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Biological activity of for-Met-Leu-Phe-OMe analogs: Relevant substitutions specifically trigger killing mechanisms in human neutrophils

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Abstract

Two analogs of the prototypical peptide for-Met-Leu-Phe-OMe (fMLP-OMe), for-Gln-Tyr-Phe-OMe (**1**) and for-Gln-Tyr-Tyr-OMe (**2**), carrying unusual hydrophilic residues, were synthesized in order to investigate whether they provoked specific biological responses, as well as intracellular calcium mobilization, in human neutrophils. Whereas neither compound stimulates chemotaxis, both are able to elicit lysosomal enzyme production. However compound **1** is able to trigger copious superoxide anion production while compound **2** only elicits minor superoxide anion production. In binding experiments on formylpeptide receptors, the newly synthesized compounds for-Gln-Tyr-Phe-OMe (**1**) and for-Gln-Tyr-Tyr-OMe (**2**) showed affinity values in the micromolar range. These derivatives demonstrate inability to find a positive contribute from single substitutions. A very important result of this research is the evidence of the ability of the formyl group alone to trigger the primary target of the human neutrophil activity, i.e. killing mechanisms, by activating the specific receptor conformation.

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

1. Introduction

Neutrophils play a key role as primary phagocytic cells as they constitute the first line of defense against invasion of bacteria and contribute to various inflammatory processes. They migrate to infected and inflamed tissues along a concentration gradient of chemoattractant molecules. The *N*-formyltripeptide for-Met-Leu-Phe-OH (fMLP), a structural analog of bacteria metabolism products, and its methylester derivative for-Met-Leu-Phe-OMe (fMLP-OMe) are the most important chemotactic peptides in the immune response (Murphy, 1994). These formylpeptides interact with specific membrane receptors and activate a highly complex signal transduction network, crucial for various neutrophil functions such as chemotaxis, superoxide anion production and lysosomal enzyme secretion (Spisani et al., 1996a,b). The concentrations of fMLP required to induce chemotaxis are lower than those required to cause superoxide anion production or secretagogue activity (Van Eeden et al., 1999).

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) (Prossnitz and Ye, 1997; Le et al., 2002; Gao et al., 1998).

Several formylpeptides, modified at position 1, have been synthesized in order to clarify the features peculiar to

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the methionine receptor pocket. The Met residue, however, seems to induce optimum chemotactic as well as killing activities. Indeed, when amino acids carrying linear or branched aliphatic side chains are inserted at position 1, a more or less marked loss of biological responses is evident (Freer et al., 1980), while other substitutions produce discordant behaviour (Torrini et al., 1991; Harvath and Aksamit, 1984; Toniolo et al., 1984; Torrini et al., 1994). We previously demonstrated (Cavicchioni et al., 1998; Spisani et al., 1998) that the substitution of Met with residues carrying a variation in electronic density, and/or a shift in its position along the side chain greatly lowers chemotactic response, together with killing mechanisms. Only the analog containing Gln residue strongly stimulates superoxide anion production even more powerfully than fMLP-OMe. We hypothesized that this analog preferentially binds to the receptor conformation able to selectively activate the transduction pathways involved in superoxide anion production, probably facing the complementary positive area of the receptor. This peptide, in fact, enhances the concentration of cytosolic Ca^{2+}, which is an obligatory second messenger for neutrophil oxidative metabolism (Fabbri et al., 1997).

Regarding position 2, a huge number of substitutions have been accomplished, introducing both natural and synthetic amino acids. The importance of these substitutions is linked to the ability of the peptide chain to properly locate the receptor pocket (Le et al., 2002; Spisani et al., 2002; Toniolo et al., 1989; Dentino et al., 1991; Formaggio et al., 1993; Sukumar et al., 1985). From the initial study (Freer et al., 1982) onwards, the receptor pocket, in which the second residue is located, has been thought to be a hydrophobic area, and all subsequent investigations have concentrated on hydrophobic sterically hindered residues. In previously reported studies, we synthesized fMLP-OMe analogs (Cavicchioni and Spisani, 2001; Cavicchioni et al., 2002) functionalized on the side chain, to verify whether there is incompatibility between the hydrophilic nature of the second residue and the receptor, as well as steric hindrance able to overcome this hypothetical incompatibility. Our results confirm that hydrophilic nature of the second residue is not vital to the biological response, which is influenced mainly by the steric hindrance of the amino acid rather than on its hydrophobic/hydrophilic nature. Analysis of the results showed that the Tyr analog, the side chain of which not only shows hydrophilicity and steric hindrance, but also a mix of hydrophilicity and hydrophobicity, strongly triggers killing mechanisms.

As a consequence, we thought it would be of interest to study the biological responses of human neutrophils to the peptide for-Gln-Tyr-Phe-OMe (**1**). Our aim was to understand if combining together these two substitutions (i.e. Gln instead of Met, and Tyr instead of Leu), as each one alone strongly triggers killing mechanisms, would lead to an even stronger response: i.e. if there is a potentiating mechanism arising from targeted multisubstitutions.

At variance with the above reported findings, we synthesized the analog for-Gln-Tyr-Tyr-OMe (**2**), introducing a Tyr residue, which has often been demonstrated to be an intriguing variation of the prototype Phe (Spisani et al., 2003), at position 3 of analog **1**. The resulting analog **2** shares with the prototype fMLP-OMe only the amino-protecting formyl group: this feature should allow clarification of whether or not the formyl group alone (independently of the three native residues) is able to bind FPR and activate neutrophil responses.

We thought comparison of the biological activities of the two analogs **1** and **2** with each other, as well as to the parent fMLP-OMe and to the references for-Gln-Leu-Phe-OMe (**3**), for-Met-Tyr-Phe-OMe (**4**), and for-Met-Leu-Tyr-OMe (**5**) would be of interest.

2. Materials and methods

2.1. Chemistry

Optical rotations were determined in methanol at 20 °C with a Perkin-Elmer Model 241 polarimeter. ^{1}H NMR spectra were recorded in deuterated chloroform ($CDCl_3$) and dimethylsulphoxide (DMSO-d_6) on a Bruker AC200 spectrometer at 200 Mz. Chemical shifts are expressed as δ (ppm) related to the tetramethylsilane signal. Melting points were determined on a Reichert–Kofler block, and are uncorrected. Thin layer chromatography (TLC) was performed on precoated silica gel F_{254} plates (Merck) with the solvent buthanol/H_2O/acetic acid 3:1:1. Satisfactory C, H, N, S microanalyses were obtained for all compounds, analytical results being within 0.4% of the theoretical values. Fura-2 AM was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Amino acids were purchased from Fluka. Removal of the Boc group was performed by treatment with a 1:1 mixture of trifluoroacetic acid (TFA)–chloroform ($CHCl_3$). Peptide coupling was achieved by the 1-hydroxy-1,2,3-benzotriazole (HOBt)-*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) hydrochloride method, while the formyl group was introduced according to the *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) method.

Crude peptides were purified by preparative reverse-phase High Performance Liquid Chromatography (HPLC) using a Waters Delta Prep 4000 system with a Waters PrepLC 40 mm Assembly column (30×4 cm, 300 Å, 15 μm spherical particle size column). The column was perfused at a flow rate of 40 mL/min with a mobile phase containing solvent A (10%, v/v, acetonitrile in 0.1% TFA), and a linear gradient from 0 to 100% of solvent B (60%, v/v, acetonitrile in 0.1% TFA) in 25 min was adopted for the elution of compounds. HPLC analysis was performed by a Beckman System Gold with a Beckman ultrasphere ODS column (5 μm, 4.6×250 mm). Retention times of the peptides were determined using HPLC conditions in the above solvent

system (solvents A and B) programmed at flow rates of 1 mL/min using the following linear gradients: from 0 to 100% B In 25 min. All peptides showed less than 1% impurities when monitored at 220 and 254 nm (Fig. 1).

2.1.1. Synthesis

For-Gln-Tyr-Phe-OMe (**1**) was synthesized following standard procedures in solution (Fig. 1). Solid (mp. 188–191 °C; Rf 0.73; $[\alpha]_D^{20}=-35.83°$ in methanol, c=1). ^{1}H NMR (dimethylsulfoxide DMSO-d6): 1.6–1.9 (2H; CH_2; m); 2.2 (2H; CH_2; m); 2.5–3.1 (4H; $2CH_2$; m); 3.55 (3H; OCH_3; s); 4.30 (1H; CH; m); 4.35–4.5 (2H; 2CH; m); 6.60 and 6.98 (4H; C_6H_4; 2d; J=8.27 Hz); 6.78 (1H; NH; s); 7.21 (5H; C_6H_5; m); 7.28 (1H; NH; s); 7.96 (1H; HCO; s) 8.05 (1H; NH; d; J=7.90 Hz); 8.17 (1H; NH; d; J=8.45 Hz); 8.47 (1H; NH; d; J=7.44 Hz); 9.16 (1H; OH; s).

For-Gln-Tyr-Tyr-OMe (**2**) was synthesized following standard procedures in solution (Fig. 1). Solid (mp. 177–180 °C; Rf 0.65; $[\alpha]_D^{20}=-18.9°$ in methanol, c=1). ^{1}H NMR (DMSO-d6): 1.55–1.75 (2H; CH_2; m); 2.15 (2H; CH_2; m); 2.50–2.95 (4H; $2CH_2$; m); 3.54 (3H; OCH_3; s); 4.28–4.50 (3H; 3CH; m); 6.62 and 6.98 (4H; C_6H_4; 2m); 6.79 (1H; NH; s); 7.25 (1H; NH; s); 7.96 (1H; HCO; s) 8.04 (1H; NH; d; J=8.24 Hz); 8.18 (1H; NH; d; J=8.57 Hz); 8.39 (1H; NH; d; J=7.32 Hz); 9.16 (1H; OH; s); 9.23 (1H; OH; s).

2.2. Biological assays

Human neutrophils were purified employing the standard techniques of dextran sedimentation of heparinized blood, followed by centrifugation on Ficoll-Paque and hypotonic lysis of contaminating red cells. The cells were washed twice and resuspended in Krebs–Ringer–phosphate containing 0.1% w/v glucose, pH 7.4. Neutrophils were 98–100% pure and ≥99% viable, as determined by trypan blue exclusion test.

2.2.1. Random locomotion and chemotaxis

Random locomotion was performed with a 48-well microchemotaxis chamber, and migration into the filter was evaluated by the leading front method (Zigmond and Hirsh, 1973). The control random movement is 32 μm±3 S.E.M. 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 Krebs–Ringer–phosphate containing 0.1% w/v glucose and 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, which is the ratio: (migration toward test attractant minus migration toward the buffer)/(migration toward the buffer).

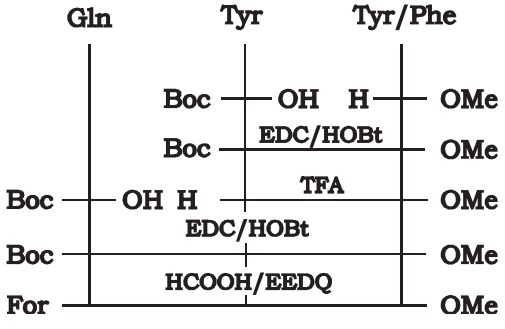

Fig. 1. Scheme of synthesis.

2.2.2. Superoxide anion production

Superoxide anion production was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome c modified for microplate-based assays. Tests were carried out in a final volume of 200 μl containing 4×10^5 neutrophils, 100 nmol cytochrome c and Krebs–Ringer–phosphate containing 0.1% w/v glucose. At zero time, different amounts (10^{-10}–10^{-4} M) of each peptide were added and the plates were incubated in a microplate reader (Ceres 900, Bio-Tek Instruments) with 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 superoxide anion produced, using molar extinction coefficient for cytochrome c of 18.5 mM^{-1} cm^{-1}. Neutrophils were preincubated with 5 μg/ml cytochalasin B for 5 min prior to activation by peptides.

2.2.3. Granule enzyme assay

Release of neutrophil granule enzymes was evaluated by determining lysozyme activity modified for microplate-based assays. Cells were incubated in microplate wells in the presence of each peptide at a final concentration of 10^{-10} to 10^{-4} M for 15 min at 37 °C. The plates were then centrifuged for 5 min at $400\times g$, and lysozyme was quantified nephelometrically by the rate of lysis of a suspension of *Micrococcus lysodeikticus* cell wall. Neutrophils were pre-incubated with 5 μg/ml cytochalasin B for 15 min at 37 °C prior to 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. Total enzyme activity was 85±1 μg/1×10^7 cells/min.

2.2.4. Cell loading and fluorescence recording

Neutrophils were loaded with 1 μM Fura 2-AM for 30 min at 37 °C. Before fluorescence recording, neutrophils were diluted to 2×10^6/ml with Krebs–Ringer–phosphate containing 0.1% w/v glucose, washed and resuspended at the same concentration. Fluorescence measurements were performed at 37 °C on 2-ml cuvette, using a Perkin-Elmer LS-5 luminescence spectrofluorimeter, as previously reported (Fabbri et al., 1997). Drug solutions (10 μl) were added to the cells and fluorescence increase was recorded until peak

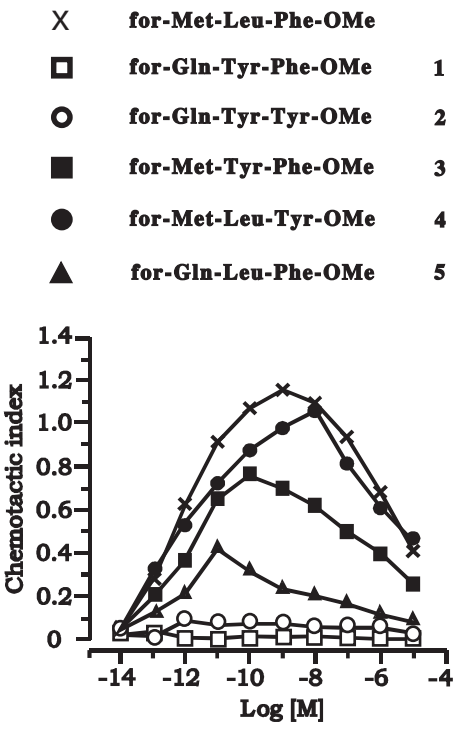

Fig. 2. Chemotactic activity towards human neutrophils of fMLP-OMe and its analogues. The data are the means of five separate experiments performed in duplicate. S.E.M. are in 0.02–0.09 chemotactic index range.

intensity was achieved; this value represents the maximum intracellular free Ca^{2+} concentration in every preparation studied. Basal and stimulated $[Ca^{2+}]_i$ were calculated according to the formula $[Ca^{2+}]$ nM$=K_d(F-F_{min})/(F_{max}-F)$, where K_d (224 nM) is the apparent dissociation constant according to Grynkiewicz et al. (1985).

2.2.5. Receptor binding

Binding experiments were carried out as previously described (Spisani et al., 1996a,b). Saturation binding experiments of [^{3}H]-fMLP (3–300 nM) to human neutrophils were performed incubating the cells for 15 min at 37 °C according to the previous time-course experiments. Competition experiments were carried out to determine the IC_{50} values using 6 nM [^{3}H]-fMLP with 100 μl of human neutrophils with different concentrations of the test compounds. Nonspecific binding was defined as that in the presence of 10 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 a Whatman GF/C glass-filter using a Micro-Mate 196 Cell Harvester (Packard Instrument Company). The filter-bound radioactivity was counted on a Top Count (efficiency 57%) with Micro-Scint-20 (30 μl in 96-well plates).

2.2.6. Statistical analysis

The non-parametric Wilcoxon test was used in the statistical evaluation of differences between groups. Differences were considered to be statistically significant if P values ≤0.05.

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: “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) were measured. 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, as well as with those of the references for-Gln-Leu-Phe-OMe (**3**), for-Met-Tyr-Phe-OMe (**4**), and for-Met-Leu-Tyr-OMe (**5**).

3.1. Chemotactic activity

The dose–response curve of fMLP-OMe is typical of chemoattractants: it rises to a peak and then decreases to zero, with ligand concentrations higher than the optimum value. Both fMLP-OMe derivatives **1** and **2** were found to be unable to induce chemotaxis (Fig. 2). Comparing these

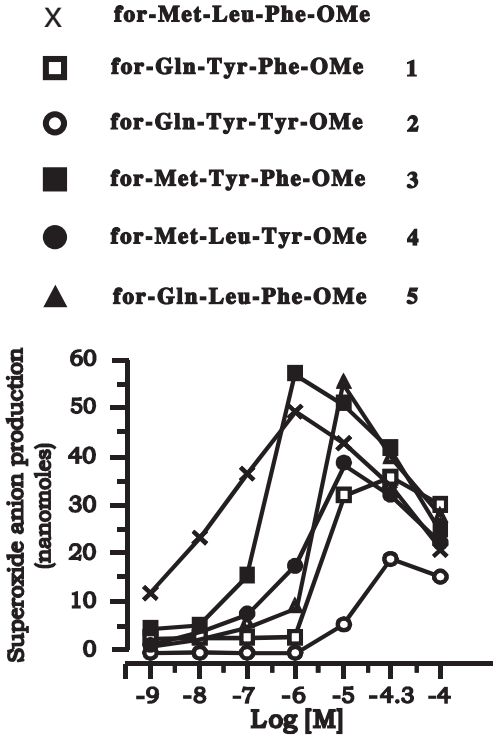

Fig. 3. Superoxide anion production of fMLP-OMe and its analogues toward human neutrophils. The data are the means of five separate experiments performed in duplicate. S.E.M. are in 0.1–4 nmol superoxide anion range.

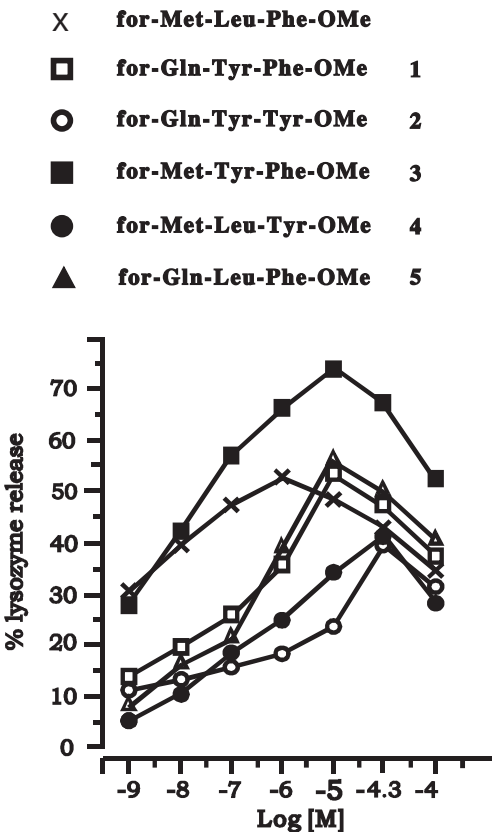

Fig. 4. Release of neutrophil granule enzymes evaluated by determining lysozyme activity induced by fMLP-OMe and its analogues. The data are the means of five separate experiments performed in duplicate. S.E.M. are in 1–6% range.

results with the chemotactic activity of the corresponding **3**, **4**, and **5**, it can be noted that analogs **4** and **5** (which maintain Met as the first residue) display marked activity while Gln at position 1 in compound **3** causes a strongly reduced affinity and compounds **1** and **2** completely lack activity, thereby demonstrating the mandatory negative presence of Gln at position 1.

3.2. Superoxide anion production

Fig. 3 shows the dose–response curves of tripeptides. Neither analog triggers respiratory burst (i.e. superoxide anion production) in the range of 10^{-9}–10^{-6} M, thereby showing less efficacy than the parent fMLP-OMe. At the concentration of 10^{-5} M, compound **1** demonstrates high potency (about three to four of the reference) reaching values of the reference at a concentration of 5×10^{-5} M. Compound **2** cannot be considered as a partial agonist because its peak is both not statistically significant ($P>0.05$) and out of the physiological range. Compound **2** is practically ineffective at all the concentrations tested and its peak is not statistically significant ($P>0.05$). Comparing these results with those of the corresponding **3**, **4**, and **5**, it can be noted that these compounds show high (analogs **3**), or moderate (analogs **4** and **5**) potency and efficacy while compounds **1** and **2** show a reduced biological activity, whose efficacy is lower than the reference **4** and decidedly lower than references **3** and **5**.

3.3. Lysozyme release

Fig. 4 shows the lysozyme release induced from granules by tripeptides. Compound **1** shows a progressive growth of potency reaching its maximum at 10^{-5} M, where it elicits a potency as high as fMLP-OMe, with a similar decrease for both slopes. Compound **2**, in this case, also triggers a response, although weaker than that of compound **1**, showing a maximum at 5×10^{-5} M, where it induces lysozyme release of the same order as the other tripeptide. Both compounds show less efficacy than fMLP-OMe. Comparing these results with the lysosomal enzyme release of the references **3**, **4**, and **5**, it can be noted that neither compound **1** nor compound **2** increase the efficacy or potency of the parent 4. Analog **2** behaves in a similar fashion to analog **5**, sharing a Tyr residue at position 3, which seems to be mandatory. Analog **1** shows the same

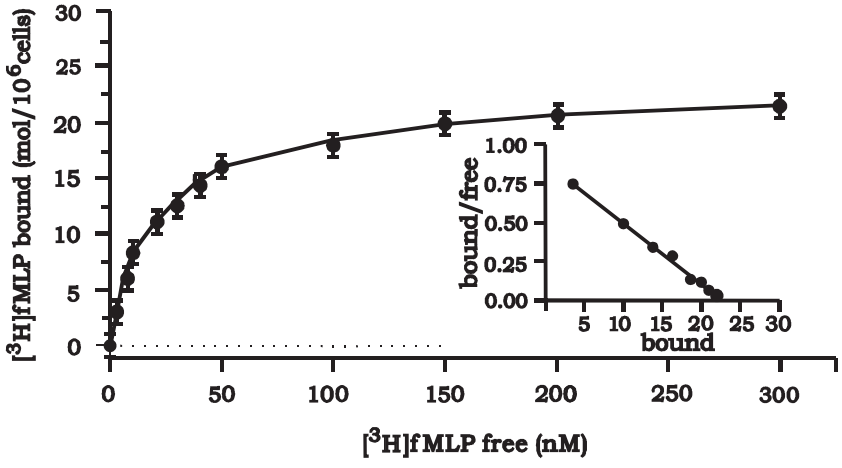

Fig. 5. Saturation of [^{3}H]fMLP binding to human neutrophils. The K_d value was 32±4 nM and the B_{max} value was 22±3 fmol/10^6 cells. Values are the mean and vertical lines are the S.E.M. of the mean of three independent experiments. The inset is a Scatchard plot of the same data.

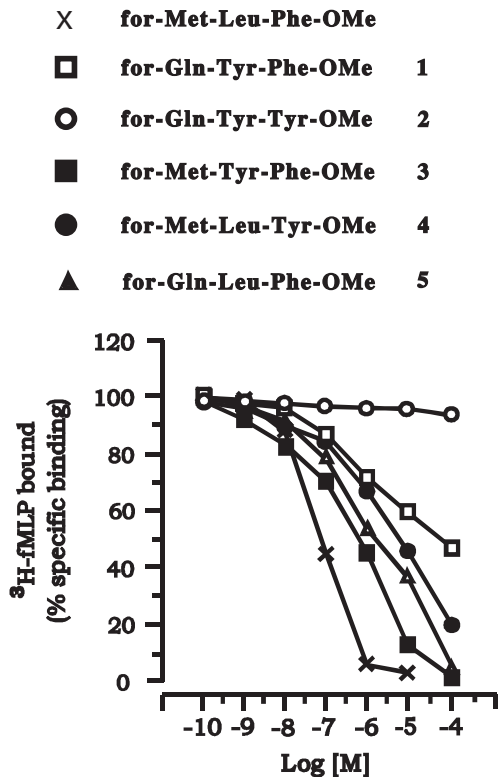

Fig. 6. Competition curves of specific [^{3}H]-fMLP-OMe binding to human neutrophils by the test compounds. Curves are representative of a single experiment taken from a series of three independent experiments. Non-specific binding was determined in the presence of 10 μl fMLP.

efficacy and potency as compound **3**, thereby highlighting the positive influence of Gln at position 1.

3.4. Effects of peptides on intracellular calcium levels

Intracellular Ca^{2+} mobilization induced by 10^{-5} M tripeptides is reported. Compound **1** significantly enhanced the levels of the intracellular calcium (approximately 3.0-fold, $P<0.05$). Compound **2** was found to be completely unable to elicit any effect. Compound **3** resulted the most efficacious, with a stimulatory effect (6.0-fold, $P<0.01$), similar to that of the parent compound fMLP-OMe. Analog **4** behaved in a similar fashion to analog **5**, both showing stimulatory effects of 2.0- and 2.5-fold, respectively.

3.5. Binding experiments

Fig. 5 shows a saturation curve of [^{3}H]fMLP and the corresponding Scatchard plot (Inset) on human neutrophils. The Scatchard plot was essentially linear, indicating that only one affinity-binding site was present under our experimental conditions. The dissociation binding constant (K_d) was 32±4 nM and the receptor density (B_{max}) was 22±3 fmol/10^6 cells. Competition-binding experiments were carried out to obtain information regarding the affinity of for-Gln-Tyr-Phe-OMe (**1**) and for-Gln-Tyr-Tyr-OMe (**2**) analogs to their receptors. Inhibitory binding constants (K_i) were derived from the IC_{50} values according to the Cheng–Prusoff equation: $K_i=IC_{50}/1+[C^*]/K_D^*$, where K_D^* is the radioligand dissociation constant, and C is the concentration of the radioligand used (Cheng and Prusoff, 1973).

Fig. 6 shows inhibition curves of the examined compounds in human neutrophils. fMLP-OMe was the most potent compound (IC_{50}=60±5 nM), while for-Gln-Tyr-Phe-OMe (**1**) and for-Gln-Tyr-Tyr-OMe (**2**) showed affinity values in the micromolar range. In particular the IC_{50} of for-Gln-Tyr-Phe-OMe (**1**) (IC_{50}=30,000±300 nM) was one order of magnitude higher than that of FMLP-OMe whereas for-Gln-Tyr-Tyr-OMe (**2**) showed a very weak affinity (IC_{50}>30,000±350 nM). It can be noted that the affinity of compounds **1** and **2** were much lower than the corresponding **3** (IC_{50}=1000±30 nM), **4** (IC_{50}=600±20 nM), and **5** (IC_{50}=3000±40 nM), thereby showing a weak ability to bind FPR.

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**–**5**. Comparisons are made between each peptide and with reference to the prototype fMLP-OMe.

4. Discussion

Formyltripeptides **1** and **2** show a selective ability in stimulating human neutrophils. In fact they are able to trigger killing mechanisms while being unable to induce chemotaxis. These data are not surprising because it has long been known

Table 1
Chemotactic activity, superoxide anion production, lysozyme release, cystosolic calcium, and binding analysis of fMLP-OMe and its analogs

Peptides	Chemotactic index	Superoxide anion production (nmol)	Lysozyme release (%)	Cytosolic calcium (%)	Receptor binding IC_{50} (nM)
fMLP-OMe	1.15±0.08 (10^{-9} M)	48±3 (10^{-6} M)	52±3 (10^{-6} M)	630±5 (10^{-5} M)	60±3
For-Gln-Tyr-Phe-OMe	0.09±0.01 (10^{-7} M)	35±2 (5×10^{-5} M)	54±3 (10^{-5} M)	309±15 (10^{-5} M)	30000±300
For-Gln-Tyr-Tyr-OMe	0.08±0.02 (10^{-9} M)	19±2 (5×10^{-5} M)	40±3 (5×10^{-5} M)	100±8 (10^{-5} M)	>30000±350
For-Met-Tyr-Phe-OMe	0.78±0.02 (10^{-10} M)	58±2 (10^{-6} M)	74±3 (10^{-5} M)	600±20 (10^{-5} M)	600±20
For-Met-Leu-Tyr-OMe	1.09±0.08 (10^{-8} M)	38±2 (10^{-5} M)	40±3 (5×10^{-5} M)	200±3 (10^{-5} M)	300±40
For-Gln-Leu-Phe-OMe	0.43±0.02 (10^{-11} M)	54±2 (10^{-5} M)	57±3 (10^{-5} M)	250±8 (10^{-5} M)	1000±30

Efficacy data of chemotaxis are expressed as chemotactic index; superoxide anion production is expressed as net nanomoles of superoxide anion production/1×10^6 cells/5 min; and lysozyme release is expressed in %. ±S.E.M. Basal calcium level is 99.7±9 nM (n=6).

that the transduction pathway underlying the chemotactic response is different from those responsible for superoxide anion production or lysozyme release (Li et al., 2000; Ferretti et al., 2001; Selvatici et al., 2003), and several previous experiments, carried out utilizing drugs (Elferink and Koster, 1993) or pharmacological manipulation of the signal transduction pathway (Mocsai et al., 1997) have highlighted the fact that distinct mechanisms are involved in each of these neutrophil responses. Moreover it has been demonstrated that each physiological function shows different requirements for receptor occupancy. Superoxide anion production requires continuous occupancy of almost 100% of the receptors to achieve and maintain an optimal response, whereas chemotaxis does not (Prossnitz and Ye, 1997).

For-Gln-Tyr-Phe-OMe (**1**), which has both formyl and Phe residues in common with the parent fMLP-OMe, possesses, as a whole, a biological activity stronger than that of for-Gln-Tyr-Tyr-OMe (**2**), and is evidently not similar enough to the parent to interact fully with the receptor.

Data obtained from our experiments on intracellular calcium levels fit well with killing mechanisms and secretagogue activity. Supporting the theory that neutrophil chemotaxis seems independent of calcium enhancement, it has been demonstrated that the murine S 100 protein, CP-10, possesses a potent chemotactic activity for phagocytes, but fails to enhance calcium levels in human or mouse neutrophils (Cornish et al., 1996). A similar conclusion has been reached by Laffafian and Hallet (1995), and by us in a previous paper (Fabbri et al., 1997), demonstrating that the cation levels, measured in human neutrophils moving towards a source of the classical chenmoattractant fMLP, do not change significantly.

Changes in the chemical structures of these new compounds probably cause the low affinity revealed in the binding experiments on formylpeptide receptors. In particular, for-Gln-Tyr-Phe-OMe (**1**) and for-Gln-Tyr-Tyr-OMe (**2**) showed affinity values in the micromolar range revealing different behaviour as compared with the reference compound fMLP-OMe.

From these data, the following conclusions can be reached:

1. chemotactic activity: compounds **1** and **2**, lacking in activity, combine the negative contribute of the single substitution in corresponding **3**, **4**, and **5**. We stress the negative contribute of Gln at position 1;
2. superoxide anion production: compounds **1** and **2** are able to trigger this function but they are unable to combine the positive contribute of the single substitution;
3. lysozyme release: compounds **1** and **2** show that the further substitutions with Gln^1 (analog **1**) and Gln^1 and Tyr^3 (analog **2**) deprives them of the strongly positive influence of the Tyr^2, analog **4**;
4. compound **1** significantly enhanced the levels of intracellular calcium. Compound **2** was found to be completely unable to elicit an effect;
5. binding experiments demonstrate the reduced ability of compounds **1** and **2** to evoke a sound biological response, showing their weak affinity to FPR: we can hypothesize that only the formyl group binds to the receptor, while the other parts of the molecules are not able to efficaciously interact with it;
6. the biological response of compound **2**, which has only the formyl group in common with fMLP, induces us to hypothesize that the formyl group is the key to recognizing the neutrophil specific receptor;
7. the greater the difference of the compound from the reference fMLP-OMe, the lower the efficacy of the biological response observed;
8. the structures of compounds **1** and **2** exploit the less specific effect of the isoform which triggers the killer mechanisms.

The aim of this research was to find synergic substitutions on the prototype fMLP-OMe, but the most important conclusion that can be drawn from this study is that: the presence of the formyl group is a condition necessary for, and sufficient to induce the recognition and binding of the ligand to FPR, thereby immediately activating the least exacting receptor isoform, i.e. the isoform that triggers killing mechanisms.

It can be reasonably hypothesized that the formyl group is the first step–"primary hooking"–in ligand binding to the FPR. After this step, it is probable that the ligand spreads itself out in the receptor, further linking and blocking itself with one or more "secondary hookings", and, the more effectively the ligand fills the receptor, the stronger and more diversified is the biological response.

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