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Properties of a novel chemotactic esapeptide, an analogue of the prototypical *N*-formylmethionyl peptide

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Abstract

The new disulphur-bridged peptide, for-Met-Leu-Cys(OMe)-Cys(OMe)-Leu-Met-for, has been synthesized and its biological properties resulting from its binding to the formyl-peptide receptor of human neutrophils characterized. Three activities resulting from this interaction were measured: directed cell migration (i.e., chemotaxis); superoxide anion production; and lysozyme enzyme release. The properties were compared with those observed for the prototypical peptide, for-Met-Leu-Phe-OMe. Chemotaxis is strongly triggered while both superoxide anion production and lysosomal enzyme release are elicited only at high concentrations and never reach the response peak observed for the prototype peptide at physiologically relevant concentrations. The derivative appears to bind with a good affinity to the formyl-peptide receptors. These results provide new information regarding the structure–activity relationship of the formyl-peptide receptor.

Keywords: *N*-Formylmethionyl peptides; Human neutrophils; Chemotaxis; Superoxide anion generation; Lysozyme release; Receptor binding

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1. Introduction

N-Formyl-peptides, potent chemoattractants for granulocytes, are released by invading microorganisms or evolve from inflamed tissues. They are known to induce directional cell migration (chemotaxis) together with a wide range of other biological functions, such as superoxide anion production and lysosomal enzyme release (killing mechanisms) through the binding to specific membrane receptors [1]. For-Met-Leu-Phe-OH (fMLP) and its derivative for-Met-Leu-Phe-OMe (fMLP-OMe) have been identified as the prototypes of these agents. The existence of multiple isoforms of the formyl-peptide receptor, which exhibit different affinities for the ligand, has been demonstrated [2]. It is thought that they may activate specific transduction pathways, thereby explaining the diversity of responses associated with cell activation by fMLP [3,4].

Numerous studies have stressed the importance of each part of the fMLP molecule. A protic amide bond between the first and the second residue [5] as well as the formyl group seem to be mandatory. The leucine residue at position 2 can be substituted by bulky and/or dialkylated hydrophobic residues [6]. Finally, the phenylalanine at position 3 is regarded as the best residue for both recognition and activation of the receptor, and a comparable effect is exerted by methionine at position 1.

In order to investigate the possibility that peptides with at least two fMLP units would stimulate a strong chemotaxis, we previously studied neutrophil stimulation by the di-tripeptides, for-Met-Ser(for-Met-Leu-Phe)-Phe-OMe and for-Met-Ser (for-Met-Leu-Phe)-Phe-OMe [7] and found that the biological response is tied to the nature of the functionalized residue. These observations and the ability of cysteines to form a disulphide bond, gave us the idea to synthesize the dimeric for-Met-Leu-Cys(OMe)-Cys(OMe)-Leu-Met-for (designated Cys–Cys) (Fig. 1).

This analog lacks the Phe residue, but its absence should be offset (i) by the Cys, which has been shown to mimic hindered amino acids [8], and (ii) by the contribution of the second fMLP moiety. The cyclo-bridged disulphur analog for-Cys-Leu-Phe-Cys-OMe has already been synthesized [9], and found to inactivate human neutrophils. However, the analog under investigation differs substantially in three ways from this molecule: it has a cyclic structure; it is a tetrapeptide monomer; and it has the essential Met substituted by Cys. The synthesis, properties, and a comparison of these properties with the prototypical peptide agonist are reported herein.

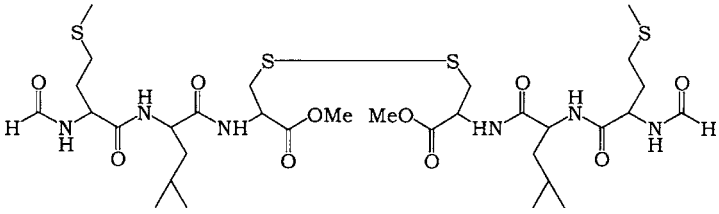

Fig. 1. Structure of the peptide, for-Met-Leu-Cys(OMe)-Cys(OMe)-Leu-Met-for.

2. Materials and methods

2.1. Chemistry

The optical rotation was determined in MeOH at 20 °C on a Perkin–Elmer Model 241 polarimeter. The melting point was determined on a Reichert–Kofler block and is uncorrected. Satisfactory C, H, N, S microanalyses were obtained for all compounds. Analytical results are within 0.4% of the theoretical values. Amino acids were purchased from Fluka (Italy). Removal of the Boc group was performed by treatment with a 1:1 mixture of trifluoroacetic acid (TFA)/$CHCl_3$. Peptide coupling was achieved by the 1-hydroxy-1,2,3-benzotriazole (HOBt)/*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) method, whereas the formyl group was introduced by the *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) method [10].

for-Met-Leu-Cys(OMe)-Cys(OMe)-Leu-Met-for was synthesized following standard procedures in solution (Fig. 2). M.p. 115–118 °C; $[\alpha]_D^{20} = -53.1°$ ($c = 1$, in MeOH), MS (M + H) 814.7.

2.2. Biological assays

Human neutrophils were purified employing the standard techniques of dextran sedimentation of heparinized blood, followed by centrifugation on Ficoll–Plaque and hypotonic lysis of contaminating red cells. The cells were washed twice and resuspended in Krebs–Ringer-phosphate containing 0.1% (w/v) glucose (KRPG), pH 7.4. Neutrophils were 98–100% pure and $\geqslant$99% viable, as determined by the Trypan blue exclusion test [7].

Random locomotion was performed with a 48-well microchemotaxis chamber, and migration into the filter was evaluated by the leading front method [11]. The actual control random movement is 32 μm ± 3 SE 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

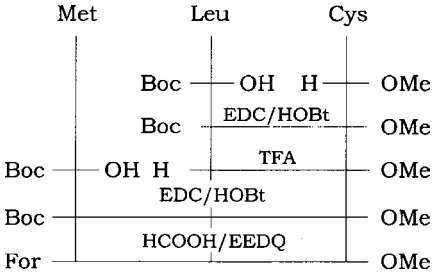

Fig. 2. Peptide synthesis. The final tripeptide was stirred throughly to give the disulphur-bridged peptide. The abbreviations are defined in Section 2.

ranging from 10^{-12} to 10^{-5} M. Data were expressed in terms of chemotactic index (CI), which is the ratio: (migration toward test attractant minus migration toward the buffer)/(migration toward the buffer).

Superoxide anion (O_2^-) production was measured by the superoxide dismutase-inhibited reduction of ferricytochrome *c* modified for microplate-based assays [7]. Tests were carried out in a final volume of 200 μl containing 4×10^5 neutrophils, 100 nmol cytochrome *c*, and KRPG. At time zero, different amounts (10^{-12}–10^{-4} M) of each peptide were added and the plates were incubated in a microplate reader (Ceres 900, Bio-Tek Instruments) with compartment temperature 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 nmoles of O_2^- produced, using the 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 activation by peptides.

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^{-12}–10^{-4} M for 15 min at 37 °C. The plates were then centrifuged for 5 min at 400*g*, and 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 activation by peptides. The 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.

Binding assays were carried out as described by Spisani et al. [12].

Saturation binding experiments of [^{3}H]fMLP (3–300 nM) to human neutrophils were performed by 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. Non-specific binding was defined as that in the presence of 10 μM fMLP, and was about 20% of total binding. The incubation time was 15 min at 37 °C. The bound and free radioactivity were 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).

2.2.1. Statistical analysis

The non-parametric Wilcoxon test was used in the statistical evaluation of differences between groups [13].

3. Results and discussion

Biological activities of the modified ligand were determined with human neutrophils and compared with those of the standard agonist tripeptide fMLP-OMe.

Directed migration (chemotaxis), superoxide anion production (O_2^-), and lysozyme release were measured as indicators of "potency" (which corresponds to the peak of each peptide: they are expressed as a chemotactic index or nmoles of O_2^- produced or % of lysozyme release), and "efficacy" (which corresponds to the activity at the optimal peptide concentration, i.e., the molar concentration). Competition-binding experiments were carried out to establish the relative ability of the peptide to compete for [^{3}H]-for-Met-Leu-Phe binding.

3.1. Chemotactic activity

For-Met-Leu-Cys(OMe)-Cys(OMe)-Leu-Met-for possesses an ability to stimulate a chemotactic response which is similar to that of the fMLP-OMe. Their representative curves show the same pattern but they do not superimpose on each other (Fig. 3). The maximum potency of the reference fMLP-OMe was 10^{-9} M, whereas the peak value of the analog was observed at a concentration of 10^{-8} M, which is 10 times higher, but within the physiological range. This result could suggest that the lack of the Phe at position 3 of for-Met-Leu-Cys-Cys-Leu-Met-for enables this analog to fit optimally into the receptor pocket isoform. We hypothesize that the Phe residue is not mandatory for strong chemotaxis providing that the rest of the molecule can properly make up for its absence.

3.2. Superoxide anion production

Fig. 4 provides evidence that the sulphur-bridged analog does not trigger a response until 10^{-6} M, reaching its maximum (30 nmol) at 10^{-5} M, one order of magnitude higher than that of reference molecule (55 nmol at 10^{-6} M). It shows a value comparable to fMLP-OMe only at a highest concentration. This shift towards high concentrations indicates weak efficacy by the analog. The derivative, clearly, is not

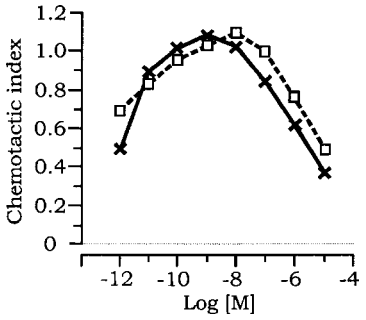

Fig. 3. Chemotactic activity of fMLP-OMe and its analog toward human neutrophils where ✖ corresponds to fMLP-OMe and □ corresponds to for-Met-Leu-Cys(OMe)-Cys(OMe)-Leu-Met-for. The data are the means of five separate experiments performed in duplicate. The SE are in 0.02–0.09% chemotactic index range.

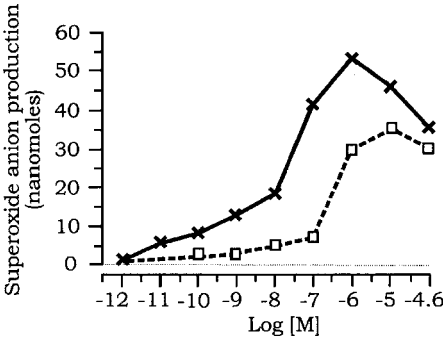

Fig. 4. Superoxide anion production of fMLP-OMe and its analog toward human neutrophils where ✖ corresponds to fMLP-OMe and □ corresponds to for-Met-Leu-Cys(OMe)-Cys(OMe)-Leu-Met-for. The data are the means of five separate experiments performed in duplicate. The SE are in 0.1–4 nmol O_2^- range.

able to properly interact and fill the receptor pocket specific isoform. We can therefore conclude that Cys–Cys is a partial agonist with regard to O_2^- production.

3.3. Lysozyme release

Fig. 5 shows the curves (as percentage) that demonstrate the effect exerted by peptides on lysozyme release. The Cys–Cys curve profile shows a lower potency as a secretagogue agent, when compared with the standard peptide, in the wide range of 10^{-10}–10^{-6} M. In fact, it shows its maximum (43%) at 10^{-5} M, which is the same concentration the parent fMLP-OMe starts to decrease. Therefore, efficacy peak is shifted 10-fold. The picture that emerges from these data is that this analogue has a weak ability to trigger efficaciously lysosomal enzyme release. The steric feature of Cys–Cys does not allow a good interaction with the specific receptor isoform.

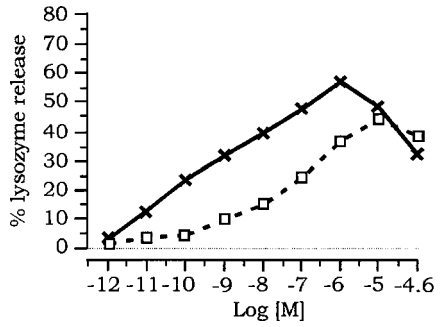

Fig. 5. Release of neutrophil granule enzymes evaluated by determining lysozyme activity induced by fMLP-OMe and its analog towards human neutrophils where ✖ corresponds to fMLP-OMe and □ corresponds to for-Met-Leu-Cys(OMe)-Cys(OMe)-Leu-Met-for. The data are the means of five separate experiments performed in duplicate. The SE are in 1–6% range.

3.4. Binding experiments

Fig. 6 shows a saturation curve of [^{3}H]fMLP and the corresponding Scatchard plot (inset) to 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 gain information regarding the affinity of the Cys–Cys analog to its receptor. 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 used radioligand [14].

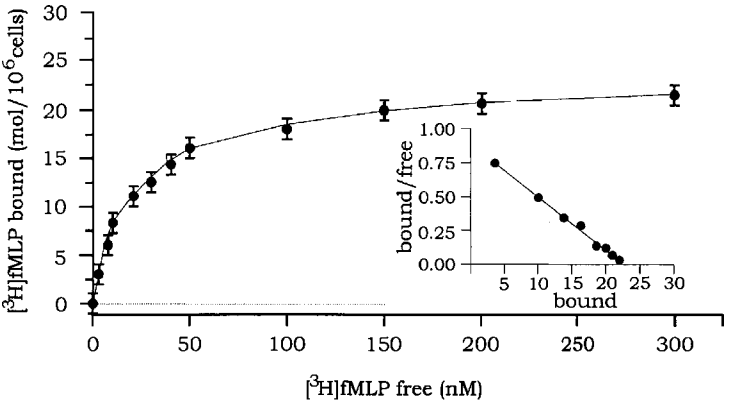

Fig. 6. Saturation of [^{3}H]fMLP binding to human neutrophils. The K_D value was 32 ± 4 nM and the B_{max} value was 22 ± 3 fmol/10^6 cells. Values are the mean and vertical lines are the SE of the mean of three independent experiments. The Scatchard plot of the same data is shown in the inset.

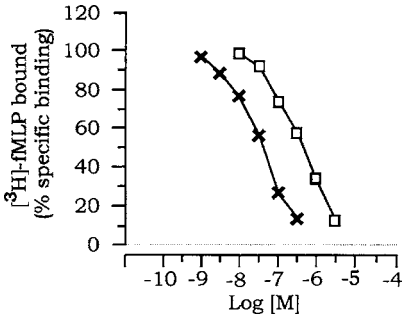

Fig. 7. Competition curves of specific [^{3}H]fMLP-OMe binding to human neutrophils by the test compounds where ✖ corresponds to fMLP-OMe and □ corresponds to for-Met-Leu-Cys(OMe)-Cys(OMe)-Leu-Met-for. Curves are representative of a single experiment taken from a series of three independent experiments. Non-specific binding was determined in the presence of 10 μl fMLP.

Fig. 7 shows inhibition curves of the examined compounds in human neutrophils. FMLP-OMe was the most potent compound ($K_i = 35 \pm 4$ nM), while the Cys–Cys analogue showed good affinity ($K_i = 238 \pm 26$ nM).

4. Conclusions

We synthesized for-Met-Leu-Cys(OMe)-Cys(OMe)-Leu-Met-for with the aim of evaluating its affinity and agonist potential toward formyl-peptide receptors on human neutrophils. With regard to the prototype fMLP or its methyl ester fMLP-OMe, it has been ascertained that the C-terminal amino acid should be aromatic with Phe being the preferred choice [15–18]. The most interesting data that emerges from our research is that the Cys–Cys analogue optimally stimulates chemotaxis though it lacks a Phe residue. The Cys can behave like a hindered amino acid [8] because it has the ability to link to another similar tripeptide, via a sulphur bridge, to give a dimeric analog. Thus, a potent chemotactic agent can be found even in formyl-peptides that have neither a Phe at position 3 nor a residue with similar characteristics [19]. The steric conformation evidently allows the molecule to interact well with the specific isoform, filling optimally the receptor pocket. In contrast, the killing mechanisms are poorly stimulated (partial agonist), even if O_2^- production is elicited to a lesser extent than lysosomal enzyme release. This observation is consistent with the literature [1] in that the killing mechanisms are mediated by the same fMLP-receptor isoform, which activates second messengers, leading to similar effect on the two biological responses. The potency and efficacy of this analog as a chemoattractant prompted us to consider that the dimeric Cys–Cys provides good specificity suggesting that it can be classified as a "pure" chemoattractant [20].

Saturation binding experiments show that human neutrophils bind fMLP with a single dissociation constant indicating, in agreement with previous data, the presence of the low affinity binding state [21]. In competition experiments fMLP-OMe and the dimeric Cys–Cys displayed a nanomolar affinity for fMLP-receptor even though the new compound appears to have less affinity than that of the reference compound. This result is in perfect agreement with the hypothesis that the concentration of the radiolabelled peptide used in the competition experiments is sufficiently high to convert the receptor conformation to the state unable to recognize pure chemoattractants [22].

In conclusion, the dimeric Cys–Cys peptide interaction with human neutrophils has been characterized by receptor binding, chemotaxis superoxide anion production and lysozyme release. The results provide new information relative to structure–activity relationships of the formyl-peptide receptor. Further studies are in progress in our laboratory to understand how to increase the biological response while developing a good selectivity. In particular, we intend to synthesize derivatives that lack the Phe residue and substitute it with a suitable structure, in order to specifically stimulate a specific receptor. In any case, the availability of a high potent ligand in well defined and distinct assays should provide useful tools in view of their potential use as diagnostic and therapeutic agents [23].

Acknowledgments

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