ELSEVIER

European Journal of Pharmacology 436 (2002) 187–196

ejp

www.elsevier.com/locate/ejphar

C- and N-terminal residue effect on peptide derivatives' antagonism toward the formyl-peptide receptor

Alessandro Dalpiaz [a], Maria E. Ferretti [b,*], Gianni Vertuani [a], Serena Traniello [c], Angelo Scatturin [a], Susanna Spisani [c]

[a] *Department of Pharmaceutical Sciences, Ferrara University, via Fossato di Mortara 19, 44100 Ferrara, Italy*
[b] *Section of General Physiology, Department of Biology, Ferrara University, via L. Borsari 46, 44100 Ferrara, Italy*
[c] *Department of Biochemistry and Molecular Biology, Ferrara University, via L. Borsari 46, 44100 Ferrara, Italy*

Received 5 July 2001; received in revised form 30 November 2001; accepted 24 December 2001

Abstract

The biological action of several X-Phe-D-Leu-Phe-D-Leu-Z (X = 3′,5′-dimethylphenyl-ureido; Z = Phe, Lys, Glu, Tyr) analogues was analysed on human neutrophils to evaluate their ability to antagonize formyl-peptide receptors. X-Phe-D-Leu-Phe-D-Leu-Phe analogues obtained as C-terminal olo or amido derivatives and T-Phe-D-Leu-Phe-D-Leu-Phe analogues (T = thiazolyl-ureido) were also analysed. The activities of pentapeptide derivatives were compared with those of X-Phe-D-Leu-Phe-D-Leu-Phe chosen as reference antagonist. Our results demonstrate that X-Phe-D-Leu-Phe-D-Leu-Phe-olo, X-Phe-D-Leu-Phe-D-Leu-Glu and X-Phe-D-Leu-Phe-D-Leu-Tyr are more active antagonists than X-Phe-D-Leu-Phe-D-Leu-Phe. The presence of Lys (X-Phe-D-Leu-Phe-D-Leu-Lys) seems, instead, to inhibit the formyl-peptide receptor antagonist properties. The presence of the N-terminal thiazolyl-ureido group seems to considerably contribute to the receptor antagonist properties of T-Phe-D-Leu-Phe-D-Leu-Phe-OH. The introduction of the C-terminal methyl ester (T-Phe-D-Leu-Phe-D-Leu-Phe-OMe) or amido group (X-Phe-D-Leu-Phe-D-Leu-Phe-NH_2) appears detrimental for the affinity and formyl-peptide receptor antagonist properties of the Phe-D-Leu-Phe-D-Leu-Phe derivatives. The examined peptides inhibit superoxide anion production and lysozyme release more efficaciously than neutrophil chemotaxis.

Keywords: Human, neutrophil; Formyl-peptide receptor; Antagonist; Neutrophil functionality

1. Introduction

Polymorphonuclear neutrophils are phagocytic cells involved in the defence against infections (Prossnitz and Ye, 1997). Evidence has shown that the peptide derivative formyl-Met-Leu-Phe-OH (fMLF), produced by bacterial sources or by disrupted mitochondria, is a potent stimulator of neutrophils (Carp, 1982; Marasco et al., 1984) and it is consequently used as a model chemoattractant for the study of phagocyte functions (Prossnitz and Ye, 1997; August et al., 1997). These researches led to the identification, on neutrophil membrane, of a G-protein-coupled formyl-peptide receptor which has since been cloned (Koo et al., 1983; Boulay et al., 1990; August et al., 1997). In addition to this receptor, which shows high affinity toward fMLF, human neutrophils express a low affinity variant (FPRL1) (Durstin et al., 1994). The binding of fMLF to its receptor triggers different functional responses, including adhesion, chemotaxis and free radical generation, which contribute to the physiological defence against bacterial infections and cell disruption (Prossnitz and Ye, 1997). On the other hand, in several pathological conditions, the inappropriate activation of neutrophils can cause tissue damage (Smith, 1994). In this context, a peptide derivative (Meera et al., 1999) and cyclosporin A (Spisani et al., 2001) have been proposed as anti-inflammatory agents on the basis of their ability to inhibit neutrophil-derived lysosome enzymes and reactive oxygen species. Moreover, annexin I peptides have been recently reported as novel, endogenous fMLF receptor ligands able to induce anti-inflammatory effects (Walther et al., 2000). It has been demonstrated that mice with a disrupted formyl-peptide receptor gene display an impaired antibacterial immunity (Gao et al., 1999), indicating that an inflammatory response of neutrophils towards chemotactic peptides also plays a role in immune responses. The im-

* Corresponding author. Tel.: +39-532-291-481; fax: +39-532-207-143.
E-mail address: clm@dns.unife.it (M.E. Ferretti).

0014-2999/02/$ - see front matter
PII: S0014-2999(01)01627-2

munomodulatory activity of cyclosporins, proposed as cancer chemotherapeutics, has been recently related to the inhibition of formyl-peptide receptor functions (Tiberghien et al., 2000). The development of fMLF receptor antagonists can be of considerable interest to better understand the role of drugs with immunomodulatory activity and potential effects in inflammation-related disorders.

Although the structural requirements for agonist interaction with formyl-peptide receptor have been extensively studied (Freer et al., 1982; Derian et al., 1996; Higgins et al., 1996; Dalpiaz et al., 2001), the information about formyl-peptide receptor antagonists appears to be relatively scarce. As a matter of fact, it has been reported that cyclosporine H (De Paulis et al., 1996), pyrazolidinedione derivatives (Levesque et al., 1991), spinorphin (Yamamoto et al., 1997), chenodeoxycholic acid (Chen et al., 2000) and peptide derivatives (Higgins et al., 1996) inhibit the fMLF binding to its receptor and some related cellular effects, but detailed information about the structural basis of this action is available for relatively few compounds (Levesque et al., 1992; Wenzel-Seifert et al., 1998; Dalpiaz et al., 1999).

As for the peptide derivatives, it was reported that the *tert*-butyloxycarbonyl (*t*-Boc) peptide derivative *t*-Boc-Phe-D-Leu-Phe-D-Leu-Phe shows formyl-peptide receptor antagonist activity on human neutrophils (Aswanikumar et al., 1977; Toniolo et al., 1990). It was also suggested that the *tert*-butyloxycarbonyl group in peptide derivatives is essential for imparting antagonist activity on rabbit and human neutrophils even if it causes a loss of binding potency (Freer et al., 1980; Derian et al., 1996). Higgins et al. (1996) have reported that *N*-ureido-Phe-D-Leu-Phe-D-Leu-Phe derivatives display an appreciable antagonist activity on human neutrophils on the basis of their observation that these peptides inhibit [^{3}H]fMLF binding and superoxide anion (O_2^-) production. We have recently confirmed and extended these studies, demonstrating that *N*-ureido-Phe-D-Leu-Phe-D-Leu-Phe peptide derivatives are able to antagonize multiple neutrophil functions evoked by fMLF, i.e. chemotaxis, O_2^- production and secretagogue activity (Dalpiaz et al., 1999; Scatturin et al., 1999). Moreover, they displace [^{3}H]fMLF from its binding sites and reduce fMLF effectiveness in enhancing the cytosolic level of Ca^{2+}, a second messenger involved in fMLF actions. The extent of inhibition of Ca^{2+} intracellular enhancement, as well as of O_2^- production and enzyme release by *N*-ureido-Phe-D-Leu-Phe-D-Leu-Phe peptide derivatives, appears related to their af-

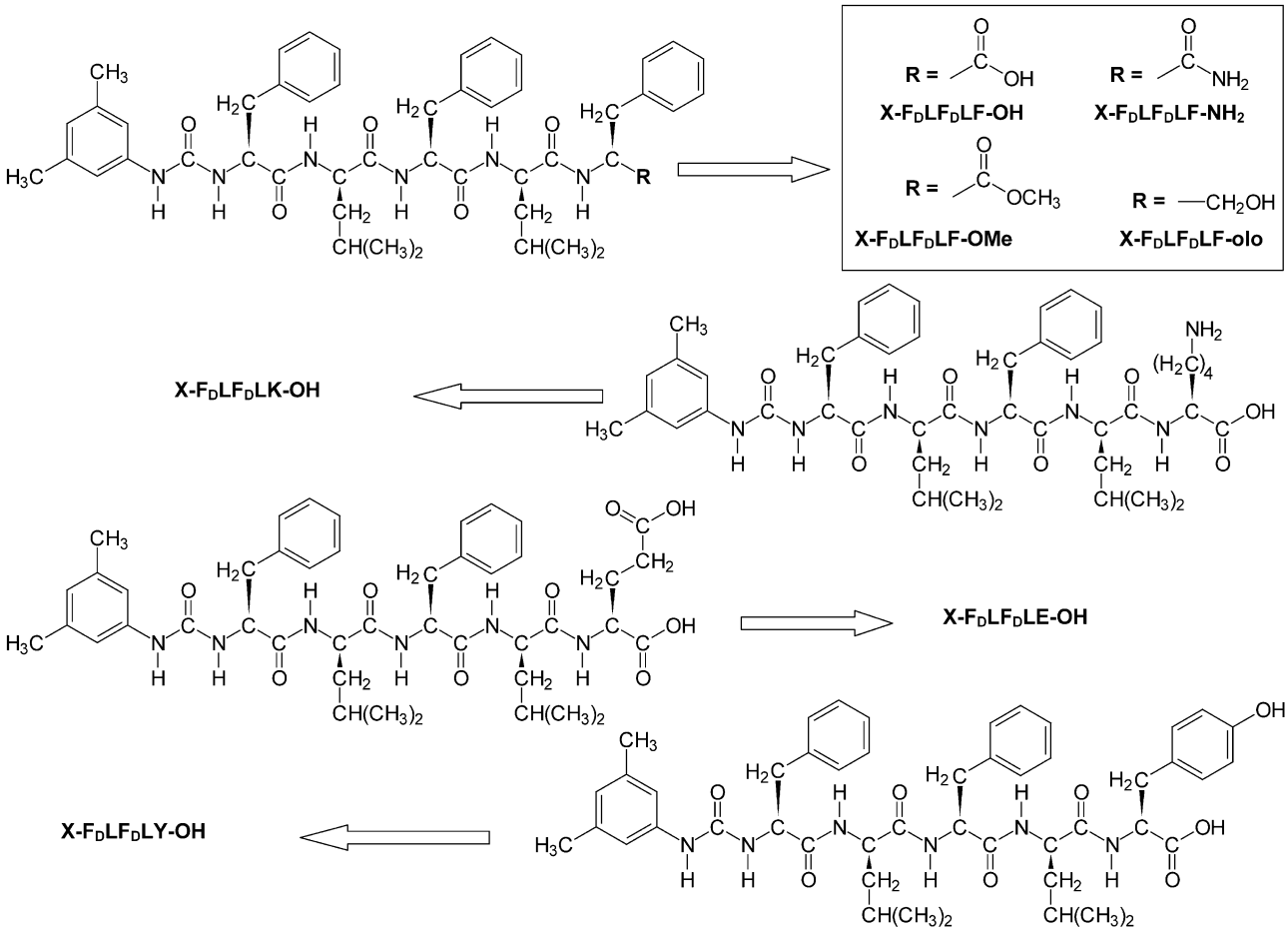

Fig. 1. Chemical formulas of the *N*-xilen-ureido pentapeptide derivatives analysed. **X** = 3′,5-dimethylphenyl-ureido; amino acids are indicated by one-letter codes.

finity toward the formyl-peptide receptor. We have also demonstrated that the C-terminal free acid peptide derivatives appear to be more active on human neutrophils than the methyl ester ones (Dalpiaz et al., 1999; Scatturin et al., 1999). The latter observation was the stimulus for our further investigations into the effect of a hydrophilic environment in the C-terminal position of *N*-ureido-pentapeptide derivatives. This paper therefore reports the synthesis and analysis of the biological properties of several X-Phe-D-Leu-Phe-D-Leu-Z [X = xilen-ureido; Z = Phe (**F**), Lys (**K**), Glu (**E**), Tyr (**Y**)] analogues with potential formyl-peptide receptor antagonist properties (Fig. 1). In particular, we have compared **X-F$_D$LF$_D$LK-OH** (L = Leu), **X-F$_D$LF$_D$LE- OH** and **X-F$_D$LF$_D$LY-OH** activities with respect to those of **X-F$_D$LF$_D$LF-OH**, which thus represents a reference compound in view of its previously demonstrated ability to act as a formyl-peptide receptor antagonist (Dalpiaz et al., 1999). In this context Lys, Glu or Tyr have been inserted in the C-terminal position of the free acid peptide chain with the aim of verifying the ability of these amino acids, more hydrophilic than Phe, to influence the antagonist behaviour of the ligands to which they belong. The derivatives **X-F$_D$LF$_D$LF-olo** and **X-F$_D$LF$_D$LF-NH$_2$** (Fig. 1) were also analysed.

The xilen-ureido substituent (**X**) has been included at the N-terminal of all the peptide analogues on the basis of our previous demonstration that this group confers appreciable formyl-peptide receptor antagonist properties to the Phe-D-Leu-Phe-D-Leu-Phe derivative (Dalpiaz et al., 1999; Scatturin et al., 1999). Moreover, the derivatives **T-F$_D$L F$_D$LF-OMe** and **T-F$_D$LF$_D$LF-OH** (T = thiazolyl-ureido, Fig. 2) have been synthesised and analysed with the aim to investigate the effects derived by a new N-terminal group.

The biological properties of the pentapeptide analogues were verified on human neutrophils by means of several in vitro assays: receptor binding and evaluation of their ability to inhibit fMLF-induced chemotaxis, O_2^- production and enzyme release.

2. Materials and methods

2.1. Peptides

The peptides were synthesised by conventional methods in the solution. Amino acid derivatives were obtained from commercial sources or synthesised according to the literature (Bodanszky and Bodanszky, 1984). The peptide derivatives 3′,5′-dimethylphenyl-ureido-(Phe-D-Leu)$_2$-Phe-OMe and 3′,5′-dimethylphenyl-ureido-(Phe-D-Leu)$_2$-Phe-OH were obtained as previously described by Dalpiaz et al. (1999). The protecting groups *tert*-butyloxycarbonyl (*t*-Boc) and *tert*-butyl ester (OtBu) were removed by treatment with a mixture of trifluoroacetic acid and CH_2Cl_2 (1:1 v/v). The benzyloxycarbonyl (Z) protecting group was removed by catalytic hydrogenation in methanol in the presence of 10% palladium on carbon and equivalent HCl using a Parr apparatus at about 60 psi. The methyl ester group was hydrolysed with 2 N NaOH and methanol (1:1 v/v). The active carbamate, thiazol-2-yl-carbamic acid 2,5-dioxo-pyrrolidin-1-yl ester, was prepared by stirring for 24 h at room

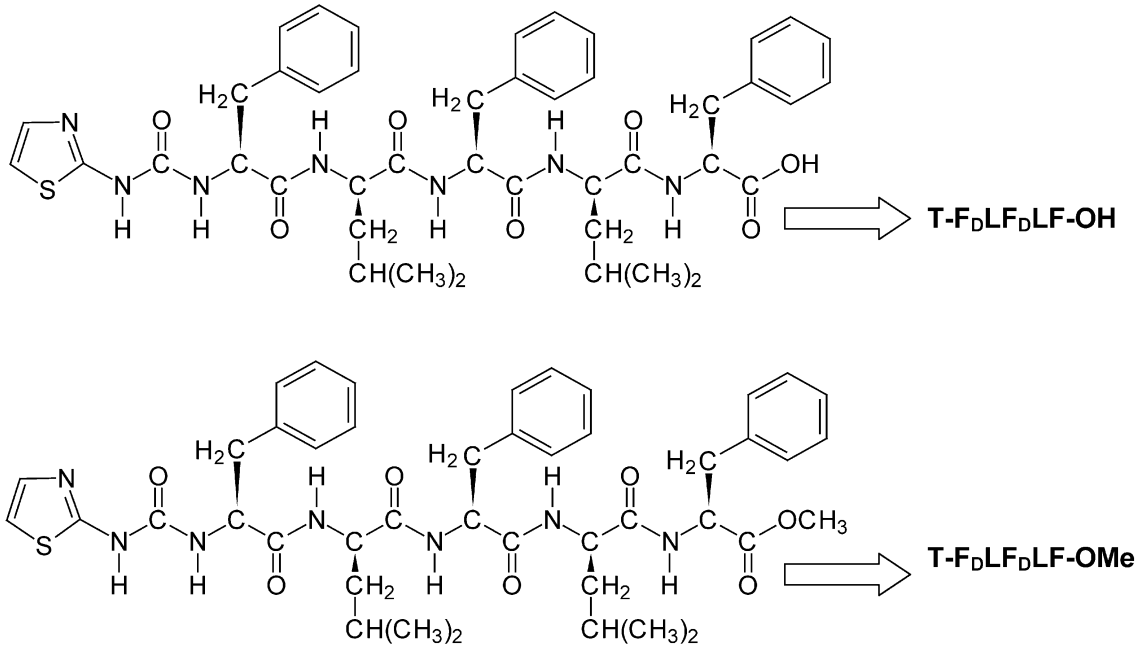

Fig. 2. Chemical formulas of the *N*-thiazolyl-ureido pentapeptide derivatives analysed. **T** = thiazolyl-ureido; amino acids are indicated by one-letter codes.

temperature 2-aminothiazol (5 mmol; from Fluka) and *N*, N^{I}-disuccinimidyl carbonate (10 mmol; from Novabiochem) in anhydrous acetonitrile (150 ml) (Takeda et al., 1983). The xilen-ureidic derivatives were obtained by the reaction of respective N-deprotected peptides with 3,5-dimethyl-phenylisocyanate in *N*,*N*-dimethylformamide in the presence of triethylamine. Using *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride and hydroxybenzotriazole, *t*-Boc-Phe-OH was coupled (on 28 mmol scale) to HCl·*H*-D-Leu-OMe in *N*,*N*-dimethylformamide containing *N*-methylmorpholine at 0 °C (1 h). After 12 h at room temperature, the dipeptide *t*-Boc-Phe-D-Leu-OMe (**1**) was obtained. The same coupling method was employed in further condensation procedures. In order to obtain the pentapeptides 3′,5′-dimethylphenyl-ureido-(Phe-D-Leu)$_2$-Lys-(H·HCl)-OH (**9**) and 3′,5′-dimethylphenyl-ureido-(Phe-D-Leu)$_2$-Tyr-OH (**10**), dipeptide **1** was then hydrolysed to *t*-Boc-Phe-D-Leu-OH (**2**). The same peptide was condensed (on a 5-mmol scale) with the two derivatives HCl·*H*-Lys-(Z)-OMe and HCl·*H*-Tyr-OMe, producing the tripeptides *t*-Boc-Phe-D-Leu-Lys-(Z)-OMe (**3**) and *t*-Boc-Phe-D-Leu-Tyr-OMe (**4**), respectively. Tripeptides **3** and **4**, *N*-deprotected, were then condensed (on a 2.9-mmol scale) with dipeptide **2**, obtaining two pentapeptides: *t*-Boc-(Phe-D-Leu)$_2$-Lys-(Z)-OMe (**5**), which was purified by chromatography on a silica gel column eluted with ethyl acetate–methanol (9:1), and *t*-Boc-(Phe-D-Leu)$_2$-Tyr-OMe (**6**). Successively, pentapeptides **5** and **6** were *N*-deprotected and added (on the 0.8-mmol scale) to the appropriate isocyanate, obtaining two xilen-ureidic derivatives: 3′,5′-dimethylphenyl-ureido-(Phe-D-Leu)$_2$-Lys-(Z)-OMe (**7**), which was purified in the same way as compound **5**, and 3′,5′-dimethylphenyl-ureido-(Phe-D-Leu)$_2$-Tyr-OMe (**8**). Peptide **7** was hydrogenated (0.4 mmol) and successively hydrolysed (0.3 mmol), producing the final peptide **9**, purified by stirring in ethyl ether. Peptide **8** was hydrolysed (0.35 mmol) to obtain the final peptide **10**, which was crystallised from ethyl acetate and petroleum ether. Dipeptide **1** was N-deprotected and condensed (on the 2.46-mmol scale) with dipeptide **2** to obtain the tetrapeptide *t*-Boc-(Phe-D-Leu)$_2$-OMe (**11**). Peptide **11**, after the cleavage of the *t*-Boc-group, was added to isocyanate (on the 1.4-mmol scale) to obtain 3′,5′-dimethylphenyl-ureido-(Phe-D-Leu)$_2$-OMe (**12**). Peptide **12** was successively hydrolysed (1.34 mmol) and condensed (on the 0.73-mmol scale) with derivative HCl·*H*-Glu-(OtBu)-OMe, obtaining pentapeptide 3′,5′-dimethylphenyl-ureido-(Phe-D-Leu)$_2$-Glu-(OtBu)-OMe (**13**). Peptide **13**, which was hydrolysed (0.6 mmol) and cleft on the side chain of the Glu residue (0.5 mmol), produced the final peptide 3′,5′-dimethylphenyl-ureido-(Phe-D-Leu)$_2$-Glu-OH (**14**) that was purified by stirring in ethyl ether. Tetrapeptide **12**, after hydrolysis, was condensed (on the 0.23-mmol scale) with derivative HCl·*H*-Phe-NH_2 to produce the final peptide 3′,5′-dimethylphenyl-ureido-(Phe-D-Leu)$_2$-Phe-NH_2 (**15**) which was purified in the same way as compound **14**. The final pentapeptide 3′,5′-dimethyl-phenyl-ureido-(Phe-D-Leu)$_2$-Phe-olo (**19**) was synthesised starting from dipeptide ***2***, which was condensed (on the 4.5-mmol scale) with derivative HCl·*H*-Phe-OMe to obtain the tripeptide *t*-Boc-Phe-D-Leu-Phe-OMe (**16**). The methyl ester group of peptide **16** (3.71 mmol) was reduced to the primary alcoholic group in ethanol/H_2O (1:1 v/v) containing $NaBH_4$ (14.84 mmol). The solution was stirred at room temperature for 1 h and heated at reflux for 5 h, giving *t*-Boc-Phe-D-Leu-Phe-olo (**17**). Tripeptide **17**, after the cleavage of the *t*-Boc-group, was coupled (on the 1.71-mmol scale) to dipeptide **2**, producing the pentapeptide *t*-Boc-(Phe-D-Leu)$_2$-Phe-olo (**18**). Peptide **18** was N-deprotected (1.16 mmol) and added (on the 0.73-mmol scale) to 3,5-dimethyl-phenylisocyanate to obtain final peptide **19**, which was crystallised from ethyl acetate and petroleum ether. The derivative T-F_DLF_DLF-OMe (**20**) was prepared by stirring for 48 h thiazol-2-yl-carbamic acid 2,5-dioxo-pyrrolidin-1-yl ester (3 mmol) with the corresponding N-deprotected pentapeptide (1.5 mmol; Dalpiaz et al., 1999) in anhydrous dioxane containing *N*-methylmorpholine (1.5 mmol). The derivative T-F_DLF_DLF-OH (**21**) was obtained from peptide **20** by treatment with NaOH in methanol (on the 1-mmol scale) and purified by stirring in ethyl ether. The structure of the purified peptides was confirmed by amino acid analysis and ^{1}H NMR spectrometry. The molecular weight of peptides was determined using a mass spectrometer Hewlett-Packard MALDI TOF model G2025A. All peptides were purified by silica gel column and were analysed by reverse phase liquid chromatography on a Vydac C_{18} column with acetonitrile gradient elution. The compounds were characterised by mass spectrometry analysis, melting point determination and polarimetric measurements.

Stock solutions, 10^{-2} M of fMLF (Sigma, St. Louis, MO, USA) and pentapeptide analogues, were prepared in dimethylsulfoxide (DMSO, Sigma) and diluted in Krebs–Ringer phosphate containing 0.1% w/v glucose (KRPG, pH 7.4) before use.

KRPG was made up as a stock solution of the following composition: NaCl, 40 g/l; KCl, 1.875 g/l; $Na_2HPO_4{\cdot}2H_2O$, 0.6 g/l; KH_2PO_4, 0.125 g/l; $NaHCO_3$, 1.25 g/l; and glucose, 10 g/l. This solution was five times the working strength. $MgCl_2$ and $CaCl_2$ (1 mM) were supplemented to the buffer before the biological test. All reagents were of the purest grade and commercially available.

Pentapeptide derivatives were assayed for their ability to cause leakage of the cytoplasmic marker lactic dehydrogenase but none of them did (data not reported). At the concentrations used, DMSO did not interfere with any of the biological assays performed.

2.2. Cell preparation

Cells were obtained from the blood of healthy subjects, and the neutrophils were purified employing the standard techniques of dextran sedimentation (Pharmacia, Uppsala,

Sweden), centrifugation on Ficoll-Paque (Pharmacia) and hypotonic lysis of contaminating red cells. The cells were washed twice, resuspended in KRPG, pH 7.4, at a final concentration of 50×10^6 cells/ml and used immediately. The percentage of the neutrophils was 98–100% pure and 99% viable as determined by the Trypan blue exclusion test.

2.3. Receptor binding assays

Neutrophils (5×10^6) were incubated in 200 μl of KRPG at 37 °C for 15 min according to previous time course experiments. Displacement experiments were performed using at least eight different concentrations of cold drugs in the presence of 10 nM [^{3}H]fMLF (specific activity = 71.5 Ci/mmol, NEN Research Products, Du Pont de Nemours, Milan, Italy). Non-specific binding was measured in the presence of 10 μM fMLF. Separation of bound from the free radioligand was performed by rapid filtration through Whatman GF/B filters, which were washed with the ice-cold buffer. Filter-bound radioactivity was measured by scintillation spectrometry after the addition of 4 ml of Packard Emulsifier Safe.

The cold drug concentrations displacing 50% of labelled ligand (IC_{50}) were obtained by the computer analysis of displacement curves. All data were analysed using the non-linear regression curve fitting computer program Graph Pad Prism (Graph Pad, San Diego, CA, USA). All the values obtained are means of three independent experiments performed in duplicate.

2.4. Neutrophil functions

Random locomotion and chemotaxis were evaluated using a 48-well microchemotaxis chamber (BioProbe, Milan, Italy), which estimates the distance in micrometers which the leading front of the cell migrated, and using the method of Zigmond and Hirsch (1973). Chemoattractant activity was determined by adding each peptide derivative to the lower compartment of the chemotaxis chamber, and it was expressed in terms of chemotactic index (CI) which is the ratio:

$$\mathrm{CI} = \frac{\text{migration toward test attractant} - \text{migration toward the buffer}}{\text{migration toward the buffer}}$$

Migration in the presence of the buffer alone was 30 μm ± 4 S.E. ($n = 15$) and the CI of 10 nM fMLF was 1.15 ± 0.1 S.E. ($n = 15$).

O_2^- production was measured by the superoxide dismutase–inhibitable reduction of ferricytochrome c (Torrini et al., 1996) modified for microplate-based assays. The tests were carried out in a final volume of 200 μl containing 4×10^5 neutrophils, 100 nmol of cytochrome *c* (Sigma) and KRPG. At time zero, 1 μM fMLF was added and the plates were incubated in a microplate reader (Ceres 900, Bio-TeK instruments) with the compartment *T* set at 37 °C. Absorbance was recorded at wavelengths of 550 and 468 nm. Differences in absorbance at the two wavelengths were used to calculate the nanomoles of O_2^- produced using a molar extinction coefficient for cytochrome *c* of 18.5 mM^{-1} cm^{-1}. Neutrophils were preincubated in the presence of 5 μg/ml cytochalasin B (Sigma) for 5 min prior to activation by fMLF. The actual control of O_2^- generation produced by 1 μM fMLF was 45 ± 4 nmol/1×10^6 cells/5 min.

The release of neutrophil granule enzymes was evaluated by determining the lysozyme activity (Torrini et al., 1996) which is modified for microplate-based assays. Cells (3×10^6) were incubated in microplate wells in the presence of 1 μM fMLF for 15 min at 37 °C. The plates were then centrifuged for 5 min at $400 \times g$ and the lysozyme was quantified nephelometrically by the rate of lysis of the cell wall suspension of *Micrococcus lysodeikticus* (Sigma). Cells were preincubated in the presence of 5 μg/ml cytochalasin B for 15 min prior to activation by fMLF. Reaction rate was measured with a microplate reader at 465 nm. Enzyme output was expressed as a net percentage of the total enzyme content released by 0.1% Triton X-100. Spontaneous release was less than 10% and total enzyme activity was 85 ± 1 μg/1×10^7 cells/min.

Antagonist activity was determined by measuring the pentapeptide's ability to inhibit chemotaxis, O_2^- production or granule enzyme release as activated by fMLF. The data of antagonists (percentage of activity) were obtained by comparing the chemotactic index, nanomoles of O_2^- or the percentage of lysozyme release in the absence (100%) and in the presence of pentapeptides. The antagonist concentrations inhibiting the fMLF-induced O_2^- production or granule enzyme release by 50% (IC_{50}) were obtained by the computer analysis of inhibition curves. All data were

Table 1
IC_{50} values for the displacement by the pentapeptide derivatives of 10 nM [^{3}H]fMLF from human neutrophils and IC_{50} values for inhibition by the same compounds of superoxide anion production and enzyme release induced by 1 μM fMLF

Ligand	IC_{50} (binding) [nM]	IC_{50} (O_2^- production) [μM]	IC_{50} (enzyme release) [μM]
X-F_DLF$_D$LF-OH	528 ± 40	2.3 ± 0.2	5.0 ± 0.3
X-F_DLF$_D$LF-OMe	11 310 ± 600	≫10 *	≫10 *
X-F_DLF$_D$LF-NH_2	6240 ± 400	≫10 *	≫10 *
X-F_DLF$_D$LF-olo	327 ± 20	1.2 ± 0.1	2.1 ± 0.1
X-F_DLF$_D$LK-OH	807 ± 50	3.3 ± 0.2	8.6 ± 0.5
X-F_DLF$_D$LE-OH	256 ± 15	1.1 ± 0.1	2.7 ± 0.2
X-F_DLF$_D$LY-OH	306 ± 18	1.5 ± 0.1	3.4 ± 0.2
T-F_DLF$_D$LF-OH	94 ± 6	0.80 ± 0.07	1.41 ± 0.06
T-F_DLF$_D$LF-OMe	5100 ± 350	≫10 *	≫10 *

Each IC_{50} value represents an average of three (binding) or six (neutrophil functions) independent experiments performed in duplicate, ± S.E.M.

* Completely unable at concentrations ranging from 10^{-10} to 10^{-5} M to significantly inhibit superoxide anion production and granule enzyme release produced by 1 μM fMLF.

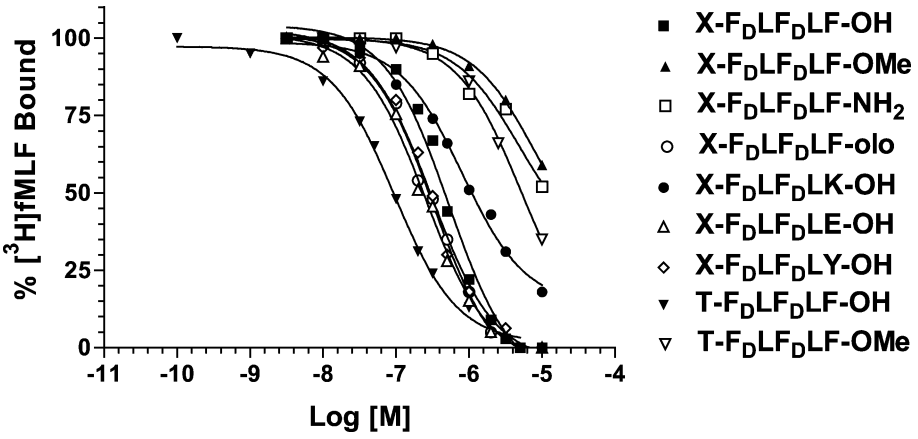

Fig. 3. Competition experiments of the pentapeptide derivatives analysed for specific [^{3}H]fMLF binding carried out at 37 °C on human neutrophils. These are single representative experiments carried out in duplicate.

analysed using the non-linear regression curve fitting computer program Graph Pad Prism, and the obtained values are means of six independent experiments performed in duplicate.

2.5. Statistical analysis

Analysis of variance was performed with SigmaStat (version 2.0, Jandel Scientific software). The difference was considered statistically significant at P values of < 0.05.

3. Results

In a first series of studies, we carried out [^{3}H]fMLF displacement experiments by incubating human neutrophils in the presence of increasing concentrations of pentapeptide derivatives. **X-F$_D$LF$_D$LF-OH** ($IC_{50} = 528$ nM, Table 1) was utilised as a reference compound in view of its ability to appreciably antagonize fMLF binding to the formyl-peptide receptor (Dalpiaz et al., 1999). As shown in Fig. 3, all analysed compounds progressively inhibited [^{3}H]fMLF binding although with varying effectiveness. Table 1 reports the IC_{50} values for 10 nM [^{3}H]fMLF displacement by the pentapeptides. It can be observed that the presence of C-terminal methyl ester (**X-F$_D$LF$_D$LF-OMe**, $IC_{50} = 11\,310$ nM; **T-F$_D$LF$_D$LF-OMe**, $IC_{50} = 5100$ nM) or amido groups (**X-F$_D$LF$_D$LF-NH$_2$**, $IC_{50} = 6240$ nM) is detrimental for the affinity of peptide analogues toward the formyl-peptide receptor. On the contrary, the presence of the C-terminal olo group exerts a beneficial effect (**X-F$_D$LF$_D$LF-olo**, $IC_{50} = 327$ nM). As for the substitution of the Phe residue in the peptide chain, an enhancement of the affinity toward the formyl-peptide receptor is observed with Glu (**X-F$_D$LF$_D$LE-OH**, $IC_{50} = 256$ nM) or Tyr (**X-F$_D$LF$_D$LY-OH**, $IC_{50} = 306$ nM), whereas a reduction is obtained with Lys (**X-F$_D$LF$_D$LK-OH**, $IC_{50} = 807$ nM). Finally, the presence of the N-terminal thiazolyl-ureido group induces a good improvement of the affinity toward the formyl-peptide receptor (**T-F$_D$LF$_D$LF-OH**, $IC_{50} = 94$ nM).

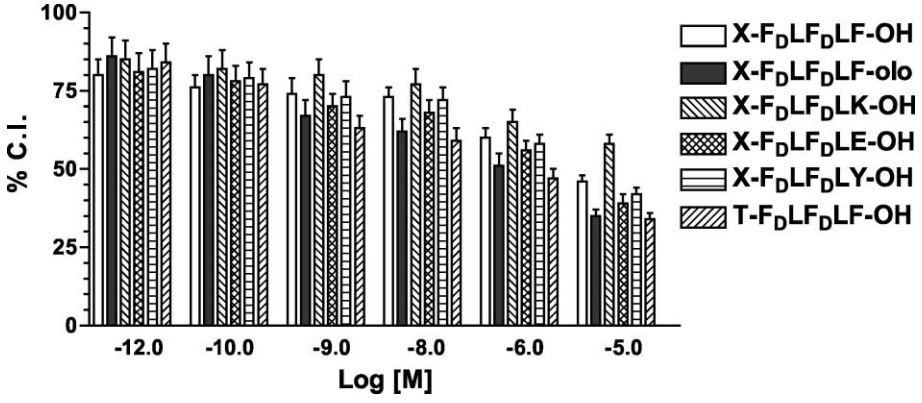

Fig. 4. Effect of the pentapeptide derivatives on chemotaxis activated by 10 nM fMLF. Data are expressed as percentage of CI. Pentapeptides were added to neutrophils 10 min before the incubation step for chemotaxis. Each value represents the mean of six separate experiments carried out in duplicate.

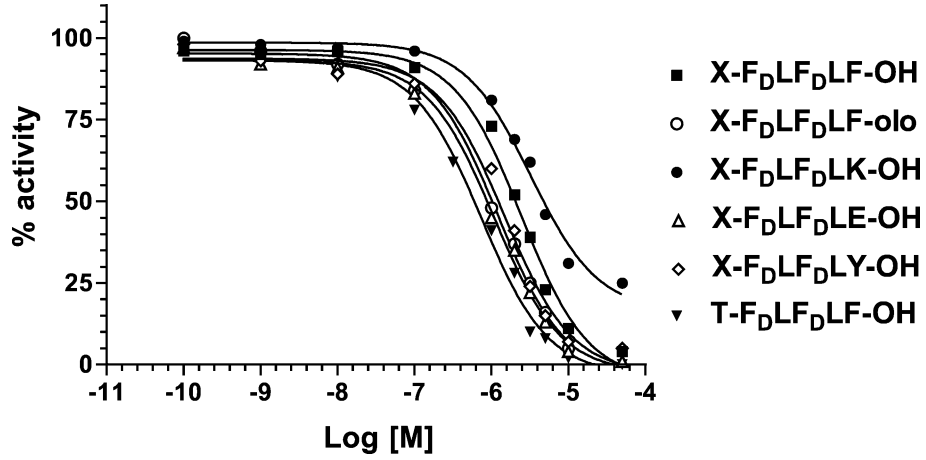

Fig. 5. Effect of the pentapeptide derivatives on superoxide anion production triggered by 1 μM fMLF. Pentapeptides were added to neutrophils 10 min before the incubation step for neutrophil functionality. These are single representative experiments carried out in duplicate.

In the first stage of functional studies, we verified whether the compounds under investigation were able to induce chemotaxis, trigger O_2^- production or lysozyme release per se. The results indicated that the peptides have no intrinsic agonist activity for human neutrophils (not shown).

In the second stage, we verified the antagonist activity of these peptides on the same neutrophil responses, once again utilising **X-F_DLF$_D$LF-OH** as a reference compound. In Fig. 4, the effect of increasing doses (from 10^{-12} to 10^{-5} M) of peptides **X-F_DLF$_D$LF-OH**, **X-F_DLF$_D$LF-olo**, **X-F_DLF$_D$LK-OH**, **X-F_DLF$_D$LE-OH**, **X-F_DLF$_D$LY-OH** and **T-F_DLF$_D$LF-OH** on the chemotactic response evoked by 10 nM fMLF is illustrated. As shown, a dose-dependent reduction of CI was observed, and inhibitions near 50% were obtained at concentrations ranging from 10^{-6} to 10^{-5} M.

Figs. 5 and 6 report the curves describing the effects exerted by the same peptides on O_2^- production or lysozyme release, respectively, triggered by 1 μM fMLF. As can be seen, all compounds were scarcely efficacious up to 10^{-7} M, while at higher concentrations, they exert a strong and dose-dependent inhibition of neutrophil responses. The IC_{50} values, which were obtained by the analysis of the curves illustrated in Figs. 5 and 6, are reported in Table 1. As in the case of binding experiments, the peptide analogues **X-F_DLF$_D$LF-olo**, **X-F_DLF$_D$LE-OH**, **X-F_DLF$_D$LY-OH** and **T-F_DLF$_D$LF-OH** were more potent antagonists than **X-P_DLP$_D$LF-OH**, whereas **X-P_DLP$_D$LK-OH** showed a weaker antagonist power.

The peptide derivatives **X-F_DLF$_D$LF-OMe**, **X-F_DLF$_D$LF-NH$_2$** and **T-F_DLF$_D$LF-OMe**, at concentrations ranging from 10^{-12} to 10^{-5} M, were almost completely unable to inhibit either the chemotaxis evoked by 10 nM fMLF or the O_2^- production and granule enzyme release evoked by 1 μM fMLF (not shown).

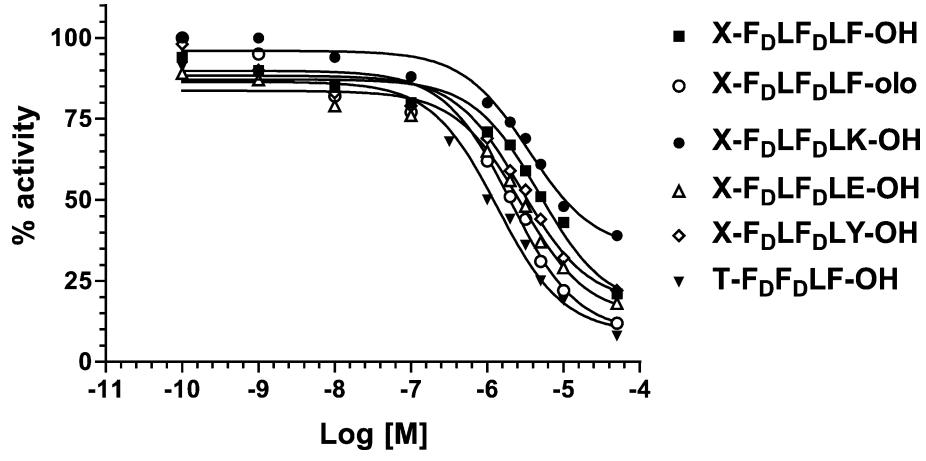

Fig. 6. Effect of the pentapeptide derivatives on lysozyme release induced by 1 μM fMLF. Pentapeptides were added to neutrophils 10 min before the incubation step for neutrophil functionality. These are single representative experiments carried out in duplicate.

4. Discussion

The various neutrophil functions are evoked when agonists, such as fMLF, interact with specific membrane receptors and activate multiple transduction pathways (Prossnitz and Ye, 1997). Many drugs exist which are able to inhibit neutrophil responses, acting by impairing some of the different steps of these transduction pathways (Bergstrand et al., 1992; Mócsai et al., 1997). A major limitation in their use as therapeutic agents for the treatment of inflammation-related diseases is that these drugs are not selective since other cellular responses could be inhibited at the same time.

Gao et al. (1999) have demonstrated that the absence of fMLF receptor impairs the antibacterial immunity in mice. Nevertheless, the development of receptor antagonists of neutrophil stimulators that are able to transiently inhibit cellular responses should improve the knowledge about leukocyte chemoattractant functions and could be of clinical relevance.

Several peptide as well as non-peptide formyl-peptide receptor antagonists have been developed but relatively few information are available about the structural requirements for such antagonism. As an example, cyclosporin H was reported as a potent formyl-peptide receptor antagonist (De Paulis et al., 1996) and very recently, it was demonstrated that it acts as an inverse agonist (Wenzel-Seifert et al., 1998). A pyrazolidinedione derivative was proposed as a selective and potent competitor of [^{3}H]fMLF binding (Levesque et al., 1992), and more recently, it was demonstrated that the endogenous ligands spinorphin (Yamamoto et al., 1997) or the chenodeoxycholic acid (Chen et al., 2000) inhibits the fMLF binding to its receptor some related cellular responses. The complete knowledge of how these ligands interact with the formyl-peptide receptor requires a more detailed analysis. As for the peptide derivatives, we have recently demonstrated that the C-terminal-free acid *N*-ureido-pentapeptide derivatives appear to be more active antagonists on human neutrophils than the methyl ester ones (Dalpiaz et al., 1999; Scatturin et al., 1999). On the basis of this observation, we synthesised the *N*-ureido-pentapeptide derivatives **X-F$_D$LF$_D$LF-olo** and **X-F$_D$LF$_D$LF-NH$_2$** with the aim of further investigating the role of the C-terminal groups in the interaction with the formyl-peptide receptor. Moreover, the analogues **X-F$_D$LF$_D$LK-OH**, **X-F$_D$LF$_D$LY-OH** and **X-F$_D$LF$_D$LE-OH** were obtained with the purpose of evaluating the influence of amino acids that are more hydrophilic than Phe in the C-terminal position on the pentapeptide antagonist behaviour. The analysis of the influence of a new N-terminal group (thiazolyl-ureido) was performed by using the derivatives **T-F$_D$LF$_D$LF-OMe** and **T-F$_D$LF$_D$LF-OH**.

As far as the C-terminal group is concerned, our results allow us to hypothesize that the presence of a hydroxyl function is essential to impart affinity to the formyl-peptide receptor. In fact, as reported in Table 1, only pentapeptide derivatives with the free acid C-terminal group (**X-F$_D$LF$_D$LF-OH**, **X-F$_D$LF$_D$LK-OH**, **X-F$_D$LF$_D$LY-OH** and **X-F$_D$LF$_D$LE-OH**) and the olo derivative (**X-F$_D$LF$_D$LF-olo**) show an appreciable affinity toward the formyl-peptide receptor, whereas the analogues with the C-terminal methyl ester (**X-F$_D$LF$_D$LF-OMe** and **T-F$_D$LF$_D$LF-OMe**) or amido groups (**X-F$_D$LF$_D$LF-NH$_2$**) scarcely bind to it. Thus, the carbonyl group does not appear to be directly involved in this interaction. These observations suggest, therefore, that an appreciable affinity to the formyl-peptide receptor could be obtained either by the presence of a negative charge in the C-terminal region of the peptide derivatives (as in the case of COO^- moiety) or via a hydrogen bond accepted by the receptor (as in the case of the olo moiety).

It is interesting to observe that the presence of the N-terminal thiazolyl-ureido greatly improves the affinity of **T-F$_D$LF$_D$LF-OH** toward the formyl-peptide receptor. In fact, the IC_{50} value obtained for this compound (94 nM) appears near to that previously reported for the endogenous ligand fMLF (IC_{50} = 41 nM; Dalpiaz et al., 2001).

The antagonist power of the pentapeptide derivatives appears similarly influenced by the molecular features of the C- and N-terminal groups. In fact, among the compounds analysed, only the analogues devoid of a hydroxyl moiety are unable to significantly antagonize the fMLF effects on human neutrophils as far as chemotaxis, O_2^- production and lysozyme release are concerned.

The substitution of Phe with more hydrophilic amino acids at the C-terminal of the pentapeptide chain seems to produce weak effects on the affinity and antagonist power toward the formyl-peptide receptor. Nevertheless, it can be observed that the presence of the Lys residue slightly attenuates the affinity and antagonist power toward the formyl-peptide receptor, whereas the insertion of Glu or Tyr residues produces opposite effects (Table 1). It can be concluded that amino acids with long and positive-charged side chain, such as Lys, are not qualified for the last position in the pentapeptide chain in view of their interaction with the formyl-peptide receptor. On the other hand, it must be underlined that **T-F$_D$LF$_D$LF-OH** shows the highest affinity and antagonist potency for the formyl-peptide receptor among the peptide derivatives analysed.

The data reported here indicate that the peptides under examination inhibit O_2^- production and lysozyme release more efficaciously than neutrophil chemotaxis (Figs. 4–6). Similar results have previously been obtained utilising different pentapeptide analogues (Dalpiaz et al., 1999). In a previous study carried out in order to evaluate the agonist activity of fMLF analogues that are able to selectively induce chemotaxis or O_2^- production, we put forward the hypothesis that the formyl-peptide receptor undergoes conformational changes, depending on the type of cellular response that it must evoke: when exposed to the high fMLF doses required for O_2^- production, it could in fact assume a conformation different from that suitable for evoking chemotaxis in response to low fMLF concentrations

(Fabbri et al., 2000). In addition, it has long been known that the transduction pathway underlying the chemotactic response is different from those responsible for O_2^- production or lysozyme release (Ferretti et al., 1994; Fabbri et al., 1997; Li et al., 2000). On the other hand, the existence of at least three or six formyl-peptide receptor subtypes for human and mouse species, respectively, has been demonstrated (Durstin et al., 1994; Gao et al., 1998). It has been reported that some of these receptor subtypes show different affinity values for fMLF and induce or not chemotactic responses requiring different optimal fMLF concentrations (Laudanna et al., 1996; Hartt et al., 1999).

On the basis of the results reported here, we can therefore hypothesize that the different antagonist activity exerted by the pentapeptide derivatives towards the various neutrophil responses could be the consequence of their interaction with different states and/or with different subtypes of the formyl-peptide receptor.

Acknowledgements

This research was supported by the Italian Ministry of University, Scientific and Technological Research (MURST) and the Italian Association for Cancer Research (AIRC). The authors thank Mrs. Linda Bruce, a qualified mother tongue teacher, for the English revision of the text and Banca del sangue of Ferrara for supplying fresh blood.

References

Aswanikumar, S., Corcoran, B.A., Schiffman, E., 1977. Peptides with agonist and antagonist chemotactic activity. In: Goodman, M., Meinhofer, J. (Eds.), Peptides. Wiley, New York, pp. 141–145.

August, J.T., Anders, M.W., Murad, F., Coyle, J., 1997. Structure and function of leukocyte chemoattractant receptors. Adv. Pharmacol. 39, 221–289.

Bergstrand, H., Eriksson, T., Hallberg, A., Johansson, B., Karabelas, K., Michelsen, P., Nybon, A., 1992. Modulation of neutrophil superoxide generation by inhibitors of protein kinase C, calmodulin, diacylglycerol and myosin light chain kinases, and peptidyl prolyl *cis-trans* isomerase. J. Pharmacol. Exp. Ther. 263, 1334–1346.

Bodanszky, M., Bodanszky, A., 1984. The Practice of Peptide Synthesis. Springer-Verlag, Berlin.

Boulay, F., Tardif, M., Brouchon, L., Vignais, P., 1990. The human *N*-formylpeptide receptor: characterization of two cDNA isolates and evidence for a new subfamily of G-protein-coupled receptors. Biochemistry 29, 11123–11133.

Carp, H., 1982. Mithocondrial *N*-formylmethionyl proteins as chemoattractants for neutrophils. J. Exp. Med. 185, 264–275.

Chen, X., Yang, D., Shen, W., Dong, H.F., Wang, J.M., Oppenhheim, J.J., Howard, M.Z., 2000. Characterization of chenodeoxycholic acid as an endogenous antagonist of the G-coupled formyl peptide receptors. Inflammation Res. 49, 744–755.

Dalpiaz, A., Ferretti, M.E., Pecoraro, R., Fabbri, E., Traniello, S., Scatturin, A., Spisani, S., 1999. Phe-D-Leu-Phe-D-Leu-Phe derivatives as formyl-peptide receptor antagonists in human neutrophils: cellular and conformational aspects. Biochim. Biophys. Acta 1432, 27–39.

Dalpiaz, A., Scatturin, A., Vertuani, G., Pecoraro, R., Borea, P.A., Varani, K., Traniello, S., Spisani, S., 2001. Met-Ile-Phe-Leu derivatives: full and partial agonists of human neutrophil formylpeptide receptors. Eur. J. Pharmacol. 411, 327–333.

De Paulis, A., Ciccarelli, A., de Crescenzo, G., Cirillo, R., Patella, V., Marone, G., 1996. Cyclosporin H is a potent and selective competitive antagonist of human basophil activation by *N*-formyl-methionyl-leucyl-phenylalanine. J. Allergy Clin. Immunol. 98, 152–164.

Derian, K., Solomon, H.F., Higgins, J.D., Beblavy, J.M., Santulli, R.J., Bridger, G.J., Pike, M.C., Kroon, D.J., Fishman, A.J., 1996. Selective inhibition of *N*-formylpeptide-induced neutrophil activation by carbamate-modified peptide analogues. Biochemistry 35, 1265–1269.

Durstin, M., Gao, J.L., Tiffany, H.L., Mc Dermott, D., Murphy, P.M., 1994. Differential expression of the members of the *N*-formylpeptide receptor gene cluster in human phagocytes. Biochem. Biophys. Res. Commun. 201, 174–179.

Fabbri, E., Spisani, S., Biondi, C., Barbin, L., Colamussi, M.L., Cariani, A., Traniello, S., Torrini, I., Ferretti, M.E., 1997. Two for-Met-Leu-Phe-OMe analogues trigger selective neutrophil responses: a differential effect on cytosolic free Ca^{2+}. Biochim. Biophys. Acta 1359, 233–240.

Fabbri, E., Spisani, S., Barbin, L., Biondi, C., Buzzi, M., Traniello, S., Pagani Zecchini, G., Ferretti, M.E., 2000. Studies on fMLP-receptor interaction and signal transduction pathway by means of fMLP-OMe selective analogues. Cell. Signal 12, 391–398.

Ferretti, M.E., Spisani, S., Pareschi, M.C., Buzzi, M., Cavallaro, R., Traniello, S., Reali, E., Torrini, I., Paglialunga Paradisi, M., Pagani Zecchini, G., Biondi, C., 1994. Two new formylated peptides able to activate chemotaxis and respiratory burst selectively as tools for studying human neutrophil responses. Cell. Signal 6, 91–101.

Freer, R.J., Day, A.R., Radding, J.A., Schiffman, E., Aswanikumar, S., Showell, H.J., Becker, E.L., 1980. Further studies on the structural requirements for synthetic peptide chemoattractants. Biochemistry 19, 2404–2410.

Freer, R.J., Day, A.R., Muthukumaraswamy, N., Pinon, D., Wu, A., Showell, H.J., Becker, E.L., 1982. Peptide chemoattractants: a model of the receptor on rabbit neutrophils. Biochemistry 21, 257–263.

Gao, J.L., Chen, H., Filie, J.D., Kozac, C.A., Murphy, P.M., 1998. Differential expansion of the *N*-formylpeptide receptor gene cluster in human and mouse. Genomics 51, 270–276.

Gao, J.L., Lee, E.J., Murphy, P.M., 1999. Impaired antibacterial host defence in mice lacking the *N*-formylpeptide receptor. J. Exp. Med. 4, 657–662.

Hartt, J.K., Barish, G., Murphy, P.M., Gao, J.L., 1999. *N*-formylpeptides induce two distinct concentration optima for mouse neutrophil chemotaxis by differential interaction with two *N*-formylpeptide receptor (FPR) subtypes: molecular characterization of FPR2, a second mouse neutrophil FPR. J. Exp. Med. 190, 741–748.

Higgins, J.D., Bridger, G.J., Derian, C.K., Beblavy, M.J., Hernandez, P.E., Gaul, F.E., Abrams, M.J., Pike, M.C., Solomon, H.F., 1996. *N*-terminus urea-substituted chemotatic peptides: new potent agonists and antagonists toward the neutrophil fMLF receptor. J. Med. Chem. 39, 1013–1015.

Koo, C., Lefkowitz, J., Snyderman, R., 1983. Guanine nucleotides modulate the binding affinity of the oligopeptide chemoattractant receptor on human polymorphonuclear leukocytes. J. Clin. Invest. 72, 748–753.

Laudanna, C., Campbell, J.J., Butcher, E.C., 1996. Role of Rho in chemoattractant-activated leukocyte adhesion through integrins. Science 271, 981–983.

Levesque, L., Gaudreault, R.C., Marceau, F., 1991. The interaction of 3,5-pyrazolidinedione drugs with receptors for f-Met-Leu-Phe on human neutrophil leukocytes: a study of the structure–activity relationship. Can. J. Physiol. Pharmacol. 69, 419–425.

Levesque, L., Gaudreault, R.C., Marceau, F., 1992. Comparison of two classes of non-peptide drugs as antagonists of neutrophil receptors for f-Met-Leu-Phe: pyrazolons and iodinated radiographic contrast agents. Biochem. Pharmacol. 43, 553–560.

Li, Z., Jiang, H., Xie, W., Zhang, Z., Smrcka, A.W., Wu, D., 2000. Roles of

PLC-β2 and -β3 and PI3Kγ in chemoattractant-mediated signal transduction. Science 287, 1046–1049.

Marasco, W.A., Phan, S.H., Krutzsch, H., Showell, H.J., Feltner, D.E., Nairn, R., Becker, E.L., Ward, P.A., 1984. Purification and identification of formyl-methionyl-leucyl-phenylalanine as the major peptide neutrophil chemotactic factor produced by *Escherichia coli*. J. Biol. Chem. 259, 5430–5439.

Meera, R., Anand, S., Ramesh, C.V., Puvankrishnan, R., 1999. Inhibition of neutrophil-derived lysosomal enzymes and reactive oxygen species by a novel tetrapeptide. Inflammation Res. 48, 479–484.

Mócsai, A., Bánfi, B., Kapus, A., Farkas, G., Geiszt, M., Buday, L., Faragó, A., Ligeti, E., 1997. Differential effects of tyrosine kinase inhibitors and an inhibitor of the mitogen-activated protein kinase cascade on degranulation and superoxide production of human neutrophil granulocytes. Biochem. Pharmacol. 54, 781–789.

Prossnitz, E.R., Ye, R.D., 1997. The *N*-formyl peptide receptor: a model for the study of chemoattractant receptor structure and function. Pharmacol. Ther. 74, 73–102.

Scatturin, A., Vertuani, G., Pecoraro, R., Dalpiaz, A., Boggian, M., Ferretti, M.E., Spisani, S., 1999. Conformational aspects of human formylpeptide receptor antagonists. Arzneim.-Forsch. 49, 873–877.

Smith, J.A., 1994. Neutrophils, host defense and inflammation: a double-edged sword. J. Leukocyte Biol. 56, 672–686.

Spisani, S., Fabbri, E., Muccinelli, M., Cariani, A., Barbin, L., Trotta, F., Dovigo, L., 2001. Inhibition of neutrophil responses by cyclosporin A: an insight into molecular mechanisms. Rheumatology 40, 794–800.

Takeda, K., Akagi, Y., Saiki, A., Tsukahara, T., Ogura, H., 1983. Convenient methods for syntheses of active carbamates, ureas and nitrosoureas using N,N^1-disuccinimido carbonate (DSC). Tetrahedron Lett. 24, 4569–4572.

Tiberghien, F., Wenandy, T., Loor, F., 2000. The potent immunosuppressive cyclosporin FR901459 inhibits the human P-glycoprotein and formyl peptide receptor functions. J. Antibiot. (Tokyo) 53, 509–515.

Toniolo, C., Crisma, M., Moretto, V., Freer, R.J., Becker, E.L., 1990. N^{α}-formylated and *tert*-butyloxycarbonylated Phe-(Leu-Phe)$_n$ and (Leu-Phe)$_n$ peptides as agonists and antagonists of the chemotactic formyl-peptide receptor of the rabbit peritoneal neutrophil. Biochim. Biophys. Acta 1034, 67–72.

Torrini, I., Pagani Zecchini, G., Paglialunga Paradisi, M., Lucente, G., Mastropietro, G., Gavuzzo, E., Mazza, F., Pochetti, G., Traniello, S., Spisani, S., 1996. Modified chemotactic peptides: synthesis, conformation, and activity of HCO-Thp-Ac_6c-Phe-OMe. Biopolymers 39, 327–332.

Walther, A., Rieheman, K., Gerke, V., 2000. A novel ligand of the formyl peptide receptor: annexin I regulates neutrophil extravasation by interacting with the FPR. Mol. Cell. 5, 831–840.

Wenzel-Seifert, K., Hurt, C.M., Seifert, R., 1998. High constitutive activity of the human formyl peptide receptor. J. Biol. Chem. 273, 24181–24189.

Yamamoto, Y., Kanazawa, T., Shimamura, M., Ueki, M., Hazato, T., 1997. Inhibitory effects of spinorphin, a novel endogenous regulator, on chemotaxis, O_2^- generation, and exocytosis by *N*-formylmethionyl-leucyl-Phenylalanine (FMLP)-stimulated neutrophils. Biochem. Pharmacol. 54, 695–701.

Zigmond, S.H., Hirsch, J.G., 1973. Leukocyte locomotion and chemotaxis: new methods for evaluation and demonstration of a cell-derived chemotactic factor. J. Exp. Med. 137, 387–391.