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Original article

N-Aryl-2-phenyl-2,3-dihydro-imidazo[1,2-*b*]pyrazole-1-carboxamides 7-substituted strongly inhibiting both fMLP-OMe- and IL-8-induced human neutrophil chemotaxis

Chiara Brullo [a,1], Susanna Spisani [b,1], Rita Selvatici [c], Olga Bruno [a,*]

[a] Dipartimento di Scienze Farmaceutiche, Università degli Studi di Genova, viale Benedetto XV, 3, 16132 Genova, Italy
[b] Dipartimento di Biochimica e Biologia Molecolare, Sezione di Biologia Molecolare, Università degli Studi di Ferrara, via Luigi Borsari, 46, 44121 Ferrara, Italy
[c] Dipartimento di Medicina Sperimentale e Diagnostica, Sezione di Genetica Medica, Università degli Studi di Ferrara, Via Fossato di Mortara 74, 44121 Ferrara, Italy

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ABSTRACT

Anomalous activation of neutrophil recruitment is one of the causes of many inflammatory diseases. The chemoattractants *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), and interleukine 8 (IL8) play a pivotal role in neutrophil chemotaxis regulation in the latter and early stages, respectively, probably by two independent mechanisms. We reported here synthesis and biological evaluation of new *N*-aryl-2-phenyl-2,3-dihydro-imidazo[1,2-*b*]pyrazole-1-carboxamides 7-substituted which were designed as possible multi-target antiinflammatory agents. Many of the title compounds showed a good inhibition, in the nano molar range, of human neutrophil chemotaxis selectively acting toward fMLP-OMe (methyl-ester of fMLP) or IL8 stimulus; whereas, two compounds showed an interesting dual activity inhibiting both fMLP-OMe and IL8-induced chemotaxis at nano molar concentration.

1. Introduction

In the last decade the over-regulation of neutrophil recruitment mechanisms has been clearly indicated as one of the cause of many inflammatory diseases.

As an example one of the most characteristic changes of inflammation in cystic fibrosis (CF) is a large infiltration by neutrophils into the lung parenchyma [1].

The chemoattractants more involved in neutrophil chemotaxis are *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and interleukine 8 (IL8 also named CXCL8) which bind on specific G-protein-coupled receptors (FPR and CXCR8, respectively). While fMLP plays a pivotal role in inflammation consequent to bacterial infections, IL8 mediates the response of neutrophils to physical injury.

Neutrophil recruitment is a complex process including rolling, adhesion, extravasation, actin polymerization and movement to inflamed site. Each step of this patho-physiological event is regulated by downstream signalling molecules, including phosphatidylinositol 3-kinase (PI3K), G-protein-coupled receptor kinases (GPKs), phospholipase C, that produce the second messengers responsible for elevation of intracellular free calcium, protein kinase C (PKC) and mitogen activated protein kinases (MAPKs) activation [2–4]. Recently, it has been demonstrated that the fMLP-OMe chemotactic response is associated with specific PKC β1 isoform translocation and p38 MAPK phosphorylation [5,6].

Heit and co-workers [7] proposed a model in which the end target chemoattractants (such as fMLP), that is of importance during the latter phase of the tracking process, function primarily by stimulating p38 MAPK, whereas intermediary (intermediate-type) chemoattractants (such as IL8), as one of importance in the early stage of neutrophil recruitment, primarily function *via* the PI3K/Akt (protein kinase B) pathway. Moreover, it has been demonstrated that a hierarchy between the two pathways exists. The same signalling hierarchy exists with regard to NADPH-oxidase activation by both formylated peptide and IL8 [8].

In the search for new antiinflammatory agents interfering with human neutrophil migration we recently reported many pyrazolyl-

* Corresponding author. Tel.: +39 010 3538367; fax: +39 010 3538358.
E-mail address: obruno@unige.it (O. Bruno).
[1] These authors contributed equally to this work.

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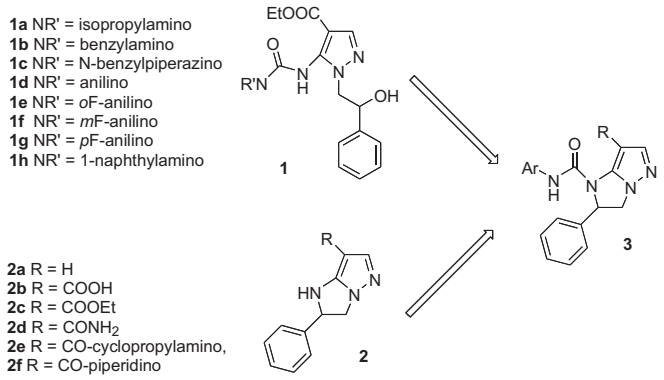


Fig. 1. General molecular structure of compounds **1**, **2** and **3**.

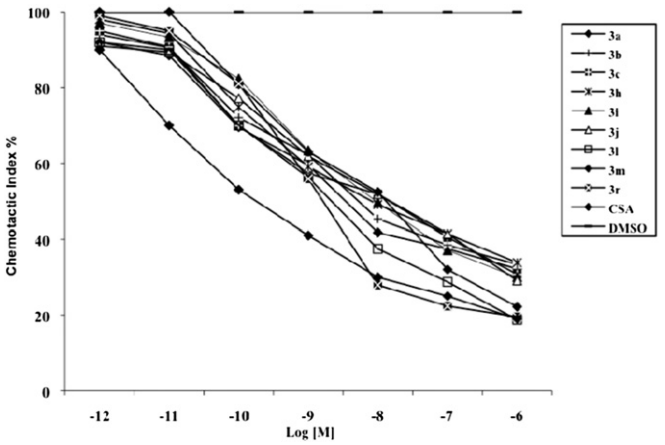


Fig. 2. Dose–response curve of compounds **3a**, **3b**, **3c**, **3h**, **3i**, **3j**, **3l**, **3m**, **3r**, CSA (positive control) and DMSO (negative control) in human neutrophil fMLP-OMe-stimulated chemotaxis. Data are expressed as a percentage of the C.I.. SEs are within 10% of the mean value.

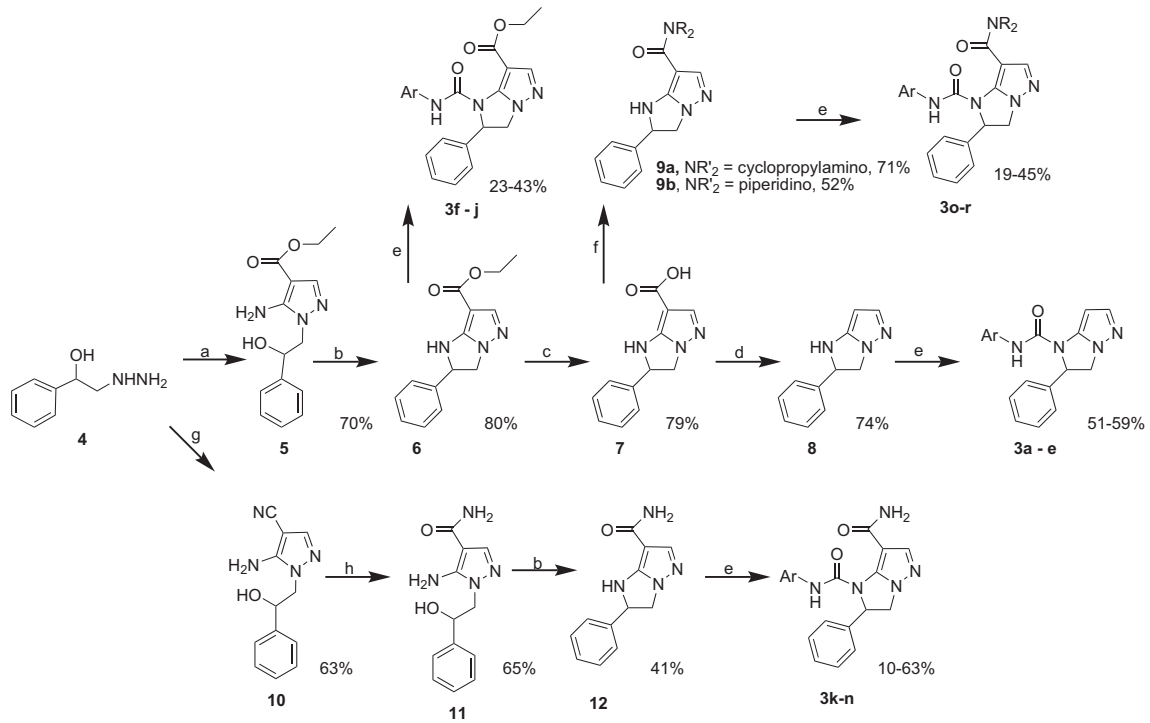
urea derivatives (compounds **1**, Fig. 1) able to block IL8-induced chemotaxis at nM concentrations [9].

The most active compounds were the 3-benzyl-, 3-(4-benzylpiperazinyl)-, 3-phenyl-, 3-fluorophenyl, and the 3-isopropylureido derivatives, having a carboxyethyl group in position 4 of the pyrazole moiety. These compounds were inactive toward the CXC receptors, while their involvement in the complex intracellular mechanisms of neutrophil recruitment was confirmed by the inhibition of tyrosine phosphorylation in the 50–70 kDa region and by a remarkable decrease of F-actin polymerization.

At the same time, we synthesized new interesting 2-phenyl-2,3-dihydro-1*H*-imidazo[1,2-*b*]pyrazoles 7-substituted (compounds **2**, Fig. 1) which, in a preliminary study, resulted in inhibiting at nM concentration the fMLP-OMe-induced chemotaxis [10]. The most active compounds are reported in Fig. 1. They were unable to displace [^{3}H]-fMLP from its specific binding site and were found to be ineffective as antagonists in superoxide anion production, as well as in granule enzyme release. For that reason they could be considered as "pure" fMLP-induced chemotaxis inhibitors [11].

A more complete understanding of intracellular signalling triggered by different chemotaxins should be of great importance in models of inflammation. In this context the development of new molecules able to discriminate between the different activation pathways of chemotaxis is of great interest for this search area.

On the other hand, in the next ten years emerged a new drug discovery paradigm which consider the development of "multiple targeted drugs" more profitable than the classic "one target-one-disease" approach [12,13]. The "polypharmacology" paradigm is aimed to increase the *in vivo* efficacy by synergic actions and to reduce the side effects by a decreasing of dose administration.



Scheme 1. Reagents and conditions: (a) (Ethoxymethylene) cyanoacetate, anhydrous toluene, 70–80 °C, 8 h; (b) conc. H_2SO_4, r.t., 15 min; (c) 2M NaOH solution, 120 °C, 4 h; (d) heating at 190 °C to complete CO_2 evolution; (e) anhydrous *N*,*N*-DMF, arylisocyanate, 100 °C, 12–18 h; (f) anhydrous *N*,*N*-DMF, excess cyclopropylamine or piperidine, anhydrous Et_3N, DPPA, 30–60 °C, 12 h g) (ethoxymethylene)malononitrile, absolute ethanol, reflux, 6 h; (h) 2M ethanol/water (50%) NaOH solution, reflux, 2 h.

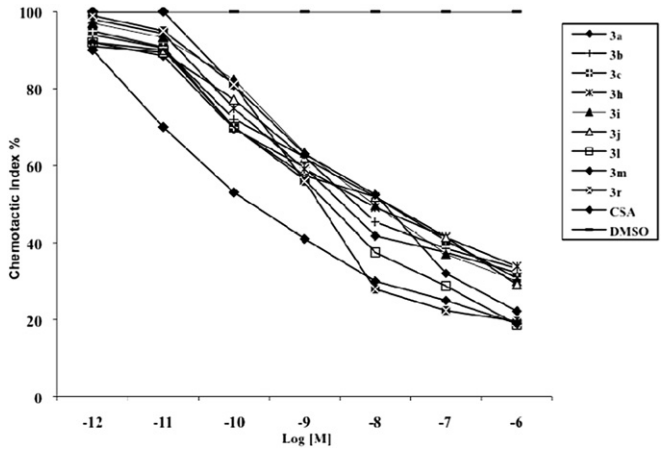


Fig. 3. Dose–response curve of compounds **3a**, **3b**, **3c**, **3h**, **3i**, **3j**, **3l**, **3m**, **3r**, CSA (positive control) and DMSO (negative control) in human neutrophil IL8-stimulated chemotaxis. Data are expressed as a percentage of the C.I.. SEs are within 10% of the mean value.

Multitarget therapies have been already tried by combining different therapeutic mechanism with drugs cocktails or with multicomponent formulations. A more recent strategy is to develop a single molecule able to simultaneously interact with different pharmacological targets.

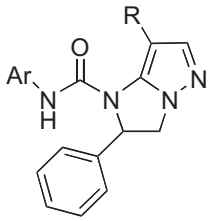
Taking into account the above considerations we designed a new series of *N*-aryl-2-phenyl-2,3-dihydro-imidazo[1,2-*b*]pyrazole-1-carboxamides 7-substituted (compounds **3**, Fig. 1) including in the same structure both the rigid scaffold of fMLP-OMe (**2**) and the peculiar urea moieties of the most active IL8-induced chemotaxis inhibitors (**1**). The substituents which were peculiar of the most active compounds **2** were maintained in position 7. Compounds **3** have been tested in *in vitro* chemotaxis assays, in human neutrophils stimulated by both fMLP-OMe and IL8 in order to obtain more structure activity relationship (SAR) information about their ability to interact with different chemoattractant pathways.

2. Chemistry

Compounds **3a**–**r** were prepared reacting the intermediates **6**, **8**, **9a**, **9b**, and **12** with the suitable arylisocyanates in anhydrous *N*,*N*-DMF, as reported in Scheme 1.

The 2-hydrazino-1-phenylethanol (**4**) is the key starting product to obtain the above intermediates. Compound (**5**), obtained by reaction of **4** with ethyl (ethoxymethylene) cyanoacetate [14], was treated with concentrated sulphuric acid at 0 °C to give the 2-phenyl-2,3-dihydro-1*H*-imidazo[1,2-*b*]pyrazole-7-carboxylic acid ethyl ester (**6**). Alkaline hydrolysis to the carboxylic acid **7**, and subsequent decarboxylation give 2-phenyl-2,3-dihydro-1*H*-imidazo[1,2-*b*]pyrazole (**8**) [10]. Acid derivative **7** was treated with an excess of the suitable amine (cyclopropilamine or piperidine) in the presence of anhydrous triethylamine and diphenylphosphorylazide (DPPA) to afford compounds **9a**–**b**. This method gave only the amide derivatives without carboxy-azide formation and subsequent Curtius rearrangement, as we have already reported [10]. By condensation of 2-hydrazino-1-phenylethanol (**4**) with ethoxymethylenemalononitrile in absolute ethanol we obtained the 5-amino-1-(2-hydroxy-2-phenylethyl)-1*H*-pyrazole-4-carbonitrile (**10**) [10] which was then hydrolysed in alkaline ethanol/water solution to 5-amino-1-(2-hydroxy-2-phenylethyl)-1*H*-pyrazole-4-carboxamide (**11**). Finally, 2-phenyl-2,3-dihydro-1*H*-imidazo[1,2-*b*]pyrazole-7-carboxamide (**12**) was prepared by the same cyclization procedure used for **6**. It is to note that all our attempts to obtain compounds **3** by direct cyclization of the pyrazolyl–ureas **1** were unsuccessful.

Table 1
Structure and potency expressed as IC_{50} values of the compounds **3a**–**r** in the chemotaxis inhibition of human neutrophils.



Comp.	R	Ar	Inhibition of fMLP-OMe-induced chemotaxis $IC_{50} \pm$ SEM (nM)	Inhibition of IL8-induced chemotaxis $IC_{50} \pm$ SEM (nM)
3a	H	C_6H_5	4.8 ± 0.43	>1000
3b	H	(o)-F-C_6H_4	5.4 ± 0.60	>1000
3c	H	(m)-F-C_6H_4	12.1 ± 1	>1000
3d	H	(p)-F-C_6H_4	95 ± 9	>1000
3e	H	α-naphthyl	>1000	>1000
3f	$COOC_2H_5$	C_6H_5	98 ± 9.1	>1000
3g	$COOC_2H_5$	(o)-F-C_6H_4	116 ± 10	7.5 ± 0.7
3h	$COOC_2H_5$	(m)-F-C_6H_4	9.2 ± 0.88	111 ± 10
3i	$COOC_2H_5$	(p)-F-C_6H_4	10.3 ± 0.92	>1000
3j	$COOC_2H_5$	α-naphthyl	13.8 ± 1.2	206 ± 18
3k	$CONH_2$	(o)-F-C_6H_4	>1000	6.2 ± 0.7
3l	$CONH_2$	(m)-F-C_6H_4	3.9 ± 0.40	1.2 ± 0.1
3m	$CONH_2$	(p)-F-C_6H_4	11.4 ± 1.1	484 ± 45
3n	$CONH_2$	α-naphthyl	110 ± 10	>1000
3o	CO–NH–cycloprop	C_6H_5	123 ± 13	13.2 ± 1.1
3p	CO–NH–cycloprop	(m)-F-C_6H_4	138 ± 14	>1000
3q	CO-Pip	C_6H_5	980 ± 90	>1000
3r	CO-Pip	(m)-F-C_6H_4	5.5 ± 0.63	1.2 ± 0.1

3. Pharmacology

Release of the cytoplasmic enzyme LDH was used as an indicator of cell viability. In none of the experiments described below 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, in the absence and in the presence of derivatives.

In Fig. 2 (graphic 1) and Fig. 3 (graphic 2) we reported the dose–response curves of the most active compounds towards the fMLP-OMe and IL8-induced chemotaxis, respectively. The antagonist concentrations inhibiting the fMLP-OMe- or IL-8-induced chemotaxis by 50% (IC_{50}) were obtained by the computer analysis of inhibition curves (Table 1). Data where compared with positive (cyclosporine A, CSA) and negative (dimethylsulfoxide, DMSO, blank) controls.

4. Results and discussion

The introduction in the rigid scaffold of compounds **2** of peculiar urea moieties of compounds **1** gave new substituted 2,3-dihydro-imidazopyrazoles **3** able to inhibit both fMLP-OMe and IL8-induced neutrophil chemotaxis. In particular **3a**–**e**, which were unsubstituted in position 7, were selective toward fMLP-OMe triggered off mechanism; **3h**–**j**, **3l**, **3m** and **3r** (bearing a carboxyethyl or a carboxamide in 7 position) were the most active having IC_{50} values in the nanomolar range.

Compounds **3o** and **3p**, having in position 7 a cyclopropylamide group and an arylcarbamoyl substituents in 1, differently than the analogue unsubstituted in 1 position compound **2e**, were inactive. Most of the active compounds (**3b**, **3c**, **3h**, **3i**, **3l**, **3m**, **and 3r**) have a fluoroanilino-carbamoyl substituent in position 1.

As concerns IL8-induced chemotaxis compounds **3g**, **3k**, **3l**, **3o** and **3r** (bearing a carboxyethyl or a carboxamide in 7 position) were strongly active having IC_{50} in the nanomolar range. All the other compounds were very poor or inactive. The fluoroanilino-carbamoyl substitution in position 1 (see **3g**, **3h**, **3l**, **3r**) gave the most active compounds in the IL8-induced chemotaxis, as well; it is noteworthy that the previous compounds **1** showed the same behaviour and the 3-fluorophenyl derivative **1b** showed a good inhibition of neutrophils chemotaxis in a preliminary *in vivo* test (mouse model of zymosan-induced peritonitis) [9].

Finally, compounds **3l** and **3r** (both having an amide substituent in position 7 and a m-fluoroanilino group in the urea moiety) showed an interesting dual activity profile being active at nanomolar concentration in both fMLP-OMe and IL8-induced chemotaxis.

5. Conclusion

In conclusion, we designed and synthesized new 1-arylcarbamoyl-2,3-dihydro-1*H*-imidazo[1,2-*b*]pyrazoles in order to obtain by a unique structure both the inhibition of IL8- and fMLP-OMe-induced chemotaxis (activities which were already showed by our previous pyrazolyl–ureas **1** and 1-unsubstituted 2,3-dihydro-1*H*-imidazo[1,2-*b*]pyrazoles **2**, respectively). Most of the new synthesized compounds were chemotaxis inhibitors, particularly toward fMLP-OMe chemoattractant, at nM concentration. Since the previously synthesized analogue pyrazolyl–ureas **1** were completely inactive as fMLP-OMe-induced chemotaxis inhibitors, we can conclude that the more rigid dihydro-imidazo-pyrazolyl scaffold is essential to inhibit fMLP-OMe-induced chemotaxis. Biological data suggest that the lack of substituents in position 7 directs the action toward fMLP-OMe signal pathway rather than IL8 one. However, in the 7-substituted compounds it is possible to selectively modulate the activity toward the two different ways by changing the steric hindrance of the carbamoyl moiety.

On the other hand, compounds **3l** and **3r**, strongly inhibiting either fMLP-OMe- and IL8- induced chemotaxis, demonstrated that these new class of arylcarbamoyl-imidazo-pyrazoles can be properly defined as multi-target neutrophil chemotaxis inhibitors.

Indeed, it is well know that fMLP stimulation is prevalent in the latter phase of the tracking process, while IL8 acts in the early stage of neutrophil recruitment. It has been demonstrated that they have two different pathways of action and that a hierarchy between the two pathways exists. Actually, these new imidazopyrazoles are able to interfere with two different steps of neutrophils recruitment. Preliminary Western blotting experiments with the compounds **31** in neutrophils activated with either fMLP or with IL8, do not show AKT phosphorylation, suggesting that PI3K/Akt pathway is not activated. A further careful investigation about its mechanism of action, particularly in the PI3K/Akt, MAPK and PKC pathways which are the primarily involved in the neutrophil recruitment cascade, has been planned.

Finally, we reported here new imidazo–pyrazoles which are the first example of compounds able to inhibit two different chemotaxis mechanisms. This good versatility represents the most important characteristic of these new molecules, particularly as concerns its pharmacological application. In fact, the more selective ones could be very useful as pharmacological tools to clearly understand the intracellular mechanism of neutrophil recruitment. Whereas, the dual active compounds are a main starting point for the development of new multi-targeted antiinflammatory drugs.

6. Experimental Protocols

6.1. Chemistry

6.1.1. General

Chiminord and Aldrich Chemical, Milan, Italy purchased all chemicals. Solvents were reagent grade. Unless otherwise stated, all commercial reagents were used without further purification. 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 and ^{13}C 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 Hz. Elemental analyses, indicated by the symbols of the elements, were within ±0.4% of the theoretical values and were determined with an Elemental Analyser EA 1110 (Fison-Instruments, Milan, Italy).

6.1.2. General procedure for compounds *3a–r*

To the starting product (**8** for compounds **3a**–**e**, **6** for compounds **3f**–**j**, **12** for compounds **3k**–**n** and **9a**,**b** for compounds **3o**–**r**) (5 mmol) solved in anhydrous *N*,*N*-dimethylformamide (DMF) (5 mL) the proper arylisocyanate (6 mmol) was added and the reaction mixture was stirred at 100 °C for 12 h (compounds

3a–**j**) or 18 h (compounds **3k**–**r**). After cooling the mixture was poured into an ice-water bath and 1 M HCl solution was added until pH 2. The solid precipitated was filtered, dried on air and the purity was verified by TLC. When it needed the final product was purified by flash chromatography (Silica gel, $CHCl_3$ or diethyl ether as eluents). The crude solids were recrystallized from absolute ethanol.

6.1.2.1. N-Phenyl-2-phenyl-2,3-dihydro-imidazo[1,2-b]pyrazole-1-carboxamide ***3a***. Yield 53%, m.p. 155 °C. ^{1}H NMR ($CDCl_3$): δ 4.22 (near q, 1 H, H3), 4.81 (near t, 1 H, H2), 5.85 (near q, 1 H, H3), 6.03 (d, $J = 2.0$, 1 H, H7), 6.53 (s, 1 H, NH, slowly disappears with D_2O), 6.98–7.50 (m, 10 H, Ar), 7.54 (d, $J = 2.0$, 1 H, H6). ^{13}C NMR (DMSO): δ 39.07, 53.56, 63.57, 87.57, 119.53, 122.61, 125.18, 127.79, 128.15, 128.56, 138.35, 140.18, 143.25, 145.25, 149.47. IR (KBr): cm^{-1} 3357 (NH), 1680 (CONH). Anal. ($C_{18}H_{16}N_4O$) C, H, N.

6.1.2.2. N-(2-Fluorophenyl)-2-phenyl-2,3-dihydro-imidazo[1,2-b]pyrazole-1-carboxamide ***3b***. Yield 59%, m.p. 149–150 °C ^{1}H NMR ($CDCl_3$): δ 4.25 (near q, 1 H, H3), 4.83 (near t, 1 H, H2), 5.87 (near q, 1 H, H3), 6.03 (d, $J = 2.0$, 1 H, H7), 6.82 (s, 1 H, NH, slowly disappears with D_2O), 6.85–7.46 (m, 8 H, Ar), 7.49 (d, $J = 2.0$, 1 H, H6), 8.09 (t, $J = 8.0$, 1 H, Ar). ^{13}C NMR (DMSO): δ (39.13, 53.57, 63.88, 87.43, 114.96, 115.35, 123.85, 125.07, 125.44, 125.60, 127.81, 128.54, 139.95, 143.25, 144.97, 149.44, 152.12, 157.01). IR (KBr): cm^{-1} 3387 (NH), 1679 (CONH). Anal. ($C_{18}H_{15}FN_4O$) C, H, N.

6.1.2.3. N-(3-Fluorophenyl)-2-phenyl-2,3-dihydro-1H-imidazo[1,2-b]pyrazole-1-carboxamide ***3c***. Yield 50%, m.p. 138–139 °C ^{1}H NMR ($CDCl_3$): δ 4.21 (near q, 1 H, H3), 4.79 (near t, 1 H, H2), 5.84 (near q, 1 H, H3), 6.01 (d, $J = 2.0$, 1 H, H7), 6.62 (s, 1 H, NH, slowly disappears with D_2O), 6.69–7.48 (m, 9 H Ar), 7.53 (d, $J = 2.0$, 1 H, H6). ^{13}C NMR (DMSO): δ (39.10, 51.71, 65.20, 99.69, 114.40, 125.32, 127.71, 128.52, 140.72, 140.80, 141.15, 142.98, 143.45, 150.40). IR (KBr): cm^{-1} 3336 (NH), 1674 (CONH). Anal. ($C_{18}H_{15}FN_4O$) C, H, N.

6.1.2.4. N-(4-Fluorophenyl)-2-phenyl-2,3-dihydro-1H-imidazo[1,2-b]pyrazole-1-carboxamide ***3d***. Yield 59%, m.p. 155–156 °C ^{1}H NMR ($CDCl_3$): δ 4.21 (near q, 1 H, H3), 4.80 (near t, 1 H, H2), 5.85 (near q, 1 H, H3), 5.99 (d, $J = 2.0$, 1 H, H7), 6.52 (s, 1 H, NH, slowly disappears with D_2O), 6.80–7.49 (m, 9 H, Ar), 7.52 (d, $J = 2.0$, 1 H, H6). ^{13}C NMR (DMSO): δ (39.13, 53.57, 63.59, 87.55, 114.48, 114.92, 121.43, 121.59, 125.17, 127.75, 128.54, 134.66, 140.17, 143.21, 145.15, 149.55, 155.25, 160.01). IR (KBr): cm^{-1} 3340 (NH), 1681 (CONH). Anal. ($C_{18}H_{15}FN_4O$) C, H, N.

6.1.2.5. N-(1-Naphthyl)-2-phenyl-2,3-dihydro-1H-imidazo[1,2-b]pyrazole-1-carboxamide ***3e***. Yield 51%, m.p. 124–125 °C ^{1}H NMR ($CDCl_3$): δ 4.24 (near q, 1 H, H3), 4.81 (near t, 1 H, H2), 5.84 (near q, 1 H, H3), 6.09 (d, $J = 2.0$, 1 H, H7), 6.70–6.85 (m, 2 H Ar), 7.15–7.83 (3m, 12 H, 10 Ar + H6 + NH, 1 H disappears with D_2O). ^{13}C NMR (DMSO): δ 39.10, 53.57, 63.78, 87.22, 126.40, 127.59, 128.23, 140.34, 143.05, 154.55). IR (KBr): cm^{-1} 3399 (NH), 1686 (CONH). Anal. ($C_{22}H_{18}N_4O$ *0,5 (C_2H_5OH) C, H, N.

6.1.2.6. Ethyl 1-{[phenylamino]carbonyl}-2-phenyl-2,3-dihydro-1H-imidazo[1,2-b]pyrazole-7-carboxylate ***3f***. Yield 43%, m.p. 141–142 °C ^{1}H NMR ($CDCl_3$): δ 1.41 (t, $J = 7.0$, 3 H, CH_3CH_2), 4.15 (near q, 1 H, H3), 4.32 (q, $J = 7.0$, 2 H, CH_3CH_2), 4.79 (near t, 1 H, H2), 6.50 (near q, 1 H, H3), 6.95–7.78 (m, 10 H, Ar), 7.91 (s, 1 H, H6), 11.23 (s, 1 H, NH, slowly disappears with D_2O). ^{13}C NMR (DMSO): δ (13.65, 39.17, 51.90, 60.61, 66.01, 96.90, 119.63, 122.71, 125.28, 127.80, 128.25, 128.56, 138.41, 140.20, 143.27, 145.35, 149.57, 163.84). IR (KBr): cm^{-1} 3260 (NH), 1697 (COOEt), 1682 (CONH). Anal. ($C_{21}H_{20}N_4O_3$) C, H, N.

6.1.2.7. Ethyl 1-{[(2-fluorophenyl)amino]carbonyl}-2-phenyl-2,3-dihydro-1H-imidazo[1,2-b]pyrazole-7-carboxylate ***3g***. Yield 23%, m.p. 122–123 °C ^{1}H NMR ($CDCl_3$): δ 1.38 (t, $J = 7.2$, 3 H, CH_3CH_2), 4.16 (near q, 1 H, H3), 4.34 (q, $J = 7.2$, 2 H, CH_3CH_2), 4.78 (near t, 1 H, H2), 6.48 (near q, 1 H, H3), 6.98–7.80 (m, 9 H, Ar), 7.91 (s, 1 H, H6), 10.83 (s, 1 H, NH, slowly disappears with D_2O). ^{13}C NMR (DMSO): δ 13.65, 39.13, 51.90, 60.61, 66.01, 96.90, 115.06, 115.45, 123.95, 124.02, 124.54, 125.35, 127.76, 128.51, 140.24, 144.44, 144.74, 150.39, 163.84. IR (KBr): cm^{-1} 3242 (NH), 1681 (CONH + COOEt). Anal. ($C_{21}H_{19}FN_4O_3$) C, H, N.

6.1.2.8. Ethyl 1-{[(3-fluorophenyl)amino]carbonyl}-2-phenyl-2,3-dihydro-1H-imidazo[1,2-b]pyrazole-7-carboxylate ***3h***. Yield 40%, m.p. 123–124 °C ^{1}H NMR ($CDCl_3$): δ 1.42 (t, $J = 7.2$, 3 H, CH_3CH_2), 4.16 (near q, 1 H, H3), 4.42 (q, $J = 7.2$, 2 H, CH_3CH_2), 4.78 (near t, 1H, H2), 6.48 (near q, 1 H, H3), 6.62–6.80 and 7.10–7.50 (2 m, 9 H, Ar), 7.91 (s, 1 H, H6), 11.44 (s, 1H, NH, slowly disappears with D_2O). ^{13}C NMR (DMSO): δ 13.60, 39.15, 51.95, 60.59, 66.12, 96.96, 114.36, 125.22, 127.51, 128.60, 140.70, 140.91, 141.15, 143.02, 143.38, 150.38, 163.86. IR (KBr): cm^{-1} 3259 (NH), 1680 (CONH + COOEt). Anal. ($C_{21}H_{19}FN_4O_3$) C, H, N.

6.1.2.9. Ethyl 1-{[(4-fluorophenyl)amino]carbonyl}-2-phenyl-2,3-dihydro-1H-imidazo[1,2-b]pyrazole-7-carboxylate ***3i***. Yield 36%, m.p. 149 °C. ^{1}H NMR ($CDCl_3$): δ 1.41 (t, $J = 7.2$, 3 H, CH_3CH_2), 4.16 (near q, 1 H, H3), 4.40 (q, $J = 7.2$, 2 H, CH_3CH_2), 4.78 (near t, 1 H, H2), 6.46 (near q, 1 H, H3), 6.85–7.05 (m, 2 H Ar), 7.15–7.60 (m, 7 H, Ar), 7.91 (s, 1 H, H6), 11.29 (s, 1 H, NH, slowly disappears with D_2O). ^{13}C NMR (DMSO): δ 13.59, 39.17, 51.95, 60.55, 66.11, 96.95, 114.70, 115.10, 120.41, 120.60, 125.31, 127.59, 128.53, 135.49, 140.81, 143.25, 143.42, 150.60, 163.84. IR (KBr): cm^{-1} 3264 (NH), 1683 (COOEt), 1672 (CONH). Anal. ($C_{21}H_{19}FN_4O_3$) C, H, N.

6.1.2.10. Ethyl 1-[(1-Naphthylamino)carbonyl]-2-phenyl-2,3-dihydro-1H-imidazo[1,2-b]pyrazole-7-carboxylate ***3j***. Yield 27%, m.p. 131–132 °C. ^{1}H NMR ($CDCl_3$): δ 1.33 (t, $J = 7.2$, 3 H, CH_3CH_2), 4.15–4.35 (m, 3 H, CH_3CH_2 + H3), 4.84 (near t, 1 H, H2), 6.52 (near q, 1 H, H3), 7.20–7.92 (m, 12 H, Ar), 7.97 (s, 1 H, H6), 11.18 (s, 1 H, NH, slowly disappears with D_2O). ^{13}C NMR (DMSO): δ 14.09, 39.12, 52.97, 58.44, 64.67, 91.33, 126.42, 127.58, 128.20, 140.31, 143.08, 154.61, 161.99. IR (KBr): cm^{-1} 3171 (NH), 1680 (CONH + COOEt). Anal. ($C_{25}H_{22}N_4O_3$) C, H, N.

6.1.2.11. N^1-(2-Fluorophenyl)-2-phenyl-2,3-dihydro-1H-imidazo[1,2-b]pyrazole-1,7-dicarboxamide ***3k***. Yield 42%, m.p. 221 °C ^{1}H NMR ($CDCl_3$): δ 1.78 (s, 2 H, NH_2 disappears with D_2O), 4.18 (near q, 1 H, H3), 4.82 (near t, 1 H, H2), 6.41 (near t, 1 H, H3), 7.00–7.70 (m, 9 H, Ar), 7.75 (s, 1 H, H6), 10.85 (s, 1 H, NH, slowly disappears with D_2O). ^{13}C NMR (DMSO): δ 39.10, 51.32, 64.80, 99.65, 115.06, 115.45, 123.95, 124.02, 124.54, 125.35, 127.76, 128.51, 140.24, 144.44, 144.74, 150.39, 165.72. IR (KBr): cm^{-1} 3345, 2773 (NH_2), 3195 (NH), 1680 (CONH), 1642 ($CONH_2$). Anal. ($C_{19}H_{16}FN_5O_2$) C, H, N.

6.1.2.12. N^1-(3-Fluorophenyl)-2-phenyl-2,3-dihydro-1H-imidazo[1,2-b]pyrazole-1,7-dicarboxamide ***3l***. Yield 63%, m.p. 206 °C ^{1}H NMR (DMSO-d_6): δ 4.02 (near q, 1 H, H3), 4.87 (near t, 1 H, H2), 6.40 (near t, 1 H, H3), 6.52–7.50 (m, 9 H, 7Ar + NH_2 which disappears with D_2O), 7.92 (s, 1 H, H6), 8.00–8.35 (m, 2 H, Ar), 12.95 (s, 1 H, NH, slowly disappears with D_2O). ^{13}C NMR (DMSO): δ 39.10, 51.31, 64.83, 99.69, 114.38, 125.20, 127.61, 128.49, 140.67, 140.89, 141.11, 142.92, 143.38, 150.36, 165.74. IR (KBr): cm^{-1} 3344, 2798 (NH_2), 3193 (NH), 1650 ($CONH_2$). Anal. ($C_{19}H_{16}FN_5O_2$).

6.1.2.13. N^1-(4-Fluorophenyl)-2-phenyl-2,3-dihydro-1H-imidazo[1,2-b]pyrazole-1,7-dicarboxamide ***3m***. Yield 41%, m.p. 224 °C ^{1}H NMR

(DMSO-d_6): δ 4.03 (near q, 1 H, H3), 4.84 (near t, 1 H, H2), 6.38 (near t, 1 H, H3), 7.00–7.55 (m, 9 H, 7 H Ar + NH_2 which disappears with D_2O), 7.90 (s, 1 H, H6), 8.00–8.35 (m, 2 H, Ar), 12.70 s, 1 H, NH, slowly disappears with D_2O. ^{13}C NMR (DMSO): δ 39.12, 51.33, 64.77, 99.67, 114.68, 115.12, 120.38, 120.54, 125.20, 127.57, 128.47, 135.47, 140.79, 143.12, 143.37, 150.57, 165.70. IR (KBr): cm^{-1} 3352, 2843 (NH_2), 3201 (NH), 1671 (CONH), 1642 ($CONH_2$). Anal. ($C_{19}H_{16}FN_5O_2$) C, H, N.

6.1.2.14. N^1-1-Naphthyl-2-phenyl-2,3-dihydro-1H-imidazo[1,2-b]pyrazole-1,7-dicarboxamide ***3n***. Yield 10%, m.p. 228–229 °C ^1H NMR (DMSO-d_6): δ 1.55 (br s, 2 H, NH_2 disappears with D_2O), 4.08 (near q, 1 H, H3), 4.91 (near t, 1 H, H2), 6.43 (near q, 1 H, H3), 7.20–8.12 (m, 12 H, Ar), 8.20 (s, 1H, H6), 12.41 (s, 1 H, NH, slowly disappears with D_2O). ^{13}C NMR (DMSO): δ 39.10, 51.31, 64.83, 99.68, 126.40, 127.36, 128.10, 140.44, 143.28, 154.42, 165.74. IR (KBr): cm^{-1} 3334, 2800 (NH_2), 3197 (NH), 1665 (CONH), 1643 ($CONH_2$). Anal. ($C_{23}H_{19}N_5O_2$) C, H, N.

6.1.2.15. N^7-Cyclopropyl-N^1,2-diphenyl-2,3-dihydro-1H-imidazo[1,2-b]pyrazole-1,7-dicarboxamide ***3o***. Yield 19%, m.p. 228–229 °C ^1H NMR ($CDCl_3$): δ 0.60–0.78 and 0.89–0.95 (2 m, 4 H, 2 CH_2-cyclop.), 2.89 (m, 1 H, CH-cyclop.), 4.14 (near q, 1 H, H3), 4.79 (near t, 1 H, H2), 6.65 (near q, 1 H, H3), 6.98–7.90 (m, 12 H, 10 Ar + H6 + NH which disappears with D_2O), 11.55 (s, 1 H, NH, slowly disappears with D_2O). ^{13}C NMR (DMSO): δ 5.25, 5.41, 22.53, 39.17, 51.27, 64.77, 99.91, 118.78, 122.40, 125.22, 127.58, 128.37, 128.47, 139.08, 140.77, 142.69, 142.93, 150.61, 164.80. IR (KBr): cm^{-1} 3308 (NH), 2686 (NH-cyclop.), 1668 (CONH-Ph), 1636 (CONH-cyclop.). Anal. ($C_{22}H_{21}N_5O_2$) C, H, N.

6.1.2.16. N^7-Cyclopropyl-N^1-(3-fluorophenyl)-2-diphenyl-2,3-dihydro-1H-imidazo[1,2-b]pyrazole-1,7-dicarboxamide ***3p***. Yield 30%, m.p. 235–236 °C ^1H NMR ($CDCl_3$): δ 0.50–0.60 and 0.70–0.80 (2 m, 4 H, 2 CH_2-cyclop.), 2.80–3.00 (m, 1 H, CH-cyclop.), 4.02 (near q, 1 H, H3), 4.82 (near t, 1 H, H2), 6.38 (near q, 1 H, H3), 6.75–6.90 (m, 2 H, 1 H Ar + NH), 7.15–7.50 (m, 7 H, Ar), 8.00 (s, 1 H, H6), 8.70 (d, 1 H, Ar), 12.65 (s, 1 H, NH slowly disappears with D_2O). ^{13}C NMR (DMSO): δ 5.20, 5.41, 22.60, 39.20, 51.35, 64.50, 99.90, 114.41, 125.25, 127.72, 128.55, 140.80, 140.95, 141.21, 142.98, 143.45, 150.42, 164.85. IR (KBr): cm^{-1} 3311 (NH), 2654 (NH-cyclop.), 1668 (CONH-Ph), 1636 (CONH-cyclop). Anal. ($C_{22}H_{20}FN_5O_2$) C, H, N.

6.1.2.17. N^7-Piperidinyl-N^1,2-diphenyl-2,3-dihydro-1H-imidazo[1,2-b]pyrazole-1,7-dicarboxamide ***3q***. Yield 22%, m.p. 224–225 °C ^1H NMR ($CDCl_3$): δ 1.45–1.75 (m, 6 H, 3 CH_2-pip.), 3.55–3.91 (m, 4 H, 2 CH_2N-pip.), 4.15 (near q, 1 H, H3), 4.76 (near t, 1 H, H2), 6.32 (near q, 1 H, H3), 6.92–7.09 (m, 1 H, Ar), 7.20–7.61 (m, 10 H, 9Ar + H6) 10.41 (s, 1 H, NH, slowly disappears with D_2O). ^{13}C NMR (DMSO): δ 23.50, 39.15, 52.35, 65.07, 98.80, 104.93, 105.40, 107.45, 108.75, 118.76, 122.46, 125.30, 127.57, 128.40, 128.50, 139.28, 140.85, 142.70, 142.97, 150.75, 195.60. IR (KBr): cm^{-1} 3251 (NH), 1686 (CONH-Ph), 1624 (CON-pip.). Anal. ($C_{24}H_{25}N_5O_2$) C, H, N.

6.1.2.18. N^7-Piperidinyl-N^1-(3-fluorophenyl)-2-diphenyl-2,3-dihydro-1H-imidazo[1,2-b]pyrazole-1,7-dicarboxamide ***3r***. Yield 45%, m.p. 192–193 °C ^1H NMR (DMSO-d_6): δ 1.35–1.65 (m, 6H, 3 CH_2-pip), 3.40–3.80 (m, 4H, 2 CH_2-N-pip), 4.07 (near q, 1 H, H3), 4.83 (near t, 1 H, H2), 6.21 (near q, 1 H, H3), 6.73–6.88 (m, 1H Ar), 7.15–7.42 (m, 8H, Ar), 7.59 (s, 1H, H6), 10.46 (s, 1H, NH, disappears with D_2O). ^{13}C NMR (DMSO): δ 23.49, 39.12, 52.24, 65.03, 98.72, 104.92, 105.44, 107.58, 108.70, 114.10, 115.38, 125.29, 127.70, 128.49, 129.89, 140.30, 140.54, 142.36, 142.62, 149.55, 163.57, 172.31, 195.46. IR (KBr): cm^{-1} 3264 (NH), 1692 (CONH), 1612 (CON-pip). Anal. ($C_{24}H_{24}FN_5O_2$) C, H, N.

6.2. Biological assays

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 (Sigma, St. Louis, MO, USA) and tested compounds (10^{-2} M) were solved in DMSO, while CXCL8 (10^{-5} M) was solved in water. Before the use, all the solutions were diluted in KRPG to obtain the final concentrations wished from the experimental protocol. 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 [15], estimating the distance in micrometers which 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 nM or CXCL8 1 nM in the lower compartment.

Each tested compound was added in the upper compartment of the chemotaxis chamber, diluted at concentrations ranging from 10^{-12} to 10^{-6}M with KRPG containing 1 mg/mL of bovine serum albumin (BSA; Orha Behringwerke, Germany).

Neutrophils were preincubated with tested compounds 10 min before the functional test. 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^{-8} M, while the value of CXCL8 is 1.02 at 10^{-9} M.

The antagonism was measured as C.I. in the absence (100%) and in the presence of the tested compounds at different concentrations. The values are means of six separate experiments performed in duplicate. Standard errors are within 10% of the mean value [16].

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. Measure of cell viability

In order to assess possible cytotoxic effects of the tested compounds, the cytoplasmic marker enzyme, lactate dehydrogenase (LDH), was determined by measuring the rate of oxidation of NADH. The absorbance change was followed at 340 nm [17].

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