, disappears with D_2O) 4.06–4.28 (m, 4H, CH_2O + CH_2N), 5.93–6.04 (m, 1H, *CH*OH), 7.14–7.90 (m, 14H, 12HAr + H_3 + 1H which disappears with D_2O).

IR (KBr): cm^{-1} 3447, 3393, 3328 (NH, OH), 1710 (COOEt), 1676 (CONH).

Anal. Calcd for ($C_{25}H_{24}N_4O_4$): C, 67.54; H, 5.45; N, 12.61. Found: C, 67.40; H, 5.74; N, 12.63.

6.1.5. General procedure for 1-(2-hydroxyalkyl)-5-(3-alkyl/cycloalkylureido)-1*H*-pyrazole-4-carboxylic acid ethyl esters 2b–d, 2i–k and for 1-methyl-5-(3-alkyl/cycloalkylureido)-1*H*-pyrazole-4-carboxylic acid ethyl esters 2p–u (Method B)

To a mixture of the suitable 5-amino-1*H*-pyrazole-4-carboxylic acid ethyl esters (**4a–d**) (10 mmol) and anhydrous CH_3COONa (2.0 g, 24 mmol) in anhydrous THF (30 mL), cooled at 0 °C, phosgene (20% in toluene, 8 mL) was added drop wise; then, the reaction mixture was heated at reflux for 4 h. After removal in vacuo of the excess phosgene, the suitable amine (25 mmol), solved in anhydrous THF, was added drop wise, at 0 °C; the mixture was stirred at room temperature overnight. After removal of the volatiles in vacuo, the crude was suspended in water (20 mL), extracted twice with CH_2Cl_2 (20 mL) and dried ($MgSO_4$). The solvent was evaporated under reduced pressure to give yellow oils, which were purified by silica gel column chromatography using diethyl ether as eluant. The white solids obtained were recrystallized from absolute ethanol.

6.1.5.1. 1-(2-Hydroxypropyl)-5-(3-isopropylureido)-1*H*-pyrazole-4-carboxylic acid ethyl ester 2b. Yield 58%, mp 93–94 °C. ^1H NMR ($CDCl_3$): δ 1.19 (d, J = 6.6, 6H, $2CH_3$ isop.), 1.30–1.46 (m, 6H, $2CH_3$), 3.72–3.90 (m, 1H, *CH*NH), 3.99–4.13 (m, 2H, CH_2N), 4.29 (q, J = 7.2, 2H, CH_2O), 4.55–4.70 (m, 1H, disappears with D_2O), 4.76–4.90 (m, 1H, *CH*OH), 5.61–5.69 (m, 2H, disappears with D_2O), 7.62 (s, 1H, H_3). IR (KBr): cm^{-1} 3456, 3357, 3322 (NH, OH), 1693 (COOEt), 1665 (CONH).

Anal. Calcd for ($C_{13}H_{22}N_4O_4$): C, 52.34; H, 7.43; N, 18.78. Found: C, 52.44; H, 7.26; N, 18.92.

6.1.5.2. 1-(2-Hydroxypropyl)-5-(3-benzylureido)-1*H*-pyrazole-4-carboxylic acid ethyl ester 2c. Yield 52%, mp 72–73 °C. ^1H NMR ($CDCl_3$): δ 1.20–1.35 (m, 6H, $2CH_3$), 3.96–4.07 (m, 2H, CH_2N), 4.19 (q, J = 7.0, 2H, CH_2O), 4.30 (d, J = 5.8, 2H, CH_2Ar), 4.74–4.90 (m, 1H, *CH*OH), 5.10–5.21 (m, 1H, disappears with D_2O), 5.41–5.76 (m, 2H, disappear with D_2O), 7.12–7.35 (m, 5H, Ar), 7.54 (s, 1H, H_3). IR (KBr): cm^{-1} 3429, 3327, 3229 (NH, OH), 1703 (COOEt) 1688 (CONH).

Anal. Calcd for ($C_{17}H_{22}N_4O_4$): C, 58.95; H, 6.40; N, 16.17. Found: C, 59.04; H, 6.69; N, 15.95.

6.1.5.3. 1-(2-Hydroxypropyl)-5-[3-(4-benzyl)piperazinylureido]-1*H*-pyrazole-4-carboxylic acid ethyl ester 2d. Yield 58%, mp 129–130 °C. ^1H NMR ($CDCl_3$): δ 1.29–1.41 (m 6H, $2CH_3$), 2.34–2.50 (m, 4H, $2CH_2N$ piperaz.), 3.42–3.57 (m, 6H, $2CH_2N$ piperaz + CH_2Ar), 3.92–4.18 (m, 2H, CH_2N piraz.), 4.28 (q, J = 7.2, 2H, CH_2O), 4.73–4.88 (m, 1H, *CH*OH), 5.61–5.69 (m, 2H, disappears with D_2O), 7.23–7.36 (m, 5H, Ar), 7.60 (s, 1H, H_3). IR (KBr): cm^{-1} 3402, 3302, 3225 (NH, OH), 1700–1680 (COOEt + CONH).

Anal. Calcd for ($C_{21}H_{29}N_5O_4$): C, 60.71; H, 7.04; N, 16.86. Found: C, 60.93; H, 7.26; N, 16.96.

6.1.5.4. 1-(2-Hydroxybutyl)-5-(3-isopropylureido)-1*H*-pyrazole-4-carboxylic acid ethyl ester 2i. Yield 51%, mp 102–103 °C. ^1H NMR ($CDCl_3$): δ 0.97 (t, J = 7.4, 3H, CH_3), 1.19 (d, J = 6.6, 6H, $2CH_3$ isop.), 1.35 (t, J = 7.0, 3H, CH_3CH_2O), 1.57–1.89 (m, 2H, CH_2CH), 3.75–3.93 (m, 1H, *CH*NH), 4.07–4.14 (m, 2H, CH_2N), 4.28 (q, J = 7.0, 2H, CH_2O), 4.52–4.74 (m, 2H, *CH*OH + 1H which disappears with D_2O), 5.72–5.85 (m, 2H, disappears with D_2O), 7.60 (s, 1H, H_3). IR (KBr): cm^{-1} 3416, 3352, 3307 (NH, OH), 1700–1680 (COOEt + CONH).

Anal. Calcd for ($C_{14}H_{24}N_4O_4$): C, 53.83; H, 7.74; N, 17.94. Found: C, 53.86; H, 7.68; N, 17.93.

6.1.5.5. 1-(2-Hydroxybutyl)-5-(3-benzylureido)-1*H*-pyrazole-4-carboxylic acid ethyl ester 2j. Yield 68%, mp 99–101 °C. ^1H NMR ($CDCl_3$): δ 0.89 (t, J = 7.4, 3H, CH_3), 1.26 (t, J = 7.2, 3H, CH_3CH_2O), 1.53–1.80 (m, 2H, CH_2CH), 4.00 (d, J = 5.4, 2H, CH_2N), 4.14 (q, J = 7.2, 2H, CH_2O), 4.30 (d, J = 6.0, 2H, CH_2Ar), 4.53–4.66 (m, 1H, CH), 5.20–5.30 (m, 1H, disappears with D_2O), 5.49–5.80 (m, 2H, disappears with D_2O), 7.14–7.33 (m, 5H, Ar), 7.56 (s, 1H, H_3). IR (KBr): cm^{-1} 3424, 3298, 3320 (NH, OH), 1698 (COOEt), 1674 (CONH).

Anal. Calcd for ($C_{18}H_{24}N_4O_4$): C, 59.99; H, 6.71; N, 15.55. Found: C, 60.32; H, 6.67; N, 15.49.

6.1.5.6. 1-(2-Hydroxybutyl)-5-[3-(4-benzyl)piperazinylureido)-1*H*-pyrazole-4-carboxylic acid ethyl ester 2k. Yield 47%, mp 117–118 °C. ^{1}H NMR ($CDCl_3$): δ 0.97 (t, J = 7.4, 3H, CH_3), 1.35 (t, J = 7.0, 3H, CH_3CH_2O), 1.60–1.92 (m, 2H, CH_2CH), 2.33–2.51 (m, 4H, $2CH_2N$ piperaz.), 3.43–3.60 (m, 6H, $2CH_2N$ piperaz. + CH_2Ar), 4.02–4.10 (m, 2H, CH_2N piraz.), 4.27 (q, J = 7.0, 2H, CH_2O), 4.50–4.63 (m, 1H, *CH*OH), 5.78–5.92 (m, 2H, disappears with D_2O), 7.21–7.41 (m, 5H, Ar), 7.59 (s, 1H, H_3). IR (KBr): cm^{-1}3418, 3331, 3231 (NH, OH), 1700–1660 (COOEt + CONH).

Anal. Calcd for ($C_{22}H_{31}N_5O_4$): C, 61.52; H, 7.27; N, 16.31. Found: C, 61.58; H, 7.29; N, 16.12.

6.1.5.7. 1-(2-Hydroxypentyl)-5-(3-isopropylureido)-1*H*-pyrazole-4-carboxylic acid ethyl ester 2p. Yield 49%, mp 70–71 °C. ^{1}H NMR ($CDCl_3$): δ 0.91 (t, J = 7.2, 3H, CH_3), 1.19 (d, J = 6.6, 6H, $2CH_3$ isop.), 1.35 (t, J = 7.2, 3H, CH_3CH_2O), 1.40–1.60 (m, 4H, $2CH_2$), 3.73–3.90 (m, 1H, *CH*NH), 4.01–4.11 (m, 2H, CH_2N), 4.28 (q, J = 7.2, 2H, CH_2O), 4.58–4.74 (m, 2H, *CH*OH + 1H which disappears with D_2O), 5.69–5.83 (m, 2H, disappears with D_2O), 7.60 (s, 1H, H_3). IR (KBr): cm^{-1} 3420, 3350, 3312 (NH, OH), 1693–1680 (COOEt + CONH).

Anal. Calcd for ($C_{15}H_{26}N_4O_4$): C, 55.20; H, 8.03; N, 17.17. Found: C, 55.29; H, 8.17; N. 17.18.

6.1.5.8. 1-(2-Hydroxypentyl)-5-(3-benzylureido)-1*H*-pyrazole-4-carboxylic acid ethyl ester 2q. Yield 50%, mp 111–112 °C. ^{1}H NMR ($CDCl_3$): δ 0.82 (t, J = 7.2, 3H, CH_3), 1.26 (t, J = 7.2, 3H, CH_3CH_2O), 1.31–1.69 (m, 4H, $2CH_2$), 3.99 (d, J = 5.4, 2H, CH_2N), 4.18 (q, J = 7.2, 2H, CH_2O), 4.30 (d, J = 5.8, 2H, CH_2Ar), 4.58–4.75 (m, 1H, *CH*OH), 5.18–5.29 (m, 1H, disappears with D_2O), 5.46–5.80 (m, 2H, disappears with D_2O), 7.13–7.34 (m, 5H, Ar), 7.51 (s, 1H, H_3). IR (KBr): cm^{-1} 3446, 3370, 3331 (NH, OH), 1724 (COOEt) 1694 (CONH).

Anal. Calcd for ($C_{19}H_{26}N_4O_4$): C: 60.95; H, 7.00; N, 14.96. Found: C, 60.87; H, 6.93; N, 15.09.

6.1.5.9. 1-(2-Hydroxypentyl)-5-[3-(4-benzyl)piperazinylureido)-1*H*-pyrazole-4-carboxylic acid ethyl ester 2r. Yield 68%, mp 128–129 °C. ^{1}H NMR ($CDCl_3$): δ 0.91 (t, J = 7.2, 3H, CH_3), 1.35 (t, J = 7.2, 3H, CH_3CH_2O), 1.40–1.81 (m, 4H, $2CH_2$), 2.37–2.51 (m, 4H, $2CH_2N$ piperaz.), 3.44–3.59 (m, 6H, $2CH_2N$ piperaz. + CH_2Ar), 4.01–4.16 (m, 2H, CH_2N piraz.), 4.28 (q, J = 7.2, 2H, CH_2O), 4.55–4.70 (m, 1H, *CH*OH), 5.80–5.89 (m, 2H, disappears with D_2O), 7.27–7.41 (m, 5H, Ar), 7.60 (s, 1H, H_3). IR (KBr): cm^{-1} 3402, 3307, 3227 (NH, OH), 1700–1680 (COOEt + CONH).

Anal. Calcd for ($C_{23}H_{33}N_5O_4$): C, 62.28; H, 7.50; N, 15.79 Found: C, 62.04; H, 7.34; N, 15.70.

6.1.6. General procedure for 1-(2-hydroxyalkyl)-5-[3-(1-naphthyl)ureido]-1*H*-pyrazole-4-carboxylic acid ethyl esters 2h, 2o (Method A)

To a suspension of the suitable 5-amino-1-(2-hydroxyalkyl)-1*H*-pyrazole-4-carboxylic acid ethyl esters (**4b** or **4c**) (2.2 mmol) in anhydrous toluene (10 mL), α-naphthylisocyanate (0.51 g, 3 mmol) was added and the reaction mixture was refluxed for 6 h. After cooling, the white solids obtained were filtered and recrystallized from absolute ethanol.

6.1.6.1. 1-(2-Hydroxypropyl)-5-[3-(1-naphthyl)ureido]-1*H*-pyrazole-4-carboxylic acid ethyl ester 2h. Yield 92%, mp 124–126 °C. ^{1}H NMR ($CDCl_3$): δ 1.26 (t, J = 7.0, 3H, CH_3CH_2), 1.35 (d, J = 6.4, 3H, CH_3CH), 3.90–4.10 (m, 2H, CH_2N), 4.18 (q, J = 7.0, 2H, CH_2O), 4.86–5.08 (m, 1H, *CH*OH), 5.20–5.60 (m, 2H, disappears with D_2O), 6.96–7.92 (m, 9H, 7HAr + H_3 + 1H which disappears with D_2O). IR (KBr): cm^{-1} 3414, 3322, 3224 (NH, OH), 1709 (COOEt), 1682 (CONH).

Anal. Calcd for ($C_{20}H_{22}N_4O_4$): C, 62.82; H, 5.80; N, 14.65. Found: C, 62.69; H, 5.96; N, 14.36.

6.1.6.2. 1-(2-Hydroxybutyl)-5-[3-(1-naphthyl)ureido]-1*H*-pyrazole-4-carboxylic acid ethyl ester 2o. Yield 60%, mp 121–123 °C. ^{1}H NMR (DMSO-d_6): δ 0.88 (t, J = 7.4, 3H, CH_3), 1.16 (t, J = 7.2, 3H, CH_3CH_2O), 1.40–1.73 (m, 2H, CH_2CH), 3.98–4.18 (m, 4H, CH_2O + CH_2N), 4.90–5.05 (m, 1H, *CH*OH), 6.10–6.23 (m, 2H, disappears with D_2O), 7.28–7.93 (m, 8H, 7HAr + H_3), 9.34 (br s, 1H, disappears with D_2O). IR (KBr): cm^{-1} 3450, 3394, 3323 (NH, OH), 1711 (COOEt), 1673 (CONH).

Anal. Calcd for ($C_{21}H_{24}N_4O_4$): C, 63.62; H, 6.10; N, 14.13. Found: C, 63.46; H, 5.98; N, 13.79.

6.1.7. General procedure for 1-(2-hydroxyalkyl)-5-[3-(2/3/4-fluorophenyl)ureido]-1*H*-pyrazole-4-carboxylic acid ethyl esters 2e–g, 2l–n (Method A)

A mixture of the suitable 5-amino-1-(2-hydroxyalkyl)-1*H*-pyrazole-4-carboxylic acid ethyl esters (**4b** or **4c**) (2.2 mmol) and the proper fluoro-phenylisocyanate (0.41 g, 3 mmol) in anhydrous toluene (30 mL) was refluxed for 6 h. After cooling, the solution was washed with 3 M HCl (2 × 20 mL), with water (20 mL), dried ($MgSO_4$) and evaporated under reduced pressure. The crude crystallized by adding a solution of diethyl ether/petroleum ether (p.eb. 50–60 °C) 1/1. The white solids obtained were recrystallized from absolute ethanol.

6.1.7.1. 1-(2-Hydroxypropyl)-5-[3-(2-fluorophenyl)ureido]-1*H*-pyrazole-4-carboxylic acid ethyl ester 2e. Yield 34%, mp 131–132 °C. ^{1}H NMR ($CDCl_3$): δ 1.27 (t, J = 7.0, 3H, CH_3CH_2), 1.35 (d, J = 6.6, 3H, CH_3CH), 3.92–4.10 (m, 2H, CH_2N), 4.20 (q, J = 7.0, 2H, CH_2O), 4.93–5.03 (m, 1H, *CH*OH), 5.36–5.55 (m, 2H, disappears with D_2O), 6.79–8.02 (m, 6H, 4HAr + H_3 + 1H which disappears with D_2O). IR (KBr): cm^{-1} 3425, 3335, 3235 (NH, OH), 1711 (COOEt), 1689 (CONH).

Anal. Calcd for ($C_{16}H_{19}N_4O_4F$): C, 54.85; H, 5.47; N, 15.99. Found: C, 54.66; H, 5.63; N, 15.93.

6.1.7.2. 1-(2-Hydroxypropyl)-5-[3-(3-fluorophenyl)ureido]-1*H*-pyrazole-4-carboxylic acid ethyl ester 2f. Yield 48%, mp 146–147 °C. ^{1}H NMR ($CDCl_3$): δ 1.20–1.36 (m, 6H, $2CH_3$), 3.90–4.07 (m, 2H, CH_2N), 4.20 (q, J = 7.2, 2H, CH_2O), 4.84–5.03 (m, 1H, *CH*OH), 5.35–5.52 (m, 2H, disappears with D_2O), 6.65–7.30 (m, 5H, 4HAr + 1H which disappears with D_2O), 7.55 (s, 1H, H_3). IR (KBr): cm^{-1} 3427, 3336, 3266 (NH, OH), 1712 (COOEt), 1689 (CONH).

Anal. Calcd for ($C_{16}H_{19}N_4O_4F$): C, 54.85; H, 5.47; N, 15.99. Found: C, 54.69; H, 5.50; N, 15.72.

6.1.7.3. 1-(2-Hydroxypropyl)-5-[3-(4-fluorophenyl)ureido]-1*H*-pyrazole-4-carboxylic acid ethyl ester 2g. Yield 50%, mp 175–177 °C. ^{1}H NMR ($CDCl_3$): δ 1.14–1.36 (m, 6H, $2CH_3$), 3.89–4.08 (m, 2H, CH_2N), 4.19 (q, J = 7.2, 2H, CH_2O), 4.82–5.00 (m, 1H, *CH*OH), 5.36–5.54 (m, 2H, disappears with D_2O), 6.84–7.32 (m, 5H, 4HAr + 1H which disappears with D_2O), 7.54 (s, 1H, H_3). IR (KBr): cm^{-1} 3413, 3321, 3215 (NH, OH), 1709 (COOEt), 1682 (CONH).

Anal. Calcd for ($C_{16}H_{19}N_4O_4F$): C, 54.85; H, 5.47; N, 15.99. Found: C, 54.96; H, 5.37; N, 15.77.

6.1.7.4. 1-(2-Hydroxybutyl)-5-[3-(2-fluorophenyl)ureido]-1*H*-pyrazole-4-carboxylic acid ethyl ester 2l. Yield 42%, mp 99–100 °C. ^{1}H NMR ($CDCl_3$): δ 0.95 (t, J = 7.6, 3H, CH_3), 1.27 (t, J = 7.0, 3H, CH_3CH_2O), 1.56–1.86 (m, 2H, CH_2CH), 4.07 (d, J = 5.8, 2H, CH_2N), 4.20 (q, J = 7.0, 2H, CH_2O), 4.63–4.80 (m, 1H, *CH*OH), 5.45–5.69 (m, 2H, disappears with D_2O), 6.82–8.06 (m, 6H,

4HAr + H_3 + 1H which disappears with D_2O). IR (KBr): cm^{-1} 3460, 3350, 3211 (NH, OH), 1706 (COOEt), 1681 (CONH).

Anal. Calcd for ($C_{17}H_{21}N_4O_4F$): C, 56.04; H, 5.81; N, 15.38. Found: C, 55.99; H, 5.87; N, 15.48.

6.1.7.5. 1-(2-Hydroxybutyl)-5-[3-(3-fluorophenyl)ureido]-1*H*-pyrazole-4-carboxylic acid ethyl ester 2m. Yield 49%, mp 128–129 °C. ^1H NMR ($CDCl_3$): δ 0.91 (t, J = 7.6, 3H, CH_3), 1.26 (t, J = 7.2, 3H, CH_3CH_2O), 1.52–1.84 (m, 2H, CH_2CH), 4.03 (d, J = 5.6, 2H, CH_2N), 4.19 (q, J = 7.2, 2H, CH_2O), 4.63–4.77 (m, 1H, *CH*OH), 5.46–5.63 (m, 2H, disappears with D_2O), 6.64–7.38 (m, 5H, 4HAr + 1H which disappears with D_2O), 7.54 (s, 1H, H_3). IR (KBr): cm^{-1} 3458, 3347, 3247 (NH, OH), 1707 (COOEt), 1680 (CONH).

Anal. Calcd for ($C_{17}H_{21}N_4O_4F$): C, 56.04; H, 5.81; N, 15.38. Found: C, 56.04; H, 5.67; N, 15.50.

6.1.7.6. 1-(2-Hydroxybutyl)-5-[3-(4-fluorophenyl)ureido]-1*H*-pyrazole-4-carboxylic acid ethyl ester 2n. Yield 50%, mp 130–131 °C. ^1H NMR ($CDCl_3$): δ 0.93 (t, J = 7.6, 3H, CH_3), 1.27 (t, J = 7.2, 3H, CH_3CH_2O), 1.53–1.86 (m, 2H, CH_2CH), 4.06 (d, J = 5.8, 2H, CH_2N), 4.19 (q, J = 7.2, 2H, CH_2O), 4.59–4.76 (m, 1H, *CH*OH), 5.41–5.77 (m, 2H, disappears with D_2O), 6.80–7.41 (m, 5H, 4HAr + 1H which disappears with D_2O), 7.54 (s, 1H, H_3). IR (KBr): cm^{-1} 3467, 3362, 3252 (NH, OH), 1710 (COOEt), 1682 (CONH).

Anal. Calcd for ($C_{17}H_{21}N_4O_4F$): C, 56.04; H, 5.81; N, 15.38. Found: C, 55.91; H, 6.08; N, 15.28.

6.2. Biological methods

6.2.1. Neutrophils preparation

Cells were obtained from the blood of healthy subjects, and human peripheral blood neutrophils were purified by using the standard techniques of dextran (Pharmacia, Uppsala, Sweden) sedimentation, centrifugation on Ficoll–Paque (Pharmacia), and hypotonic lysis of contaminating red cells. Cells were washed twice and resuspended in Krebs–Ringer phosphate (KRPG), pH 7.4, at a final concentration of 50×10^6 cells/mL and kept at room temperature until used. Neutrophils were 98–100% viable, as determined using the Trypan blue exclusion test. Local Ethics Committee approved the study and informed consent was obtained from all participants.

6.2.2. Preparation of chemoattractants and tested compounds

fMLP-OMe and tested compounds (10^{-2} M) were solved in dimethylsulfoxide (DMSO), while IL8 (10^{-5} M) was solved in water. Before the use, all the solutions were diluted in KRPG containing 1 mg/mL of bovine serum albumin (BSA; Orha Behringwerke, Germany) to obtain the final concentrations ranging from 10^{-15} to 10^{-5} M. At the concentrations used, DMSO did not interfere with any of the biological assays performed.

6.2.3. Random locomotion

Random locomotion was performed with 48-well microchemotaxis chamber (Bio Probe, Milan, Italy) and migration into the filter was evaluated by the leading-front method,[21] estimating the distance in micrometers that the leading edge of the cell migrated. The actual control random movement is 35 ± 3 μm SE of six separate experiments performed in duplicate.

6.2.4. Chemotaxis

Directional movement or chemotaxis was measured with the same chamber used for the random locomotion, adding fMLP-OMe 10^{-9} M or IL8 10^{-8} M in the lower compartment.

Data were expressed in terms of chemotactic index (C.I), which is the ratio: [(migration toward test attractant-migration toward the buffer)/migration toward the buffer]. The C.I. of fMLP-OMe is 1.2 at 10^{-9} M, while the value of IL8 is 1.02 at 10^{-8} M.

Neutrophils were preincubated with tested compounds 10 min before the functional test.

The antagonism was measured as C.I. in the absence (100%) and in the presence of the tested compounds at different concentrations, following the methods already reported.[10]

IC_{50} values were calculated from the sigmoid dose–response curve by a non-linear regression analyses (Graph Pad Prism, San Diego, USA).

6.2.5. Measurement of cell viability

In order to assess possible cytotoxic effects of the tested compounds, the cytoplasmatic marker enzyme, lactate dehydrogenase (LDH), was determined by measuring the rate of oxidation of NADH. The absorbance change was followed at 340 nm.[22]

6.2.6. Induction of peritonitis, cell count, cell composition and determination of protein concentration in the peritoneal exudate

The experiments were performed using male Swiss mice (25–30 g) fasted 16 h before the experiment, but with free access to water. Mice were randomly assigned to groups of 12 animals orally treated with vehicle or the compounds under examination (50 mg/kg) 1 h before the induction of peritonitis. Dexamethasone (3 mg/kg) was used as reference drug. Peritonitis was induced following a modification of Thurmond's method.[23] Briefly, 5 mg/mL zymosan or phosphate-buffered-saline (PBS) was injected into the peritoneal space of mice (final volume 0.2 mL). After 4 h, the animals were euthanized; the peritoneal cavities were washed with 3 mL of PBS containing 3 mM EDTA and the volume collected with automatic pipettes. Total leukocyte counts were performed in a Neubauer chamber by means of optical microscopy after diluting a sample of the peritoneal fluid with Türk solution (1:10). Differential cell counts were performed using a light microscope. Their chromatic characteristics and the shape of the nucleus relative to the cytoplasm easily differentiated neutrophils.

Protein content was spectrophotometrically determined following the bicinchonate method with a commercial kit (Pierce, BCA protein assay kit). Experiments were carried out in accordance to the Italian law (DL 116/92) and approved by 'Ministry of Health'.

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