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Original article

Oligomeric formylpeptide activity on human neutrophils

Giorgio Cavicchioni [a,*], Anna Fraulini [a], Sofia Falzarano [b], Susanna Spisani [b]

[a] Department of Pharmaceutical Sciences, University of Ferrara, Via Fossato di Mortara 17/19, Ferrara 44100, Italy
[b] Department of Biochemistry and Molecular Biology, University of Ferrara, Via L. Borsari 46, Ferrara 44100, Italy

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ABSTRACT

A series of oligomeric formylpeptides were synthesized by cross-linking the prototype fMLP using a Lys residue. They were then investigated for their ability to stimulate chemotaxis, superoxide anion production, and lytic enzyme release in human neutrophils.

Although active in stimulating the different receptor isoforms, leading to the different biological responses, these analogues showed a lesser potency and affinity than the standard peptide. On the basis of the results reported here, we can hypothesise that: (i) the increased bulk of these molecules seems to hinder their correct positioning into the receptor pocket, thereby hindering favourable receptor interaction; and that: (ii) fMLP space positions do not seem to allow the ligand to increase biological responses.

1. Introduction

Human neutrophils are phagocytic cells specialized in innate host defence and they represent the largest subset of circulating leukocytes. This cell type also has major associations with acute inflammatory reactions.

Neutrophils circulate in the bloodstream, and can be recruited in high numbers in infected or inflamed tissues along the concentration gradients of chemoattractants released either by the infecting microorganisms or arising from inflammatory reactions in the infected tissue. These cells express a number of different chemoattractant receptors which enable them to sense invading microbes and approach the site of infection by direct migration. Invaders are subsequently phagocytized then degraded by toxic oxygen radicals (respiratory burst) and/or hydrolytic enzyme release. The potent bactericidal compounds produced by neutrophils are non-selective agents, and their production must therefore be strictly regulated in order to be efficient and safe for the host organism [1,2].

Various inflammatory mediators, including the N-formylmethionyl peptides derived from newly synthesised bacterial (or mitochondrial) proteins, are capable of activating neutrophils via specific receptors [3]. A distinct hierarchy of neutrophil responses to varying concentrations of the prototype N-formyl-methionyl-leucyl-phenylalanine (fMLP) has been clearly demonstrated; the concentrations of fMLP required to induce chemotaxis are lower than those required to cause superoxide anion production or lysosomal enzyme release [1]. fMLP acts by binding to classical G-protein-coupled receptors, first identified in 1976 and subsequently classified as high-affinity (FPR) or low-affinity (FPRL-1, FPR-like 1) fMLP receptors [4–6].

Downstream of this interaction, a number of signalling systems are triggered: the intracellular FPR-cascade includes activation of phosphoinositide 3-kinases (PI3Ks), phospholipase A (PLA), PLD, and mitogen-activated protein kinases (MAPKs) [7–9].

Receptor-specific ligands may act as useful tools for studying such complex signal transduction systems. A bivalent ligand, displaying two copies of the pharmacophores, would be expected to exhibit a considerably greater potency than the monomer, due to entropic factors triggered when the linker possesses sufficient length to bridge proximal recognition sites of different receptor subtypes. Dimeric ligands of bioactive peptide molecules have previously been synthesized and demonstrated to be specific and selective. It has also been established that dimeric enkephalins, neurokinins, and bradykinin, cross-linked by methylene chains, display remarkable activities and high receptor subtype selectivity [10–13]. These results suggest that: (i) dimeric ligands may exhibit increased affinity, reliant on the length of the cross-linking spacers, as compared to the corresponding monomeric analogues, and (ii) the spacer length which produces a peak increase in affinity is dependent upon the receptor type [10,13].

In the specific field of fMLP derivatives, Miyazaki et al. studied dimeric fMLPs and found that (i) the length of the linker portion has

* Corresponding author. Tel.: +39 532 455278; fax: +39 532 455948.
E-mail address: g5z@unife.it (G. Cavicchioni).

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a determining influence on its activities and receptor selectivity, (ii) lower activity may result from linkers with a branched structure, probably due to an increased rigidity in the overall conformation, (iii) a short linker triggers maximal activity, (iv) dimeric analogues are highly selective ligands for chemotactic activity, which is elicited far more potently than superoxide anion production, and (v) the length of the dimeric analogue discriminates between the two different receptor subtypes for chemotaxis and superoxide anion production [14].

Because a short linker triggers maximal activity, we synthesized cross-linked di-fMLP analogues by binding the carboxylic terminal group of fMLP to a functional group from the side chain of a suitable second residue from an fMLP-OMe analogue. The aim was to ascertain whether (and in this case to what extent), the cross-linked fMLP was able to contribute to enhance the biological response.

Our data was found to confirm the common conviction that the isoform responsible for killing mechanisms is less exactly than that responsible for chemotaxis, as superoxide anion production and secretagogue activity, unlike chemotaxis, benefit from the presence of the cross-linked tripeptide, particularly if sterically hindered. Even though it is less potent than monomeric fMLP, this dimeric analogue shows high receptor affinity [15,16].

By taking into account the above considerations, we report here biological studies on human neutrophils of the following newly synthesized oligo-fMLP cross-linked analogues: for-Met-Leu-Phe-Lys[γAsp(OMe)-Phe-Leu-Met-for]-OMe **1**, for-Met-Leu-Phe-Lys(Phe-Leu-Met-for)-OMe **2**, for-Met-Leu-Phe-Lys(Phe-Leu-Met-for)-εLys(OMe)-Phe-Leu-Met-for **3**, and for-Met-Leu-Phe-Lys(-Phe-Leu-Met-for)-Lys(Phe-Leu-Met-for)-OMe **4**.

2. Results and discussion

The biological activity of the new compounds (**1–4**) under examination was determined on human neutrophils and compared to that of the reference ligand fMLP-OMe. Directed migration (chemotaxis), superoxide anion production and lysozyme release were measured as "efficacy" (which corresponds to the maximum effect of a ligand) and "potency" (which corresponds to the concentration of a ligand at which 50% of its maximum effect is reached).

Chemotactic activity elicited by the reference compound and ligands **1–4** are reported in Fig. 1. The dose response curves, as usually found for chemoattractants, all increased to reach a peak and then decreased rapidly as the concentration of the ligand increased. The presence of fMLP/fMLP-OMe in the same molecule failed to enhance the activation of chemotaxis, irrespective of the number inserted. Moreover, both potency and efficacy were found to be lower than those of the parent peptide. Specifically, the potency of the analogues was ten times lower than that of fMLP-OMe, and their chemotactic index was marginally lower than that fMLP-OMe (C.I. = 1.05) at 10^{-8} M, although in the same narrow range (C.I. = 0.90–1.01). Considering the other concentrations, we can see that the efficacy of analogues was **4** ≈ **1** ≈ **3** (C.I. ~ 0.95 *vs* 1.15 of fMLP-OMe), while compound **2** showed the lowest efficacy.

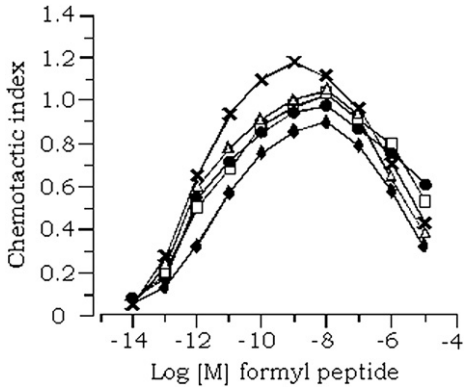

Fig. 1. Chemotactic activity in human neutrophils provoked by fMLP-OMe and its analogues. The data are the means of five separate experiments performed in duplicate. S.E.M. are in 0.02–0.09 no % chemotactic index range.

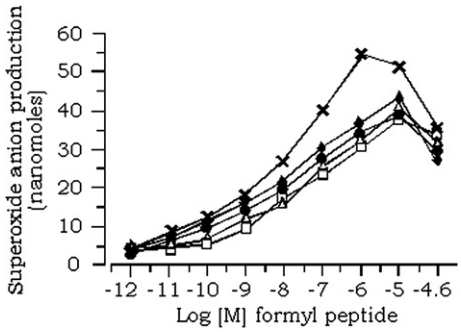

Fig. 2. Superoxide anion production in human neutrophils provoked by fMLP-OMe and its analogues. The data are the means of five separate experiments performed in duplicate. S.E.M. are in 0.1–4 nmol O_2^- range.

Analysing superoxide anion production (Fig. 2), all four derivatives were found to have the same order of efficacy at increasing concentrations. This was consistently lower than that of fMLP-OMe, and reached its greatest difference at 10^{-6} M.

Lysozyme release activity (Fig. 3) showed a particular trend; the efficacy was found to be incrementally lower than the prototype in the concentration range 10^{-10} to 10^{-6} M, reaching an efficacy of about 40% (compounds **2** and **4**) and 20% (compounds **1** and **3**) *vs* about 60% of fMLP-OMe at 10^{-6} M. The lysozyme release activity of the derivatives peaked at the concentration of 10^{-5} M, once again a concentration ten times higher than that of the monomer prototype.

3. Conclusions

As a part of a broad strategy in the study of oligomeric formylpeptides, a series of fMLP analogues cross-linked by a Lys residue were synthesized, in order to specifically investigate their ability to better stimulate and/or selectively trigger neutrophil functions.

From the data presented here, the following items can be drawn:

Compounds **1**, **2**, **3**, and **4** are active in stimulating the different receptor isoforms, leading to the different biological responses, even though with a lesser potency and affinity than the standard peptide.

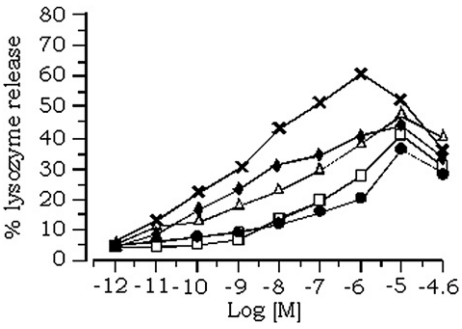

Fig. 3. Release of neutrophil granule enzymes evaluated by determining lysozyme activity induced by fMLP-OMe and its analogues. The data are the means of five separate experiments performed in duplicate. S.E.M. are in 1–6% range.

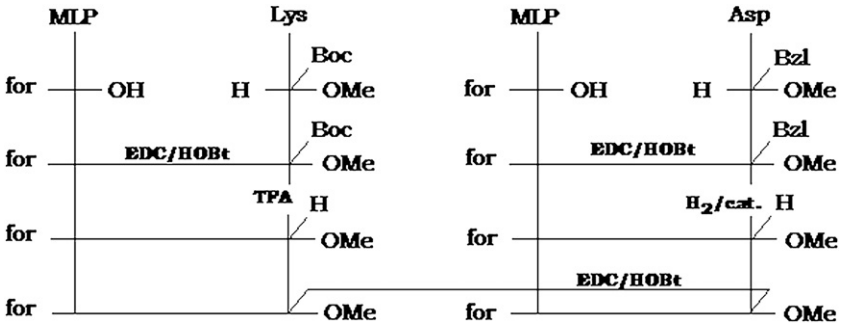

Fig. 4. – Scheme of synthesis and structural formula of *for-Met-Leu-Phe-Lys[γAsp(OMe)-Phe-Leu-Met-for]-OMe.*

These analogues have a very similar common behaviour with an activity peak centered at 10^{-8} M and 10^{-5} M for chemotaxis and superoxide anion production, respectively. Concerning lysozyme release the four derivatives exhibit a profile of activity with a maximum centered at 10^{-5} M and a rank of potency **4** > **2** = **1** > **3**.

On the basis of the above reported results, we can argue that (i) our polymeric analogues are unable to stimulate more than just one receptor pocket, (ii) the increased bulk of these molecules seems to hinder their correct allocation so avoiding to establish requirements for optimal ligand–receptor interaction.

In conclusion, the here described molecules provide new informations on structure–activity relationship concerning chemotactic peptides and formyl-peptide receptors, taking into account the recent identification of novel FPRs agonists which broadens the spectrum of functional significance of such receptors. Further model possessing different steric hindrances and different fMLP steric positions are in progress in our laboratories to explore this topic.

4. Experimental protocols

4.1. Chemistry

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.

Thin-layer chromatography (TLC) was performed on pre-coated silica gel F254 plates (Merck) with the solvent system methylene chloride/methanol 5:1.

Satisfactory C, H, N, S microanalyses were obtained for all compounds, analytical results being within 0.4% of the theoretical values.

Amino acids were purchased from Fluka. Peptides were synthesized following standard procedures in solution [16]. Removal of the Boc group was performed by the treatment with a 1:1 mixture of TFA/CHCl3. Peptide coupling was achieved by the 1-hydroxy-1,2,3-benzotriazole (HOBt)/N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) method [17], whereas the formyl group was introduced according the N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) method [18].

Molecular weight of compounds was determined by an ESI Micromass, ZMD2000 mass spectrometer.

Crude peptides were purified by preparative reverse-phase 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 elution of the 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 and 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.

4.2. General procedure for the synthesis

for-Met-Leu-Phe-Lys[γAsp(OMe)-Phe-Leu-Met-for]-OMe **1** (Fig. 4) – Colourless solid (mp 107–110 °C; Rf 0.31; $[\alpha]_D = -17.3°$, $c = 1$, MeOH; Mass: Na^+ adduct 1151.34; K^+ adduct 1167.34).

for-Met-Leu-Phe-Lys(Phe-Leu-Met-for)-OMe (Fig. 5) **2** – Colourless solid (mp 96–100 °C; Rf 0.45; $[\alpha]_D = -15.2°$, $c = 1$, MeOH; Mass: Na^+ adduct 1022.1; K^+ adduct 1038.1).

for-Met-Leu-Phe-Lys(Phe-Leu-Met-for)-εLys(OMe)-Phe-Leu-Met-for **3** (Fig. 6) **3** – Colourless solid (mp 182–185 °C; Rf 0.48; $[\alpha]_D = -20.4°$, $c = 1$, MeOH; Mass: molecular ion 1547; Na^+ adduct 1570; K^+ adduct 1586).

for-Met-Leu-Phe-Lys(Phe-Leu-Met-for)-Lys(Phe-Leu-Met-for)-OMe (Fig. 7) **4** – Colourless solid (mp 193–197 °C; Rf 0.58; $[\alpha]_D = -23.7°$, $c = 1$, MeOH; Mass: molecular ion 1547; Na^+ adduct 1570; K^+ adduct 1586).

4.3. Biological activity

4.3.1. Peptide dilution

A 10^{-2} M stock solution of fMLP-OMe (Sigma Chemical Co. St. Louis MO, U.S.A.) and the new 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 five-times-working-strength stock solution with the

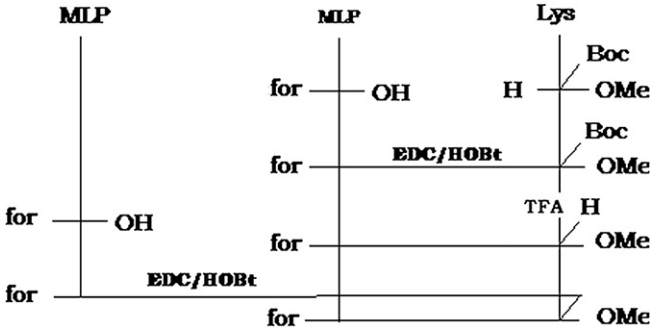

Fig. 5. – Scheme of synthesis and structural formula of *for-Met-Leu-Phe-Lys (Phe-Leu-Met-for)-OMe.* **X,** for-Met-Leu-Phe-OMe; □ **1** for-Met-Leu-Phe-Lys[γAsp(OMe)-Phe-Leu-Met-for]-OMe; ◆ **2** for-Met-Leu-Phe-Lys(Phe-Leu-Met-for)-OMe; ● **3** for-Met-Leu-Phe-Lys(Phe-Leu-Met-for)-εLys(OMe)-Phe-Leu-Met-for; △ 4 for-Met-Leu-Phe-Lys (Phe-Leu-Met-for)-Lys(Phe-Leu-Met-for)-OMe.

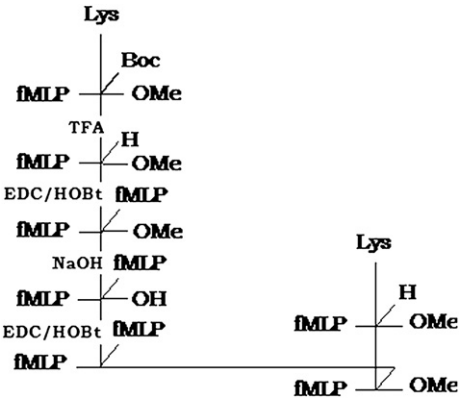

Fig. 6. – Scheme of synthesis and structural formula of *for-Met-Leu-Phe-Lys(Phe-Leu-Met-for)-εLys(OMe)-Phe-Leu-Met-for.* **X,** for-Met-Leu-Phe-OMe; □ **1** for-Met-Leu-Phe-Lys [γAsp(OMe)-Phe-Leu-Met-for]-OMe; ◆ **2** for-Met-Leu-Phe-Lys(Phe-Leu-Met-for)-OMe; ● **3** for-Met-Leu-Phe-Lys(Phe-Leu-Met-for)-εLys(OMe)-Phe-Leu-Met-for; △ 4 for-Met-Leu-Phe-Lys(Phe-Leu-Met-for)-Lys(Phe-Leu-Met-for)-OMe.

following composition: NaCl, 40 g/l; KCl, 1.875 g/l; $Na_2HPO_4.2H_2O$, 0.6 g/l; KH_2PO_4, 0.125 g/l; $NaHCO_3$, 1.25 g/l; glucose, 10 g/l. 1 mM $MgCl_2$ and $CaCl_2$ supplemented the buffer before biological tests. All reagents were of the purest grade commercially available.

4.3.2. Purification of human neutrophils

Cells were obtained from heparinized (10 U/ml) peripheral blood from fasting healthy subjects. 20 ml of blood was supplemented with 12 ml of a solution consisting of 6% (by weight) Dextran T70 (Pharmacia, Upsala, Sweden) and was settled in a 50 ml polypropylene tube at room temperature for 45 min. The leukocyte-containing turbid upper supernatant was carefully removed and layered onto 10 ml of Ficoll-Paque (Pharmacia, density gradient for lymphocyte isolation), and centrifuged at 250*g* for 20 min at room temperature. The pellet containing the neutrophils was further purified by hypotonic lysis of erythrocytes (0.86% NH_4Cl for 10 min). The cells were then washed twice and resuspended using KRPG, pH 7.4, at a final concentration of 50×10^6 cells/ml and used immediately. The neutrophils were 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. All experiments were carried out according to the guidelines set out by local and regional ethics committees.

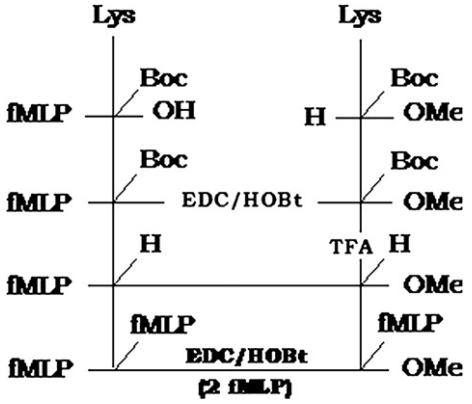

Fig. 7. – Scheme of synthesis and structural formula of *for-Met-Leu-Phe-Lys(Phe-Leu-Met-for)-Lys(Phe-Leu-Met-for)-OMe.* **X,** for-Met-Leu-Phe-OMe; □ **1** for-Met-Leu-Phe-Lys [γAsp(OMe)-Phe-Leu-Met-for]-OMe; ◆ **2** for-Met-Leu-Phe-Lys(Phe-Leu-Met-for)-OMe; ● **3** for-Met-Leu-Phe-Lys(Phe-Leu-Met-for)-εLys(OMe)-Phe-Leu-Met-for; △ 4 for-Met-Leu-Phe-Lys(Phe-Leu-Met-for)-Lys(Phe-Leu-Met-for)-OMe.

4.3.3. Random locomotion and chemotaxis

Random locomotion and chemotaxis studies were performed with a 48-well microchemotaxis chamber (BioProbe, Milan, Italy), and migration into the filter was evaluated by the leading-front method, according to Zigmond and Hirsch [19]. The random movement, used as control, was 32 μm ± 3 SE of ten 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 are expressed in terms of the chemotactic index (C.I.) ratio as follows: (migration toward test attractant minus migration toward the buffer)/(migration toward the buffer).

4.3.4. Superoxide anion (O_2^-) production

Superoxide anion production was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome *c* modified for microplate-based assays [15]. Tests were carried out in a final volume of 200 μl containing 4×10^5 neutrophils, 100 nmol cytochrome *c* and KRPG. At zero time, different amounts (10^{-10} to 10^{-4} M) of each peptide were added and the plates were incubated in a microplate reader (Ceres 900, Bio-Tek Instruments, Inc) with T set to 37 °C. Absorbance was recorded at wavelengths of 550 and 468 nm. Differences in absorbance at the two wavelengths were used to calculate the nmoles of O_2^- produced, using a molar extinction coefficient for cytochrome *c* of 18.5 $mM^{-1}\,cm^{-1}$. Neutrophils were pre-incubated with 5 μg/ml cytochalasin B for 5 min prior to the activation by peptides.

4.3.5. Granule enzyme assay

Neutrophil granule enzyme release was evaluated by determining the modified lysozyme activity in microplate-based assays [15]. 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*g*, and the amount of lysozyme was quantified nephelometrically by the rate of lysis of a cell wall suspension of *Micrococcus lysodeikticus.* Neutrophils were pre-incubated with 5 μg/ml cytochalasin B for 15 min at 37 °C prior to the activation by peptides. The reaction rate was measured using a microplate reader at 465 nm. Enzyme release is expressed as a net percentage of the total enzyme content released by 0.1% Triton X-100. Total enzyme activity was 85 ± 1 μg/1×10^7 cells/min.

4.4. Statistical analysis

Data are given as mean ± S.E.M. The significance of the differences between oligo-fMLP analogues and the reference fMLP-OMe was assessed by the non-parametric Wilcoxon test. Differences between groups were judged to be statistically significant at $P \leq 0.05$.

Acknowledgments

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