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# Biological Activity in Human Neutrophils of Di-tripeptides, Analogues of the Chemotactic fMLP

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**Abstract**—for-Met-Ser(for-Met-Leu-Phe)-Phe-OMe **1** and for-Met-Lys(for-Met-Leu-Phe)-Phe-OMe **2** were synthesized in order to investigate biological activities on human neutrophils of crosslinked di-tripeptides. Our results seem to highlight that the tested di-tripeptides (i) do not step up chemotaxis, (ii) can elicit superoxide anion production which is dependent on the nature of the residue at position 2, chosen in the tripeptide that is crosslinked to the fMLP-OMe. 

*N*-Formyl-peptides are known as potent chemoattractants for granulocytes. Released by invading microorganisms or arising from inflamed tissues, they induce directional cell migration together with a wide range of other biological functions, such as superoxide anion ($O_2^-$) production and lytic lysosomal enzyme release through binding to specific membrane receptors; 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 (FPR), which exhibit different affinities for the ligand, has been demonstrated.[1] These peptides may activate specific transduction pathways, thus explaining the diversity of responses associated with cell activation.[2]

A huge number of studies have stressed the importance of each part of the fMLP molecule. Essential conditions seem to be a protic amide bond between the first and second residues,[3] as well as the presence of a formyl group; the phenylalanine at position 3 is known to be the best residue in both recognition and activation of the receptor, and a comparable effect is exerted by methionine at position 1. Furthermore, the leucine residue at position 2 can be substituted by bulky and/or dialkylated hydrophobic residues.[4]

Since it has been ascertained that various dimeric peptides, such as bradykinins, enkephalins, neurokinins, and chemotaxins,[5] crosslinked by spacers of different length, show an increase of reactivity together with some better selectivity, crosslinked di-fMLP analogues were synthesized by binding the carboxylic terminal group of fMLP to a functional group of the side chain of a suitably chosen second residue of a fMLP-OMe analogue. We obtained for-Met-Ser(for-Met-Leu-Phe)-Phe-OMe **1** and for-Met-Lys(for-Met-Leu-Phe)-Phe-OMe **2**; these were characterized and their biological activity on human neutrophils was evaluated. Both Ser- and Lys-tripeptide analogues were chosen (in spite of the hydrophilicity of their side chains and the small dimensions of the Ser, conditions which do not trigger an optimal biological response)[6] because they possess functional groups which allow crosslinked di-tripeptides to be synthesized. The aim was to ascertain whether (and in this case to what extent), the crosslinked fMLP can contribute to enhancing the biological response of the Ser- and Lys-tripeptides alone, or else whether (and in this case to what extent), the Ser- and Lys-crosslinked analogues can contribute to enhancing the biological response of the fMLP alone. for-Met-Ser(for-Met-Leu-Phe)-Phe-OMe **1**, colorless solid (mp 183–185 °C; $[\alpha] = +2.85°$ in methanol, $c = 1$), MS $(M+H)^+$ 846.8, and for-Met-Lys(for-Met-Leu-Phe)-Phe-OMe **2**, colorless solid (mp 163–165 °C; $[\alpha] = -21.25°$ in methanol, $c$ 1), MS $(M+H)^+$ 887.7, were synthesized following standard procedures in solution (Fig. 1) and purified by HPLC.

*Human neutrophils* were purified employing the standard techniques of dextran sedimentation of heparinized

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blood, followed by centrifugation on Ficoll–Paque and hypotonic lysis of red cells. The cells were washed twice and resuspended in Krebs–Ringer-phosphate containing 0.1% w/v glucose (KRPG), pH 7.4. The percentage of neutrophils was 98–100% pure and $\geq$99% viable as determined by Tripan blue exclusion test.

*Random locomotion* was performed with a 48-well microchemotaxis chamber, and migration into the filter was evaluated by the leading-front method. The actual control random movement is 32 μm±3 SE of 10 separate experiments done 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.) which is the ratio: (migration toward test attractant minus migration toward the buffer)/(migration toward the buffer).

*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 $4\times10^5$ neutrophils, 100 nmols cytochrome c and KRPG. At zero time, different amounts ($10^{-10}$–$10^{-4}$ M) of each peptide were added and the plates were incubated into a plastic microplate reader (Ceres 900, Bio-Tek instruments, INC) with the compartment T set at 37 °C. Absorbance was recorded at wavelengths of 550 and 468 nm. Differences in absorbance at the two wavelengths were used to calculate the nmoles of $O_2^-$ produced, using the millimolar 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.

*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 in a final concentration of $10^{-10}$–$10^{-4}$ 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*. Neutrophils were preincubated with 5 μg/mL cytochalasin B for 15 min at 37 °C prior to activation by 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/L×$10^7$cells/min.

*Statistical analysis*. The non parametric Wilcoxon test was used in the statistical evaluation of differences between groups.

As shown in Figure 2, both compounds, **1** and **2**, evidence a chemotactic index which is less than the reference. Only the Lys analogue **2** reaches the fMLP-OMe potency at concentrations higher than $10^{-7}$ M: this response, stronger than that of analogue **1**, could be due to its major sterical hindrance. The results on the whole can be explained in two ways: either (i) the trigger of chemotaxis, and true ligand, is the fMLP, while the crosslinked (Ser or Lys)-tripeptide analogues do not allow a full interaction with the receptor, or (ii) the trigger of chemotaxis, and true ligand, is the methyl-ester tripeptide analogue, while the crosslinked fMLP acts as a sterically hindered second residue, lowering the efficacy of the analogue. In any case, this does not seem the best way to find molecules in order to reach a C.I. higher than the parent fMLP-OMe.

A completely different pattern emerges from the study of superoxide anion production (Fig. 3). The di-tripeptide **1** containing the Ser residue, in spite of the small hydrophilic side chain, exhibits a good activity, comparable with that of the parent fMLP-OMe. The Lys containing compound **2** triggers a biological response, in terms of affinity, decidedly stronger than either **1** or the parent fMLP-OMe, over a broad range of concentrations with a peak at $10^{-7}$ M. This picture seems to indicate that di-tripeptides can be useful as $O_2^-$ generating agents. One way to read these results is to hypothesize that the two tripeptides for-Met-Ser-Phe-OMe and for-Met-Lys-Phe-OMe are strengthened in their action by the crosslinked fMLP. Our data agree

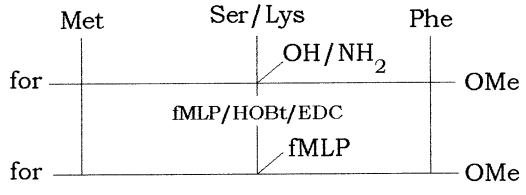


**Figure 1.** Synthesis of compounds **1** and **2**.

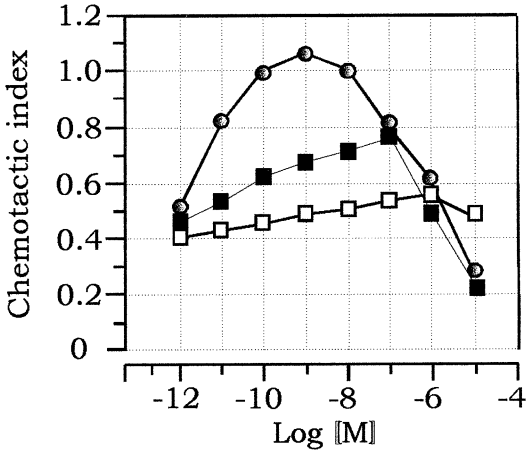


**Figure 2.** Chemotactic activity toward human neutrophils.

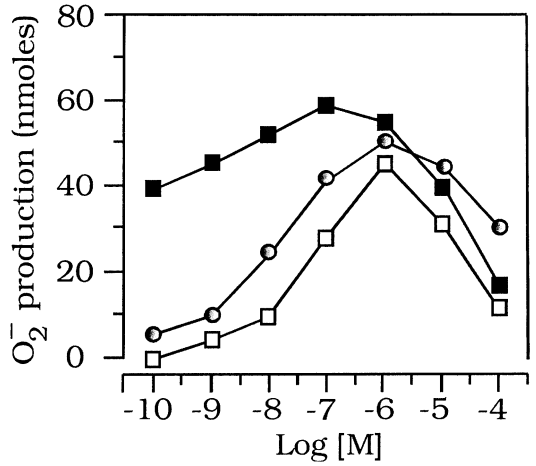


**Figure 3.** Superoxide anion production toward human neutrophils.

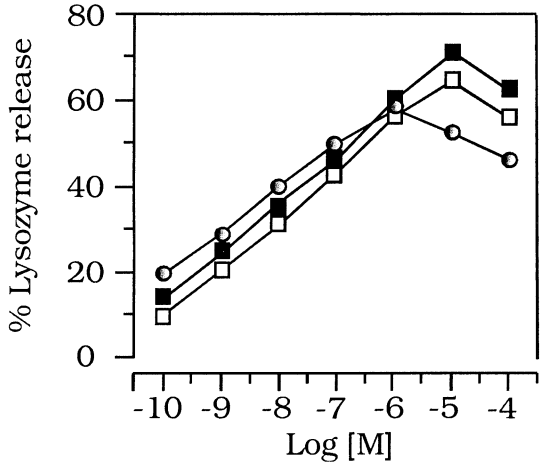


**Figure 4.** Release of neutrophil granule enzymes evaluated by determining lysozyme activity.

with the common conviction that the isoform responsible for $O_2^-$ production is less exacting than that responsible for chemotaxis.

Concerning lysozyme release (Fig. 4), a good picture emerges from our results. Both compounds **1** and **2** stimulate the segretagogue activity with a dose-response curve which is as high as the parent formylpeptide, particularly at physiological concentrations:[7] for-Met-Ser-Phe-OMe and for-Met-Lys-Phe-OMe show to be strengthened, in potency as well as in efficacy, by the crosslinked fMLP. In this case, the receptor isoform responsible for granule enzyme release is the least exacting in triggering the biological response.

We can conclude by suggesting that (i) the chemotactic response of these new di-tripeptides depends on the nature of the second residue carrying the functional group to which the fMLP is crosslinked; in any case it never exceeds that of fMLP-OMe alone; (ii) superoxide anion production and secretagogue activity benefit from the presence of the crosslinked tripeptide, particularly if sterically hindered.

These di-tripeptides could represent a basis of study to find fMLP analogues that efficaciously and preferentially stimulate killing mechanisms of human neutrophils.

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 di-tripeptides linked by spacers of varying length, in order to stimulate different receptors simultaneously.

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