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Hydrophilic residues at position 3 highlight unforeseen features of the fMLP receptor pocket

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Abstract

The peptides for-Met-Leu-Tyr-OMe, for-Met-Leu-Glu-OMe, for-Met-Leu-Asp-OMe and for-Met-Leu-Ser-OMe were synthesized to investigate the importance of a hydrophilic side chain of the residue at position 3 on biological activities of human neutrophils. A number of in vitro essays were carried out, including: chemotaxis, superoxide anion production, lysozyme release and receptor binding. Our results highlight that for-Met-Leu-Asp-OMe acts as a full agonist with a higher efficacy than formyl-Met-Leu-Phe-OMe, the tripeptide normally used as a model chemoattractant for the study of cell functions. The other analogs show efficacies that are in the same range or a little less than the prototype. The main point emerging from this study is that the role of Phe substitution needs to be re-hypothesised.

Keywords: *N*-formylmethionyl peptide; Neutrophil, human; Chemotaxis; Superoxide anion generation; Lysozyme release; Receptor binding

1. Introduction

Neutrophils are phagocytic cells, which play a key role in host defence against pathogens. They circulate in the bloodstream, and can be recruited at high numbers in infected or inflamed tissues along with concentration gradients of chemoattractants either released by microorganisms themselves, or arising from inflammatory reactions in the infected tissue. The invaders are subsequently phagocytized, then degraded by toxic oxygen radicals (respiratory burst) (Baggiolini et al., 1993; Wenzel-Seifert and Seifert, 2001; Babior et al., 2001) and/or hydrolytic enzyme release. The mechanism of migration to the site of infection is thought to be sensitive to low peptide concentration; in contrast, phagocytosis and superoxide anion (O_2^-) production, which are thought to begin when chemotaxis is finished and neutrophils are in contact with the target, are expected to require a higher threshold concentration to prevent aberrant triggering (Iizawa et al., 1995; Van Eeden et al, 1999).

N-formyl-Met-Leu-Phe-OH (fMLP), which derives from bacterial sources or disrupted mitochondria (Carp, 1982; Marasco et al., 1984), is the reference chemotactic peptide together with its synthetic methyl ester derivative for-Met-Leu-Phe-OMe (fMLP-OMe) (Schiffmann et al., 1975; Ho et al., 1978). Two human genes, termed *FPR1* and *FPRL1*, encode two formyl peptide receptor subtypes, commonly referred as formyl peptide receptor (FPR) and FPR-like 1 (FPRL1) (Koo et al., 1982; Boulay et al., 1990; Su et al., 1999). These proteins show a high degree of amino acid sequence identity. FPR binds fMLP with high affinity and is activated by picomolar to low nanomolar concentrations of fMLP; FPRL1 is defined as a low-affinity fMLP receptor, based on its activation only by high concentrations of fMLP (micromolar range) (Durstin et al., 1994; Murphy, 1996; Prossnitz and Ye, 1997; Gao et al., 1998; Le et al., 2002). In addition, it has long been known that the transduction pathway underlying the chemotactic response is different from those responsible for cytotoxic functions (Fabbri et al., 1997; Li et al., 2000; Ferretti et al., 2001, 1994). This can be rationalized on the basis of the existence of at least two different functional receptor subtypes or isoforms (Dalpiaz

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et al., 2002, 2003; Le et al., 2002); low doses of a full agonist (or a "pure" chemoattractant) are required to interact with a high-affinity receptor subtype that activates transduction pathway responsible for chemotactic response; while the increase of the full agonist concentration allows binding with the low-affinity subtype, able to activate the transduction pathways responsible for O_2^- production and lysozyme release (Murphy, 1994; Ye and Boulay, 1997; Selvatici et al., 2003).

With regard to the prototype fMLP or its methyl ester (fMLP-OMe), it has been ascertained that (i) methionine at position 1 is optimal for binding to and activating the receptor (Freer et al., 1980; Harvath and Aksamit, 1984; Toniolo et al., 1988; Torrini et al., 1991, 1994; Spisani et al., 1998), even though its substitution with a Gln residue leads to a strong production of superoxide anion (O_2^-) (Cavicchioni et al., 1998); with the presence of a sulfur atom, there is an increase in the side-chain length of the N-terminal amino acid and the peptide activity; (ii) concerning position 2, a huge number of substitutions have been accomplished with the introduction of both natural and synthetic amino acids (Freer et al., 1982; Sukumar et al. 1985; Toniolo et al., 1989; Dentino et al., 1991; Formaggio et al., 1993; Dugas et al., 1993; Spisani et al., 2002): it has previously been stated that the receptor pocket, in which the second residue is allocated, is probably a hydrophobic area, and all subsequent investigations have been concentrated on hydrophobic sterically hindered residues. In previously reported studies, in which we introduced hydrophilic residues (Cavicchioni and Spisani, 2001), we showed that chemotaxis needs a hydrophobic residue, while killing mechanisms (superoxide anion production and lysosomal enzyme release) can be triggered even in the presence of a hydrophilic residue sterically hindered; (iii) the C-terminal amino acid should be aromatic, Phe being the preferred choice (Freer et al., 1980, 1982; Toniolo et al., 1984; Prossnitz and Ye, 1997).

To conclude our investigations on the nature of the substitutions for each of the three amino acids of the fMLP-OMe peptide chain, we introduced hydrophilic residues in place of the Phe. Thus, we synthesized the following peptides: for-Met-Leu-Tyr-OMe (**1**), for-Met-Leu-Glu-OMe (**2**), for-Met-Leu-Asp-OMe (**3**) and for-Met-Leu-Ser-OMe (**4**). The biological properties of the peptide derivatives have been determined on human neutrophils by means of several in vitro assays: receptor binding, chemotaxis, O_2^- production and lysozyme release, and compared with that of the reference peptide fMLP-OMe.

2. Materials and methods

2.1. Chemistry

The [^{1}H]nuclear magnetic resonance ([^{1}H]NMR) spectra were recorded in deuterated chloroform ($CDCl_3$) and dimethylsulfoxide (DMSO-d6) on a Bruker AC200 spectrometer at 200 MHz. Chemical shifts are expressed as δ (ppm) related to the TMS signal. Optical rotations were determined in MeOH at 20 °C with a Perkin-Elmer Model 241 polarimeter. Melting points were determined on a Reichert-Kofler block and are uncorrected. Satisfactory microanalyses were obtained for all compounds, analytical results being within ±0.4% of the theoretical values.

Purification of all the final products were achieved by reverse-phase high-performance liquid chromatography (HPLC) analysis on a Waters Delta Prep 3000 and revelation with UV spectrophotometer Waters 484 at 220 nm using, as stationary less polar phase, a Delta Pack C 18-300 A column (0.3 × 30 cm, particles 15 μm) with a proper eluting system.

2.1.1. Synthesis

t-Boc-Met-OSu, dissolved in distilled *N*,*N*-dimethylformamide (DMF), was coupled to H-Leu-OMe in distilled DMF at −5 °C (1 h) and kept at room temperature overnight. The resulting *t*-Boc-Met-Leu-OMe was saponified with 1 N NaOH, treated with 98% formic acid and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), and kept at room temperature (3 h) (Lajoie and Kraus, 1984) producing the dipeptide for-Met-Leu-OH.

Tripeptide **1** was prepared coupling for-Met-Leu-OH to H-Tyr-OMe using 1-hydroxy-benzotriazole (HOBT) and *N*-dimethylaminopropyl-*N*′-ethylcarbodiimide (EDC) in distilled DMF at −5 °C (1 h) and kept at room temperature overnight. Solid, mp 100–105 °C; $[\alpha]_D^{20} = 12.5°$ c = 1 in MeOH. ^{1}H-NMR ($CDCl_3$): 0.82–0.98 (6 H; 2 CH_3; m); 1.48–1.72 (3 H; CH + CH_2; m); 1.90–2.12 (2 H; CH_2; m); 2.11 (3 H; SCH_3; s); 2.41–2.65 (2 H; CH_2; m); 2.85–3.20 (2 H; CH_2; m); 3.75 (3 H; OCH_3; s); 4.35–4.50 (1 H; CH; m); 4.60–4.72 (1 H; CH; m); 4.74–4.85 (1 H; CH; m); 6.10–6.18 (1 H; NH; br); 6.58–6.61 (1 H; NH; br); 6.71–7.10 (4 H; C_6H_4; dd; J = 8.33 Hz); 8.13 (1 H; HCO; s).

Tripeptide **2** was prepared coupling for-Met-Leu-OH to H-Glu-OMe using the previous coupling method. Solid, mp 180–183 °C; $[\alpha]_D^{20} = -2.5°$ c = 1 in MeOH. ^{1}H-NMR ($CDCl_3$): 0.90–1.00 (6 H; 2 CH_3; m); 1.53–1.68 (5 H; CH + 2 CH_2; m); 1.85–2.10 (2 H; CH_2; m); 2.12 (3 H; SCH_3; s); 2.25–2.35 (2 H; CH_2; m); 2.53–2.68 (2 H; CH_2; m); 3.74 (3 H; OCH_3; s); 4.41–4.65 (2 H; 2 CH; m); 4.68–4.82 (1 H; CH; m); 6.58 (1 H; NH; d; J = 7.31 Hz); 6.77 (1 H; NH; d; J = 8.03 Hz); 6.92 (1 H; NH; d; J = 7.04 Hz); 8.21 (1 H; HCO; s).

Tripeptide **3** was prepared coupling for-Met-Leu-OH to H-Asp-OMe using the previous coupling method. Solid, mp 176–178 °C; $[\alpha]_D^{20} = -3°$ c = 1 in MeOH. ^{1}H-NMR ($CDCl_3$): 0.8–1.0 (6 H; 2 CH_3; m); 1.55–1.68 (3 H; CH + CH_2; m); 1.87–2.10 (2 H; CH_2; m); 2.14 (3 H; SCH_3; s); 2.40–2.63 (2 H; CH_2; m); 2.70–2.93 (2 H; CH_2; m); 3.71 (3 H; OCH_3; s); 4.32–4.48 (1 H; CH; m); 4.50–4.65 (1 H; CH; m); 4.67–4.73 (1 H; CH; m); 7.96 (1 H; NH; d; J = 8.04 Hz); 8.02 (1 H; NH; d; J = 8.16 Hz); 8.14 (1 H; HCO; s); 8.21 (1 H; NH; d; J = 8.23 Hz).

Tripeptide **4** was prepared coupling for-Met-Leu-OH to H-Ser-OMe using the previous coupling method. Solid, mp 120–125 °C; $[\alpha]_D^{20} = -43.7°$ c = 1 in MeOH ^{1}H-NMR ($CDCl_3$): 0.90–1.10 (6 H; 2 CH_3; m); 1.60–1.85 (3 H; CH + CH_2; m); 1.95–2.10 (2 H; CH_2; m); 2.12 (3 H; SCH_3; s); 2.55–2.70 (2 H; CH_2; m); 3.75–4.00 (2 H; CH_2; m); 3.79 (3 H; OCH_3; s); 4.45–4.85 (3 H; 3 CH; m); 7.06 (1 H; NH; d; J = 7.47 Hz); 7.41 (1 H; NH; d; J = 7.90 Hz); 7.52–7.61 (1 H; NH; br); 8.19 (1 H; HCO; s).

2.2. Biological assay

2.2.1. Peptides

Stock solutions, 10^{-2} M of fMLP-OMe (Sigma, St. Louis, MO, USA) and tripeptide analogs, 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; glucose, 10 g/l. This solution was five times working strength. One millimolar $MgCl_2$ and 1 mM $CaCl_2$ were supplemented to the buffer before the biological test. All reagents were of the purest grade commercially available.

2.2.2. Cell preparation

Cells were obtained from peripheral 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 blood cells. The cells were washed twice and resuspended in Krebs–Ringer–phosphate containing 0.1% w/v glucose (KRPG), pH 7.4, at a final concentration of 50×10^6 cells/ml and used immediately. The percentage of neutrophils was 98–100% pure and ≥ 99% viable as determined by the Trypan blue exclusion test. The study was approved by the local Ethics Committee, and informed consent was obtained from all participants.

2.2.3. Random locomotion and chemotaxis

Random locomotion and chemotaxis were performed with a 48-well microchemotaxis chamber (BioProbe, Milan, Italy) and the migration into the filter was evaluated by the method of leading-front according to Zigmond and Hirsh (1973). The actual control random movement is 32 μm ± 3 S.E. of 10 separate experiments performed in duplicate. Chemotaxis was studied by adding each peptide to the lower compartment of the chemotaxis chamber. Peptides were diluted from a stock solution (10^{-2} M in DMSO) with KRPG containing 1 mg/ml of bovine serum albumin and used at concentrations ranging from 10^{-12} to 10^{-5} M. Data were expressed in terms of chemotactic index (C.I.), ratio as follows: (migration toward test attractant minus migration toward the buffer)/(migration toward the buffer).

2.2.4. Superoxide anion production

Superoxide anion production was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome *c* modified for microplate-based assays. The tests were carried out in a final volume of 200 μl containing 4×10^5 neutrophils, 100 nmol cytochrome *c* (Sigma) and KRPG. At zero time, different amounts (10^{-10}–5×10^{-5} M) of each peptide were 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 nanomoles of O_2^- produced, using a molar extinction coefficient for cytochrome *c* of 18.5 mM^{-1} cm^{-1}. Neutrophils were preincubated with 5 μg/ml cytochalasin B (Sigma) for 5 min before activation by peptides.

2.2.5. Granule enzyme assay

Release of neutrophil granule enzymes was evaluated by determining lysozyme activity modified for microplate-based assays. Cells (3×10^6) were incubated in microplate wells in the presence of each peptide with a final concentration of 10^{-10}–5×10^{-5} M for 15 min at 37 °C. The plates were then centrifuged for 5 min at 400 × *g* and the lysozyme was quantified nephelometrically by the rate of lysis of cell wall suspension of *Micrococcus lysodeikticus* (Sigma). Neutrophils were preincubated with 5 μg/ml cytochalasin B for 15 min at 37 °C before activation by the peptides. Reaction rate was measured with a microplate reader at 465 nm. Enzyme was expressed as net percentage of 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.

2.2.6. Receptor binding

Binding assays were carried out according essentially to Spisani et al. (1996). In competition, experiments were carried out to determine the IC_{50} (IC_{50} = inhibitory concentration 50%) values, 6 nM [^{3}H]fMLP (specific activity = 71.5 Ci/mmol, NEN Research Products, Du Pont de Nemours, Milan, Italy) was incubated with 100 μl of human neutrophils (5×10^6) at different concentrations of the test compounds at 37 °C for 15 min. Non-specific binding was measured in the presence of 100 μM fMLP, and was about 20% of total binding. Incubation time was 15 min at 37 °C. Bound and free radioactivity was separated by filtering the assay mixture through Whatman GF/C glass-filters using a Micro-Mate 196 Cell Harvester (Packard Instrument). The filter-bound radioactivity was counted on Top Count (efficiency 57%) with Micro-Scint-20 (30 μl in 96-well plates). The cold drug concentrations displacing 50% of labelled ligand (IC_{50}) were obtained by 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 the mean of three independent experiments performed in duplicate.

2.2.7. Statistical analysis

The nonparametric Wilcoxon test was used in the statistical evaluation of differences between groups.

3. Results

fMLP-OMe analogs were examined for their potential ability to induce chemotaxis and to trigger both superoxide anion production and lysozyme release from the granules of human neutrophils: they were measured as ‘‘efficacy’’ (which corresponds to maximum effect of a ligand) and ‘‘potency’’ (which corresponds to the concentration of a ligand at which 50% of its maximum effect is reached). In addition, the receptor affinity of the derivatives was determined in binding studies and data were expressed as IC_{50} values. The biological activities were compared with those of the standard agonist fMLP-OMe.

3.1. Chemotaxis

Chemotactic responses of human neutrophils to analogs **1 2 3 4** are shown in Fig. 1. The dose–response curves rise to a peak and then decline to zero, with ligand concentrations higher than the optimal value. This behaviour is typical of chemoattractants (Rot et al., 1987; Vertuani et al., 1987). The peak value of the reference fMLP-OMe was observed at a concentration of 10^{-9} M, whereas the optimum concentration value of the analogs was 10^{-8} M, at a concentration 10 times higher. The efficacy values of analogs **1**, **2** and **4** were in the same narrow range (C.I. = 1.02–1.12) and showed similarly height that of fMLP-OMe (C.I. = 1.05). Furthermore, their ability to induce chemotaxis at the other concentrations did not significantly change with respect to the control. Concerning **3**, this peptide showed an efficacy that was by far the highest (C.I. = 1.32), and with its C.I. values over that of the fMLP-OMe curve profile. These results indicate on the whole that the tripeptide derivatives are full agonists like the prototype fMLP-OMe.

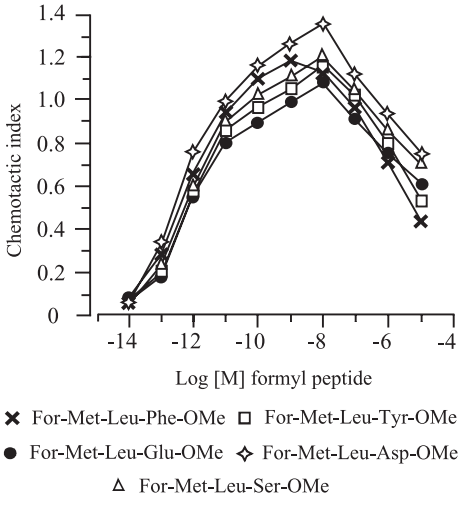

Fig. 1. Chemotactic activity of fMLP-OMe and its analogs toward human neutrophils. The data are the means of five separate experiments performed in duplicate. S.E.M. are in 0.02–0.09% chemotactic index range.

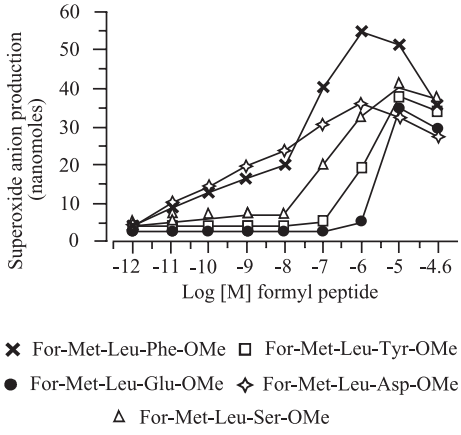

Fig. 2. Superoxide anion production of fMLP-OMe and its analogs toward human neutrophils. The data are the means of five separate experiments performed in duplicate. S.E.M. are in 0.1–4 nmol O_2^- range.

3.2. Superoxide anion production

Fig. 2 shows nanomoles of superoxide anion elicited by analogs **1 2 3 4**. It can be observed that the potency of analogs **1**, **2** and **4** is at 10^{-5} M, which is a concentration one order of magnitude higher than that of fMLP-OMe (10^{-6} M). At this physiological concentration, the order of efficacy is fMLP-OMe>**4** ≥ **1**>**2**. The difference of nanomoles between the analogs and fMLP-OMe rises as the concentration decreases, reaching to fMLP-OMe values only at a concentration of 10^{-5} M. The Asp containing

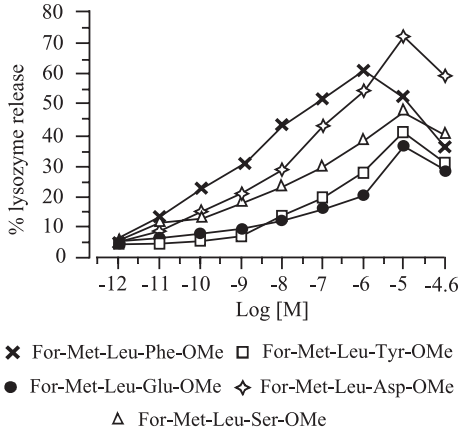

Fig. 3. Release of neutrophil granule enzymes evaluated by determining lysozyme activity induced by fMLP-OMe and its analogs. The data are the means of five separate experiments performed in duplicate. S.E.M. are in 1–6% range.

compound **3** does not show a peak decidedly stronger than the others, but triggers nanomoles of O_2^- production, which are in a restricted range (22 ≤ efficacy ≤ 33 nmol) for all the concentrations.

3.3. Lysozyme enzyme release

Fig. 3 shows the curves that demonstrate the effect exerted by the same peptides on lysozyme release. The optimal responses are found at 10^{-5} M which is 10 times lower than fMLP-OMe. Efficacy values of compounds **1**, **2** and **4** (34 ≤ efficacy ≤ 44% release) are significantly lower ($P < 0.05$) than the value of the prototype (57% release) at its optimal concentration. Interesting is the behaviour of compound **3** whose response is lower than fMLP-OMe at the concentration range 10^{-10}–10^{-7} M; it reaches a comparable value to fMLP-OMe at 10^{-6} M overcoming it at 10^{-5} M ($P < 0.01$).

3.4. Binding experiments

The order of potency in [^{3}H]-fMLP displacement assays for the test peptides is: for-Met-Leu-Phe-OMe>**3**>**4**>**1**>**2**. fMLP-OMe is the most potent peptide, with its affinity in the nanomolar range (60 nM), while **3**, **4**, **1** and **2** display affinities in the micromolar range (1000 to >10,000 nM) (Fig. 4). These data fit perfectly with (i) the chemotactic behaviours of analogs at 10^{-8} M, (ii) O_2^- production, (iii) and the percentage of lysosomal enzyme release at their activity peak 10^{-5} M. In particular, the peptide containing Asp **3** is the most efficacious concerning both chemotaxis and lysozyme release and the most potent concerning O_2^- production, while the peptide containing Glu **2** is the weakest agonist amongst the derivatives.

A comprehensive picture of all biological data is summarized in Table 1: it shows efficacy values of the chemotactic index, the nanomoles of superoxide anion production, the percentage of lysozyme release and IC_{50} values obtained from receptor binding experiments of compounds **1 2 3 4**. Comparisons are made between each peptide and with reference to the prototype fMLP-OMe.

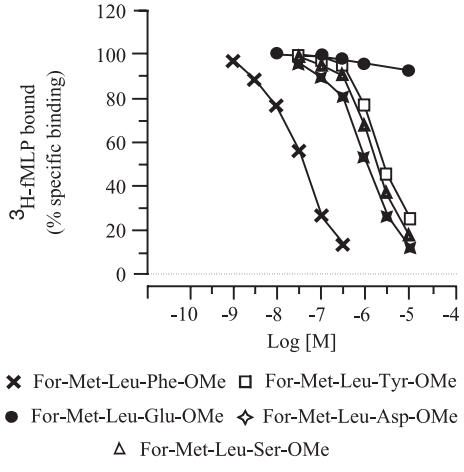

Fig. 4. Competition curves of specific [^{3}H]fMLP binding to human neutrophils by the test compounds. Curves are representative of a single experiment taken from a series of three independent experiments. Non-specific binding was determined in the presence of 10 μl fMLP.

Table 1
Chemotactic activity, superoxide anion production, lysozyme release and binding analysis of fMLP analogs

Peptides	Chemotaxis (C.I.)	Superoxide production (nmol)	Lysozyme release (%)	Receptor binding IC_{50} (nM)
fMLP	1.15 ± 0.08 (10^{-9} M)	52 ± 3 (10^{-6} M)	58 ± 3 (10^{-6} M)	60 ± 4
Tyr^3 **1**	1.09 ± 0.08 (10^{-8} M)	37.9 ± 2 (10^{-5} M)	40 ± 3 (10^{-5} M)	3000 ± 500
Glu^3 **2**	1.02 ± 0.06 (10^{-8} M)	33.2 ± 3.2 (10^{-5} M)	33 ± 3 (10^{-5} M)	>10,000
Asp^3 **3**	1.32 ± 0.10 (10^{-9} M)	31 ± 2 (10^{-5} M)	69 ± 3 (10^{-5} M)	1000 ± 120
Ser^3 **4**	1.12 ± 3.00 (10^{-8} M)	40.9 ± 3 (10^{-5} M)	45 ± 3 (10^{-5} M)	2000 ± 260

Efficacy data of chemotaxis are expressed as chemotactic index; superoxide anion production is expressed as net nanomoles of $O_2^-/1 \times 10^6$ cells/5 min; and lysozyme release is expressed in percent ± S.E.M. All data are represented by the mean of six independent experiments performed in duplicate.
Receptor binding values (IC_{50}) represent the mean ± S.E.M. of three independent determinations performed in duplicate.

4. Discussion

We synthesized a series of formyltripeptides to specifically investigate the features of the receptor pocket, which is allocated in the third residue of the neutrophil activator for-Met-Leu-Phe. Previously, we have introduced hydrophilic residues at positions 1 and 2 to clarify the features of the receptor parts in which first and second fMLP residues allocated. The evidence resulted in that Met can be substituted by Gln (Cavicchioni et al., 1998) and Leu by an encumbered residue (even if hydrophilic) (Cavicchioni and Spisani, 2001), affording to the peptide mechanisms to trigger killing while having no chemotactic effects. Thus, substitution of the Phe residue with hydrophilic amino acids should have led to molecules able to elicit selectively superoxide anion production and lysozyme release but not to stimulate chemotaxis. Unexpectedly, we obtained agonists, which efficaciously activated chemotaxis. The results reported in Fig. 1 clarify that the presence of hydrophilic residues at position 3 is compatible, with a good interaction with the receptor isoform, to stimulate chemotaxis. The different steric hindrance is not a crucial factor since the encumbered **1** shows an efficacy and potency similar to that of the small **4**. Probably, the strong activity of **3** is due to its steric hindrance, not too big but also not too small, fitting optimally into the receptor. The killing mechanisms cannot be considered optimally stimulated because their efficacy

peak (10^{-5} M) corresponds to a concentration 10 times higher than that of fMLP-OMe.

As a general consideration, **3** proved to be the strongest hydrophilic full agonist in all biological activities, while **2** was demonstrated to be the weakest (partial) agonist among the test derivatives: these data have been well confirmed by competition binding experiments.

From these data emerges a new picture of the receptor pocket in which the third residue is accommodated: it can accept even hydrophilic residues and is indifferent to residue steric hindrance.

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