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A formyl peptide substituted with a conformationally constrained phenylalanine residue evokes a selective immune response in human neutrophils

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ABSTRACT

Formyl-Met-Leu-Phe-OH (fMLP) binds to formyl peptide receptors, FPR1 and FPR2, and evokes migration and superoxide anion production in human neutrophils. To obtain a more effective and selective ligand, fMLP analogs in which the Phe residue was substituted with four isomers of cyclopropanephenylalanine were synthesized. While *Z*-isomer peptides induced both migration and superoxide anion production, *E*-isomer peptides elicited only chemotaxis. Homologous receptor desensitization experiments revealed that *E*-isomer peptides bound to FPR2. Although a selective agonist of chemotaxis also binds to FPR2 without increasing intracellular calcium concentration, *E*-isomer peptide elevated the concentration to the same level as fMLP. Understanding of mechanisms responsible for the selectivity of the reported selective agonists and ∇Phe-substituted analogs should prove useful for revealing the relationship between receptor–ligand interactions and biological responses of human neutrophils.

1. Introduction

Human neutrophils represent 50–60% of the total leukocytes in circulation, and are highly specialized for their primary function, that is, phagocytosis and destruction of microorganisms through generation of superoxide anion and release of protease-containing granules. Leukocyte recruitment to the sites of inflammation and infection is dependent upon the presence of a gradient of locally produced chemotactic factors. The bacterial peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) is one such chemotactic factor and is a highly potent leukocyte chemoattractant. It interacts with receptors on the membranes of neutrophils, activating these cells through G-protein-coupled pathways. Two functional fMLP receptors in human neutrophils are characterized, namely formyl peptide receptor (FPR1) and its subtype (FPR2) with high and low affinities for fMLP, respectively.[1]

The tripeptide fMLP and its methyl ester analog fMLP-OMe are generally adopted as the reference models for evaluating the activity of newly synthesized analogs. While the binding of the formyl peptides to the receptors causes multiple immune responses such as superoxide production and chemotaxis, some agonists elicit a selective response. For example, Torrini et al. identified formyl-Thp-Leu-Ain-OMe ([Thp[1],Ain[3]]fMLP-OMe, Fig. S2), characterized by the presence of an unusual α-amino acid in which the native Met and Phe external residues were substituted with 4-amino-tetrahydrothiopyran-4-carboxylic acid (Thp) and 2-aminoindane-2-carboxylic acid (Ain), as a selective agonist which only induces the chemotactic response in human neutrophils.[2,3] As another example of a selective agonist, Pagani Zecchini et al. found that formyl-Met-Δ^{Z}Leu-Phe-OMe ([Δ^{Z}Leu[2]]fMLP-OMe, Fig. S2), in which the central Leu residue was replaced by (*Z*)-2,3-dihydroleucine (Δ^{Z}Leu), was only able to elicit superoxide anion production and degranulation.[4] These results seem to indicate that incorporation of an amino acid surrogate with conformationally constrained side chains is crucial for the design of highly selective and potent peptide analogs with enhanced properties and has provided valuable insights into how peptides interact with their receptors.[5]

Amino acids containing a cyclopropane skeleton are found in a wide class of naturally occurring products, and are of interest for peptide design because of their steric hindrance due to the α,α-disubstitution. The presence of the tensioned three-membered

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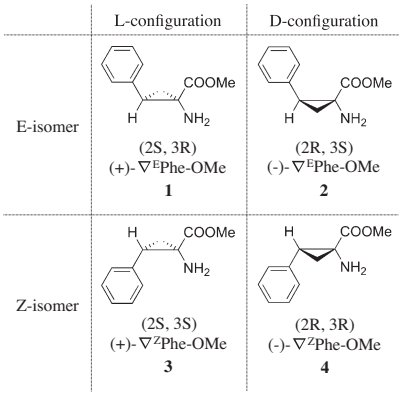


Figure 1. Chemical structures of cyclopropane phenylalanine methyl esters (∇Phe-OMe).[52,53]

ring introduces severe constrains in the proximal backbone torsion angles. Cyclopropane phenylalanine (∇Phe, Fig. 1) has been the most widely studied. For example, all four stereoisomers were incorporated into enkepharin[1–3,6–9] and aspartame.[4,10] In addition, some peptides containing the ∇Phe residue were proven to be much more resistant to enzymatic degradation by chymotrypsin.[5,11] In the present study, to obtain a more effective and selective ligand, fMLP analogs in which the Phe residue was substituted with four stereoisomers of ∇Phe were synthesized (Table 1), and the effects of the peptides on chemotaxis and superoxide anion production in human neutrophils were evaluated.

2. Results and discussion

2.1. Synthesis

Racemic ∇^{E}Phe-OMe and ∇^{Z}Phe-OMe were synthesized by a diazoaddition method[12] and malonate method,[13,14] respectively. The *E*-isomers (**1** and **2**) and the *Z*-isomers (**3** and **4**) were resolved by formation of diastereomeric salts with brucine[15] and *O*,*O*-dibenzoyltartaric acid,[10] respectively (Fig. 1). The optical purities of the stereoisomers were evaluated by comparison of reported $[\alpha]_D$ values.[16] The synthetic route and details are described in the Supporting Information.

Peptide synthesis was performed by a conventional solution phase method (Scheme 1). The coupling of Boc-Leu-OH with ∇Phe-OMe was achieved by a mixture anhydride (MA) method to give dipeptide **9**. After the Boc group was removed by the treatment of trifluoroacetic acid (TFA), formyl-Met-OH was introduced to dipeptide **9** utilizing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as a coupling reagent. Peptides **5**, **6**, **7** and **8** were purified by silica gel chromatography.

Table 1
Structures of fMLP-OMe and its derivatives containing cyclopropane phenylalanine (∇Phe)

Peptide	Sequence
fMLP-OMe	Formyl-Met-Leu-Phe-OMe
5	Formyl-Met-Leu-(+)-∇^{E}Phe-OMe
6	Formyl-Met-Leu-(−)-∇^{E}Phe-OMe
7	Formyl-Met-Leu-(+)-∇^{Z}Phe-OMe
8	Formyl-Met-Leu-(−)-∇^{Z}Phe-OMe

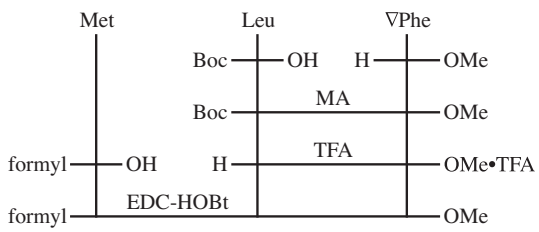
2.2. ∇^{E}Phe-containing fMLP analogs only evoked chemotaxis in human neutrophils

The biological activities of synthetic peptides were evaluated in human neutrophils. fMLP was used as a control peptide. Figure 2A shows the concentration dependence of chemotaxis in human neutrophils toward ∇Phe-containing synthetic peptides **5**–**8** and fMLP-OMe. The chemotactic activity EC_{50} value of fMLP-OMe was 2.5 ± 0.1 nM (Table 2). The other ∇Phe-containing peptides also showed chemotactic activity in human neutrophils but they were about 2- to 8-fold lower as compared with fMLP (Fig. 2A and Table 2). The chemotactic activities of peptides **6** and **8**, in which the side chains are D-configuration, were approximately 3- and 2-fold higher than that of the L-configuration peptides **5** and **7**, respectively.

Superoxide anion production in human neutrophils stimulated by ∇Phe-containing peptides is shown in Figure 2B. fMLP-OMe induced superoxide anion production with an EC_{50} value of 16 ± 0.2 nM (Table 2). In contrast to chemotactic response, only peptide **8**, which contains the (−)-Z isomer, evoked superoxide anion production with an EC_{50} of 250 ± 80 nM. Peptide **7** containing the (+)-Z isomer showed only 45% activity relative to fMLP at 1.0 μM. On the other hand, *E*-isomer analogs **5** and **6** did not show definite activity although the peptides induced migration of human neutrophils. Thus, peptide **5** and **6** seemed to be selective agonists of chemotaxis in human neutrophils.

2.3. Both *E*-isomer and *Z*-isomer analogs induced intracellular calcium mobilization in human neutrophils

[Thp^{1}, Ain^{3}]fMLP-OMe (Fig. S2), a selective agonist for chemotaxis,[2] did not increase cytoplasmic calcium concentration,[3,17] whereas [Δ^{Z}Leu^{2}]fMLP-OMe (Fig. S2), which evoked superoxide production and degranulation,[4] increased the concentration.[3,17,18] In addition, superoxide anion production by the addition of [Δ^{Z}Leu^{2}]fMLP-OMe was reduced in the presence of a calcium ion chelator.[3] Likewise, superoxide anion production by human neutrophils stimulated by fMLP-OMe was also affected by the chelator. These results suggested that an increase of cytoplasmic calcium concentration is required for NADPH oxidase in human neutrophils. In contrast, substance P, transforming growth factor β1, and the murine S100 protein, only induced chemotactic responses and did not influence calcium concentration.[19,20] These results suggested that intracellular calcium mobilization could be a good indicator for investigating the mechanisms of selective agonists. Figure 3 shows the maximum concentrations of intracellular calcium ion induced by the addition of fMLP-OMe and ∇Phe-containing peptides **5**–**8**. The cytoplasmic calcium concentrations induced by the stimulation of ∇Phe-containing peptides **5**–**8** were similar to that of fMLP-OMe although the biological activities of peptides **5**–**8** were lower than fMLP-OMe. Previous studies of receptor occupancy–response relationships demonstrated that whereas almost 100% of the receptors needed to be occupied to achieve maximal superoxide anion production, less than 1% receptor occupancy was required to generate maximal actin polymerization,[21,22] which represents an essential facet of neutrophil migration.[23] If the selectivity of the *E*-isomer analogs **5** and **6** was due to their low binding affinities to the receptors as compared with the affinities of the *Z*-isomer analogs **7** and **8**, the calcium concentrations induced by peptides **5** and **6** may be lower than that of fMLP-OMe and peptides **7** and **8**. WKYMVm binds to both FPR1 and FPR2, and elevated intracellular calcium concentration, leading to chemotaxis, and endocytosis. In contrast, WKXMVm (X = G or R) binds to only FPR2, and evoked chemotaxis, while cytoplasmic calcium concentration was not increased.[24] Although the mechanisms responsible for the selectivity of *E*-isomer peptides are unclear, it is possible that peptides **5** and **6** might bind to a specific receptor, FPR1 or FPR2.



Isomer of ∇Phe			
E-isomer		Z-isomer	
(+)	(-)	(+)	(-)
9a	**9b**	**9c**	**9d**
5	**6**	**7**	**8**

Scheme 1. Synthesis of ∇Phe-containing peptides **5**–**8**.

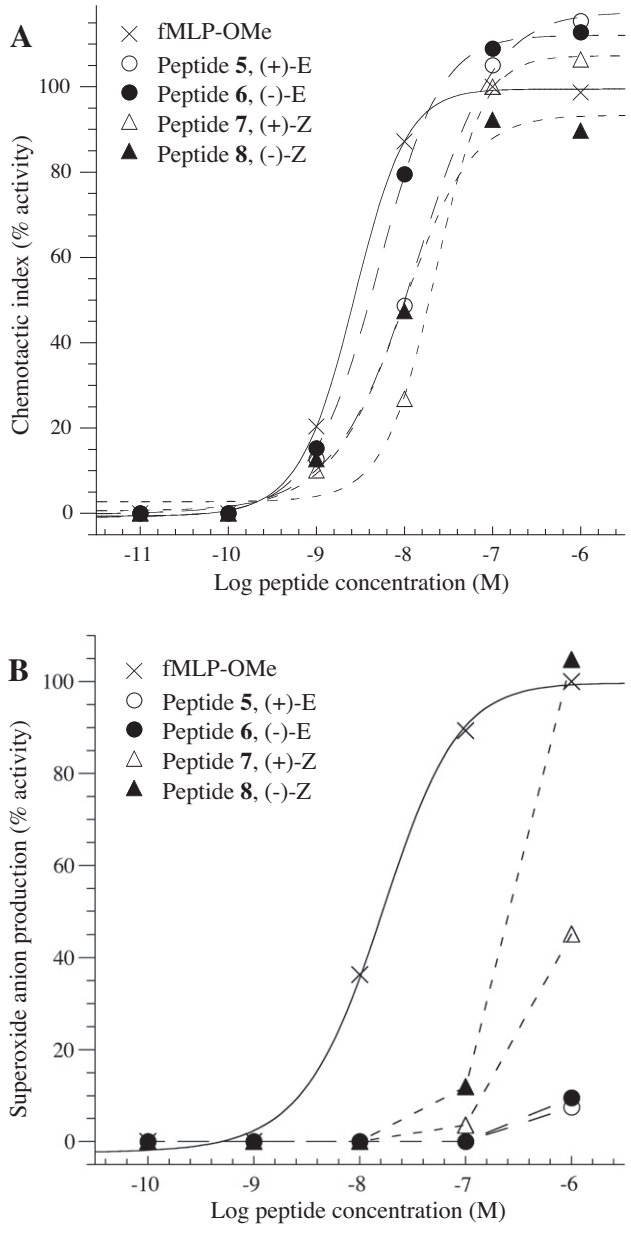


Figure 2. Chemotaxis (A) and superoxide production (B) of human neutrophils stimulated by fMLP-OMe and its derivatives (cross, fMLP; open circle, peptide **5**; closed circle, peptide **6**; open triangle, peptide **7**; closed triangle, peptide **8**). The data expressed are the means of five separate experiments performed in triplicate.

2.4. Human neutrophils were primed by *Z*-isomer peptides, but not *E*-isomer peptides

Exposure of human neutrophils to fMLP-OMe at concentrations insufficient to cause superoxide anion production nevertheless primes these cells for exaggerated superoxide anion production after subsequent addition of an agonist,[25–27] a process known as neutrophil priming.[28–30] Treatment with calcium ion ionophores such as A23187 and ionomycin that do not bind to specific receptors also causes neutrophil priming, suggesting that an increase of cytoplasmic calcium ion leads to pre-activation of human neutrophils. ∇Phe-containing fMLP-OMe analogs **5**–**8** increased intracellular calcium concentration (Fig. 3) while only *Z*-isomers **7** and **8** initiated superoxide anion production in human neutrophils. Thus, we thought that examination of the neutrophil priming activity of the synthetic peptides could provide insight into the mechanisms of the selective agonists. Figure 4 and Table 2 indicate the priming effects of the synthetic peptides. Interestingly, enhanced superoxide anion production was observed only when the cells were pretreated with *Z*-isomer peptides **7** and **8**. These results suggested that *Z*-isomer peptides **7** and **8** might have high binding affinity to a specific receptor that functions in superoxide anion production in human neutrophils.

2.5. *E*-Isomer peptide binds to FPR2

When neutrophils encounter increasing concentration of a chemoattractant, they gradually become non-responsive to further stimulation by the same agonist.[31] This process, known as homologous desensitization, is due to a decreased affinity of receptors for G-proteins by association with arrestin family and internalization.[32] Thus, we performed receptor desensitization experiments to investigate ∇Phe-containing peptides-binding receptors.[33–39] To measure such homologous desensitization in the case of FPRs, we recorded intracellular calcium mobilization in response to fMLP-OMe and ∇Phe-containing peptides. The peptide concentrations required for homologous receptor desensitization was determined (Fig. S3). As shown in Figure 5A and 5B, no significant second rise of intracellular calcium concentration was observed by stimulation with *E*- and *Z*-isomer peptide. These results indicate that both peptides bind to FPRs. Similarly, intracellular calcium concentration did not increased by addition of *E*-isomer peptide in human neutrophils pre-stimulated with *Z*-isomer peptide (Fig. 5D). Conversely, the second stimulation with *Z*-peptide raised intracellular calcium concentration in human neutrophils pre-activated with *E*-isomer peptide (Fig. 5C). These results suggested that *E*-isomer peptide bound to either FPR1 or FPR2 while *Z*-isomer peptide interacted with both receptors. To verify the receptor interacting with *E*-isomer peptide, desensitization experiments were carried out utilizing the FPR2 selective agonist, MMK-1.[35] The desensitization of FPR2 by MMK-1 inhibited *E*-isomer peptide-induced intracellular calcium mobilization (Fig. 6A) although the mobilization elicited by *Z*-isomer peptide was observed (Fig. 6B). In addition, repression of an increase in the concentration evoked by MMK-1 was observed in human neutrophils pretreated with *E*- and *Z*-isomer peptide (Fig. 6C and 6D). These results strongly suggest that *E*-isomer peptide associates with FPR2 while *Z*-isomer peptide binds to both FPR1 and FPR2.

Table 2
Biological activities of fMLP-OMe and ∇Phe-containing peptides

Peptide	Isomer of ∇Phe	Chemotaxis		O_2^- Production		Priming activity[a] (%)
		EC_{50} (nM)	Relative activity (%)	EC_{50} (nM)	Relative activity (%)	
fMLP-OMe		2.5 ± 0.1	100	16 ± 2	100	
5	(+)-E	14 ± 2	18	10%[b]	—	114
6	(−)-E	4.7 ± 0.2	53	10%[b]	—	114
7	(+)-Z	21 ± 6	12	10%[b]	—	175
8	(−)-Z	9.1 ± 2.4	26	250 ± 80	6.4	200

[a] Human neutrophils were preincubated with PBS or 0.1 μM of Phe-containing peptides for 5 min at 37 °C, followed by stimulated with 0.1 μM of fMLP-OMe. The priming activity was estimated by comparison with superoxide anion production in human neutrophils pretreated with PBS.
[b] The mean activity at 1.0 μM.

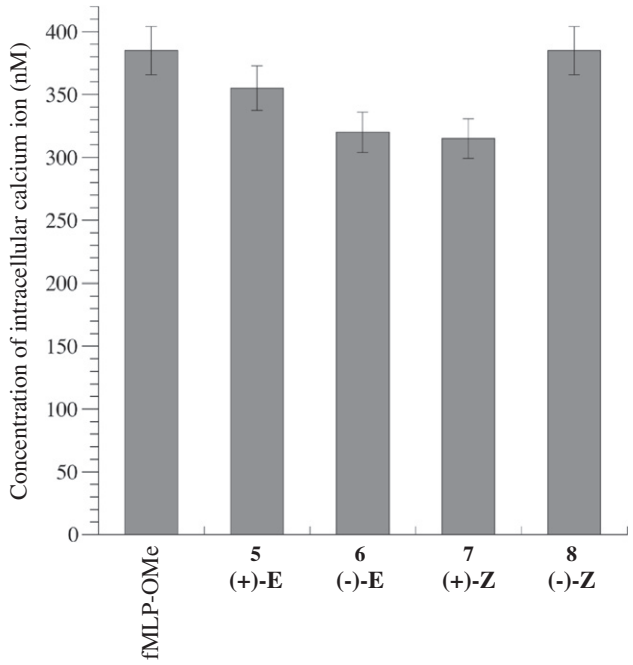


Figure 3. Maximum concentrations of intracellular calcium ion in human neutrophils stimulated by fMLP-OMe and its derivatives (0.10 μM). The basal level of intracellular calcium concentration was approximately 140 nM. The data expressed are the means of five separate experiments performed in triplicate.

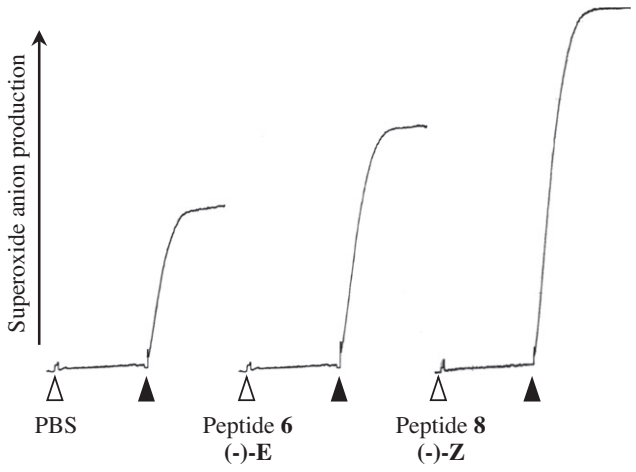


Figure 4. Representative enhanced superoxide anion production in human neutrophils pre-incubated with ∇Phe-containing fMLP-OMe analog. Human neutrophils were pre-incubated with PBS, peptide **6** or peptide **8** (0.1 μM) for 5 min at 37 °C (open arrowhead) before stimulation by fMLP-OMe (1.0 μM) (closed arrowhead).

Jesaitis and co-worker investigated the binding site of fMLP in FPR1 using receptor mutants[40,41] and a photoaffinity labeled fMLP peptide.[42] They proposed that (1) the positioning of fMLP in the binding pocket of FPR1 was parallel to the fifth transmembrane (TM) helix, (2) the formyl group formed hydrogen bonds to Asp106 and Arg 201, (3) the carboxyl group made an ion-pair with Arg205, (4) Leu side chain was positioned around residues 83–85 (VRK), which is near the extracellular surface of the second TM helix. The importance of Arg84 and Lys85 for the interaction between fMLP and FPR1 was also reported by Radel et al.[43] and Lala et al.[44] The molecular modeling of FPR1-the photoaffinity labeled peptide complex suggested that the Phe side chain bound to the pocket consisting of the third, the fifth, the sixth, and the seventh TM helices of FPR1. We showed that FPR1 coupled with only *Z*-isomer peptide while FPR2 recognized both *E*- and *Z*-isomer peptides. Thus, if the ∇Phe also interacts with the pocket recognizing the Phe of fMLP, the pocket of FPR1 might be smaller than that of FPR2.

3. Conclusions

Herein, we found that fMLP-OMe analogs in which the Phe residue was substituted with the conformationally constrained amino acid, cyclopropane phenylalanine (∇Phe, Fig. 1), elicited a selective biological response in human neutrophils. Among the four different isomers of ∇Phe studied (Fig. 1), the analogs replaced by *E*-isomer ∇Phe (peptides **5** and **6**) showed only chemotactic activity while the *Z*-isomer ∇Phe-containing peptides **7** and **8** evoked both migration and superoxide anion production (Fig. 2 and Table 2). Homologous receptor desensitization experiments revealed that *E*-isomer peptide interacted with FPR2 while *Z*-isomer peptide bound to both FPR1 and FPR2. Some agonists such as [Thp^1,Ain^3]fMLP-OMe and [$\Delta^Z Leu^2$]fMLP-OMe (Fig. S2), are known to specifically evoke biological responses in human neutrophils, that is, [Thp^1,Ain^3]fMLP-OMe and [$\Delta^Z Leu^2$]fMLP-OMe are selective agonists of migration and superoxide anion production, respectively.[45] Interestingly, while pure chemoattractant [Thp^1,Ain^3]fMLP-OMe does not affect the intracellular calcium concentration, peptides **5** and **6** caused intracellular calcium mobilization as well as fMLP-OMe and *Z* - isomer peptides **7** and **8**. Fabbri et al. determined the binding affinities of fMLP-OMe, [Thp^1,Ain^3]fMLP-OMe, and [$\Delta^Z Leu^2$]fMLP-OMe utilizing a competition assay with [^{3}H]-fMLP in human neutrophils.[46] The binding affinity of [Thp^1,Ain^3]fMLP-OMe was about 50- and 600-fold lower than that of [$\Delta^Z Leu^2$]fMLP-OMe and fMLP-OMe, respectively. These results seem to suggest that the selectivity of [Thp^1,Ain^3]fMLP-OMe was due to its significant low binding affinity to receptors because less than 1% occupancy of FPRs was suggested to be required for migration in human neutrophils. In contrast to [Thp^1,Ain^3]fMLP-OMe, despite the fact that intracellular calcium mobilization seems to be important for superoxide anion production in human neutrophils, *E*-isomer peptides **5** and **6** were selective agonists of migration while increasing intracellular calcium concentrations. Understanding of mechanisms responsible

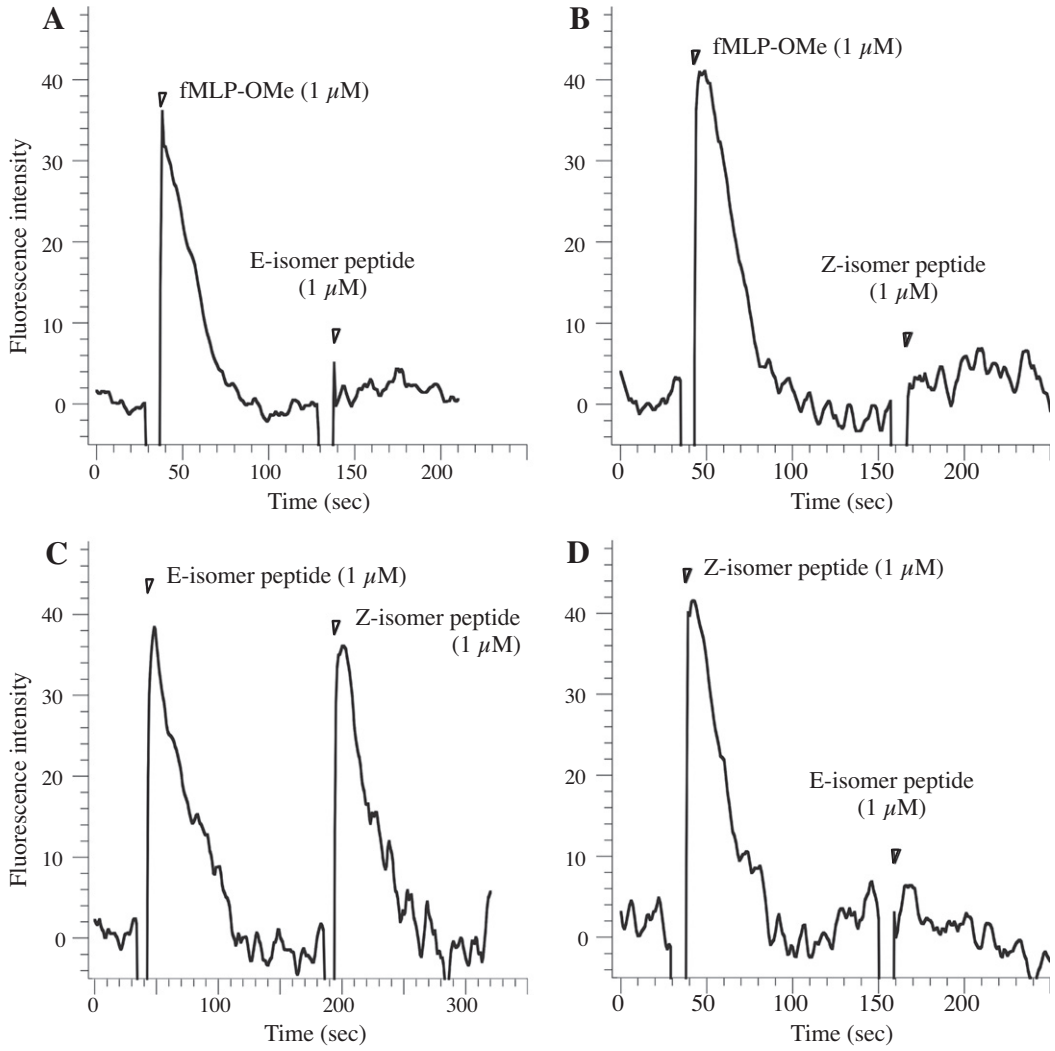


Figure 5. Desensitization of intracellular calcium mobilization in human neutrophils triggered with fMLP-OMe, *E*-isomer peptide (the equal quantity mixture of peptides **5** and **6**), and *Z*-isomer peptide (that of peptides **7** and **8**) at the concentration indicated. Cells were stimulated with fMLP-OMe, followed by the addition of *E*- (A) and *Z*-isomer peptide (B). Cells re-stimulated with *Z*- (C) and *E*-isomer peptide (D) after activation with *E*- and *Z*-isomer peptide, respectively. Curves show representative experiments. Open triangles indicate the time point of the agonist addition.

for the selectivity of the reported selective agonists and ∇Phe-substituted analogs should prove useful for revealing the relationship between receptor–ligand interactions and biological responses of human neutrophils.

4. Experimental

4.1. Materials and methods

Amino acids, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrogen chloride (EDC·HCl), and *N*-hydroxybenzotriazole (HOBt) were purchased from the Peptide Institute (Osaka, Japan). Other chemicals for organic synthesis were obtained from Wako Chemicals (Osaka, Japan) or Katayama Chemicals (Osaka, Japan), and used without further purification. ^{1}H NMR spectra were recorded with a JEOL-JMN GX 270 spectrophotometer (JEOL, Tokyo) using tetramethylsilane as an internal standard, and signals were assigned by 2D-correlation spectroscopy. Description of cyclopropane protons is defined in Figure S1 Supplementary data. Optical rotations were recorded with JEOL-JMS 300 (JEOL, Tokyo) at 25 °C. Thin-layer chromatography (TLC) was performed on silica gel GF254 (Merck) with the following solvent systems (v/v); R_f^2, chloroform–methanol (9:1); R_f^4, chloroform–methanol–acetic acid (95:5:1). Components on a TLC plate were colorized by carbonization with sulfuric acid, *o*-tolidine, iodine, and ninhydrin.

4.2. Peptide synthesis

4.2.1. General procedure of Boc-Leu-∇Phe-OMe (9)

A solution of Boc-Leu-OH (72 mg, 0.30 mmol) and *N*-methylmorpholine (33.6 μl, 0.30 mmol) in THF (1.0 ml) was cooled to −15 °C. Isobutylchloroformate (40 μl, 0.30 mmol) was added to the solution slowly, and then the mixture was stirred for 10 min at −15 °C. A solution of ∇Phe-OMe salt (0.30 mmol) and triethylamine (42 μl, 0.30 mmol) in dichloromethane (3.0 ml) was added to the anhydride mixture. The reaction was stirred for 15 min at −15 °C, and then at room temperature for 5 h. After removing the solvent using a rotary evaporator, the residue obtained was dissolved in ethyl acetate (10 ml). The organic layer was washed successively with water, 0.5 M sodium bicarbonate aqueous solution, and 5% potassium hydrogen sulfate aqueous solution. The solution was dried over anhydrous sodium sulfate. The solvent was evaporated, and the residue obtained was crystallized by the addition of petroleum ether to give a white solid.

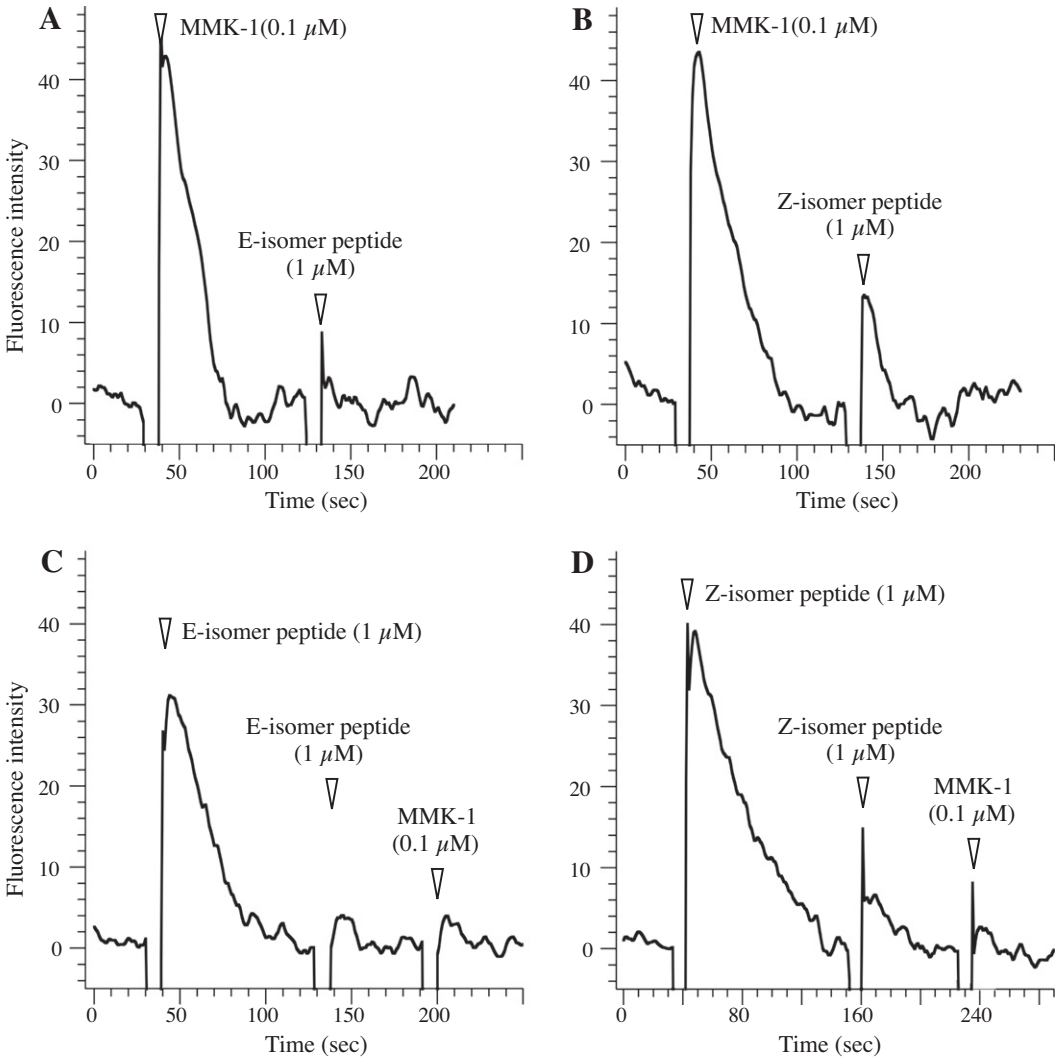


Figure 6. Simultaneous desensitization of FPR2 by MMK-1 inhibited *E*-isomer peptide-induced intracellular calcium mobilization. The intracellular calcium mobilization elicited by *E*- (A) and *Z*-isomer peptide (B) in human neutrophils pre-stimulated with the FPR2 selective agonist MMK-1. Cells were pretreated with *E*- (C) and *Z*-isomer peptide (D) before stimulation with MMK-1. Curves show representative experiments. Open triangles indicate the time point of the agonist addition.

Boc-Leu-(+)-∇^{E}Phe-OMe (**9a**) yield, 72 mg (60%). $[\alpha]_D$ +22.0°, (*c* 0.20, methanol). TLC R_f^2 = 0.79, TLC R_f^4 = 0.610.44. ^{1}H NMR ($CDCl_3$); δ = 7.41–7.18 (5H, m, aromatic protons), 6.87 (1H, s, NH of (+)-∇^{E}Phe), 4.87 (1H, d, NH of Leu), 4.12 (1H, m, α-proton of Leu), 3.33 (3H, s, methyl ester), 2.86 (1H, dd, H_x), 2.21 (1H, m,H_a), 1.59 (1H, m, H_b), 1.57 (9H, s, Boc group), 1.49 (1H, m, γ-protons of Leu), 1.73 (2H, m, β-protons of Leu), 0.97 (6H, dd, δ-protons of Leu). Found; C, 64.88; H, 7.93; N, 6.87. Calcd for $C_{22}H_{32}N_2O_5$; C, 65.32; H, 7.79; N, 6.93.

Boc-Leu-(−)-∇^{E}Phe-OMe (**9b**) yield, 82 mg (68%). $[\alpha]_D$ −35.3°, (*c* 0.20, methanol). TLC R_f^2 = 0.87, TLC R_f^4 = 0.610.61. ^{1}H NMR ($CDCl_3$); δ = 7.39–7.21 (5H, m, aromatic protons), 6.91 (1H, s, NH of (−)-∇^{E}Phe), 4.93 (1H, d, NH of Leu), 4.14 (1H, m, α-proton of Leu), 3.33 (3H, s, methyl ester), 2.83 (1H, dd, H_x), 2.23 (1H, m,H_a), 1.60 (1H, m, H_b), 1.61 (9H, s, Boc group), 1.46 (1H, m, γ-protons of Leu), 1.73 (2H, m, β-protons of Leu), 0.97 (6H, dd, δ-protons of Leu). Found; C, 64.82; H, 7.97; N, 6.93. Calcd for $C_{22}H_{32}N_2O_5$; C, 65.32; H, 7.79; N, 6.93.

Boc-Leu-(+)-∇^{Z}Phe-OMe (**9c**) yield, 75 mg (62%). $[\alpha]_D$ +54.5°, (*c* 0.20, methanol). TLC R_f^2 = 0.75, TLC R_f^4 = 0.610.40. ^{1}H NMR ($CDCl_3$); δ = 7.35–7.13 (5H, m, aromatic protons), 5.95 (1H, s, NH of (+)-∇^{Z}Phe), 4.67 (1H, d, NH of Leu), 3.85 (1H, m, α-proton of Leu), 3.73 (3H, s, methyl ester), 3.02 (1H, dd, H_x), 2.13 (1H, m,H_a), 1.72 (1H, m, H_b), 1.57 (9H, s, Boc group), 1.46 (1H, m, γ-protons of Leu), 1.24 (2H, m, β-protons of Leu), 0.82 (6H, dd, δ-protons of Leu). Found; C, 65.28; H, 7.89; N, 6.93. Calcd for $C_{22}H_{32}N_2O_5$; C, 65.32; H, 7.79; N, 6.93.

Boc-Leu-(−)-∇^{Z}Phe-OMe (**9d**) yield, 85 mg (70%). $[\alpha]_D$ −88.5°, (*c* 0.20, methanol). TLC R_f^2 = 0.77, TLC R_f^4 = 0.610.42. ^{1}H NMR ($CDCl_3$); δ = 7.35–7.14 (5H, m, aromatic protons), 5.98 (1H, s, NH of (−)-∇^{Z}Phe), 4.68 (1H, d, NH of Leu), 3.90 (1H, m, α-proton of Leu), 3.73 (3H, s, methyl ester), 2.99 (1H, dd, H_x), 2.18 (1H, m,H_a), 1.69 (1H, m, H_b), 1.57 (9H, s, Boc group), 1.47 (1H, m, γ-protons of Leu), 1.25 (2H, m, β-protons of Leu), 0.80 (6H, dd, δ-protons of Leu). Found; C, 65.31; H, 7.96; N, 6.90. Calcd for $C_{22}H_{32}N_2O_5$; C, 65.32; H, 7.79; N, 6.93.

4.2.2. General procedure of preparation of formyl-Met-Leu-∇Phe-OMe (5, 6, 7, and 8)

Boc-protecting dipeptide **9** (81 mg, 0.20 mmol) was treated with trifluoroacetic acid (1.0 ml) for 30 min in an ice-bath. Trifluoroacetic acid was evaporated using nitrogen gas flashing. Diethyl ether was added to the residue obtained, resulting in white solid. The solid was dissolved in dimethylformamide (0.50 ml), and then

triethylamine (70 μl, 0.50 mmol) was added to the solution. Formyl-Met-OH (35 mg, 0.20 mmol) and HOBt (32 mg, 0.24 mmol) was added to the solution. After the mixture was cooled to 0 °C, EDC·HCl (42 mg, 0.22 mmol) was added. The mixture was stirred for 2 h in an ice-bath, and then for 10 h at room temperature. The solvent was removed using a rotary evaporator in vacuo. The residue obtained was dissolved in ethyl acetate (15 ml), and the organic layer was washed with water, 0.5 M sodium bicarbonate aqueous solution, 5% potassium hydrogen sulfate, and brine. After the solution was dried over anhydrous sodium sulfate, the solvent was evaporated. Diethyl ether was added to the residue obtained, resulting in white solid as the formyl peptide.

Formyl-Met-Leu-(+)-∇^{E}Phe-OMe (**5**) yield, 72 mg (77%). $[\alpha]_D$ +15.6°, (*c* 0.50, methanol). TLC R_f^2 = 0.42, TLC R_f^4 = 0.610.46. ^{1}H NMR (DMSO-d_6); δ = 8.78 (1H, s, NH of (+)-∇^{E}Phe), 8.27 (1H, d, NH of Met), 8.05 (1H, d, NH of Leu), 8.03 (1H, s, formyl group), 7.28–7.20 (5H, m, aromatic protons), 4.44 (1H, m, α-proton of Met), 4.31 (1H, m, α-proton of Leu), 3.23 (3H, s, methyl ester), 2.76 (1H, dd, H_x), 2.44 (2H, t, γ-protons of Met), 2.09 (1H, m,H_a), 2.04 (3H, s, methyl protons of Met), 1.86 (2H, m, β-protons of Met), 1.64 (1H, m, γ-protons of Leu), 1.50 (2H, m, β-protons of Leu), 1.36 (1H, m, H_b), 0.90 (6H, dd, δ-protons of Leu), Found; C, 59.57; H, 7.11; N, 9.07. Calcd for $C_{23}H_{33}N_3O_5S$; C, 59.59; H, 7.18; N, 9.07.

Formyl-Met-Leu-(−)-∇^{E}Phe-OMe (**6**) yield, 72 mg (77%). $[\alpha]_D$ −116.8°, (*c* 0.50, methanol). TLC R_f^2 = 0.43, TLC R_f^4 = 0.610.48. ^{1}H NMR (DMSO-d_6); δ = 8.79 (1H, s, NH of (−)-∇^{E}Phe), 8.05 (1H, d, NH of Met), 8.27 (1H, d, NH of Leu), 7.99 (1H, s, formyl group), 7.32–7.17 (5H, m, aromatic protons), 4.44 (1H, m, α-proton of Met), 4.31 (1H, m, α-proton of Leu), 3.23 (3H, s, methyl ester), 2.72 (1H, dd, H_x), 2.43 (2H, t, γ-protons of Met), 2.07 (1H, m,H_a), 2.02 (3H, s, methyl protons of Met), 1.82 (2H, m, β-protons of Met), 1.61 (1H, m, γ-protons of Leu), 1.50 (2H, m, β-protons of Leu), 1.37 (1H, m, H_b), 0.89 (6H, dd, δ-protons of Leu), Found; C, 59.41; H, 7.19; N, 9.00. Calcd for $C_{23}H_{33}N_3O_5S$; C, 59.59; H, 7.18; N, 9.07.

Formyl-Met-Leu-(+)-∇^{Z}Phe-OMe (**7**) yield, 70 mg (76%). $[\alpha]_D$ +26.4°, (*c* 0.50, methanol). TLC R_f^2 = 0.47, TLC R_f^4 = 0.610.51. ^{1}H NMR (DMSO-d_6); δ = 8.13 (1H, s, NH of (+)-∇^{Z}Phe), 7.70 (1H, d, NH of Met), 8.18 (1H, d, NH of Leu), 7.99 (1H, s, formyl group), 7.25–7.12 (5H, m, aromatic protons), 4.38–4.32 (1H, m, α-proton of Met), 4.24–4.15 (1H, m, α-proton of Leu), 3.62 (3H, s, methyl ester), 3.17 (1H, dd, H_x), 2.35 (2H, t, γ-protons of Met), 2.03 (3H, s, methyl protons of Met), 1.81–1.59 (4H, m, β-protons of Met, H_a, H_b), 1.45 (1H, m, γ-protons of Leu), 1.21 (2H, m, β-protons of Leu), 0.77(6H, dd, δ-protons of Leu), Found; C, 59.19; H, 7.13; N, 8.80. Calcd for $C_{23}H_{33}N_3O_5S$; C, 59.59; H, 7.18; N, 9.07.

Formyl-Met-Leu-(−)-∇^{Z}Phe-OMe (**8**) yield, 70 mg (76%). $[\alpha]_D$ −111.0°, (*c* 0.50, methanol). TLC R_f^2 = 0.46, TLC R_f^4 = 0.610.52. ^{1}H NMR (DMSO-d_6); δ = 8.29 (1H, s, NH of (−)-∇^{Z}Phe), 7.80 (1H, d, NH of Met), 8.14 (1H, d, NH of Leu), 8.00 (1H, s, formyl group), 7.26–7.14 (5H, m, aromatic protons), 4.39 (1H, m, α-proton of Met), 4.16 (1H, m, α-proton of Leu), 3.61 (3H, s, methyl ester), 3.01 (1H, dd, H_x), 2.40 (2H, t, γ-protons of Met), 2.03 (3H, s, methyl protons of Met), 1.87–1.65 (4H, m, β-protons of Met, H_a, H_b), 1.19 (1H, m, γ-protons of Leu), 0.99–0.78 (2H, m, β-protons of Leu), 0.69 (6H, dd, δ-protons of Leu), Found; C, 59.50; H, 7.14; N, 9.03. Calcd for $C_{23}H_{33}N_3O_5S$; C, 59.59; H, 7.18; N, 9.07.

4.3. Isolation of human neutrophils

Human neutrophils were isolated from heparinized venous blood obtained from healthy volunteers. Standard isolation techniques involving Ficoll/Hypaque gradients were used,[47] followed by dextran sedimentation and hypotonic lysis to remove erythrocytes. Cells were suspended in PBS without calcium chloride.

4.4. Chemotaxis

The chemotactic assay was performed in a micro chemotaxis chamber.[48] Cells were suspended in Krebs–Ringer phosphate buffer containing 1 mM $CaCl_2$, 5 mM D-glucose, and 0.1% bovine serum albumin, at a concentration of 1.0×10^6 cells/ml. Peptide solutions or buffer were placed in the lower wells. The polyvinylpyrrolidone-free polycarbonate filter was assembled and incubation was carried out at 37 °C for 60 min in humidified air.[49] After incubation, the filter was stained with Diff-Quick (Kokusai Siyaku, Kobe) solution and counted at 1000× magnification for 5 fields. The chemotactic index was estimated as the migrated cell number. Data are reported as the mean value ± standard error of three independent experiments.

4.5. Superoxide anion production

Superoxide anion production was measured as superoxide dismutase inhibitable reduction of ferricytochrome c.[50,51] The reduction of the ferricytochrome c concentration was monitored with a Shimadzu UV-3000 dual-wavelength spectrophotometer (Kyoto, Japan) at 540–550 nM. A cell suspension (1×10^6 cells/ml), containing 1 mM $CaCl_2$, 5 mM D-glucose, and 100 mM ferricytochrome c was preincubated at 37 °C for 5 min before the addition of various concentrations of peptides. The superoxide anion release was calculated based on a molar absorption coefficient of 19.1×10^3 M^{-1} cm^{-1}. Data are reported as the mean value ± standard error for at least three independent experiments.

4.6. Intracellular calcium mobilization

Intracellular calcium mobilization was measured by the Fura-2 method.[50,51] Cells (2×10^6 cells/ml) were incubated with 4 mM Fura-2 AM at 37 °C for 30 min, washed twice with PBS, and then suspended in PBS containing 1 mM $CaCl_2$ and 5 mM D-glucose. Changes in the fluorescence emission intensity at 490 nM (excitations at 340 and 380 nM) were monitored with a Shimadzu RF-5000 spectrofluorophotometer. The cells were lysed by the addition of Triton X-100 for the determination of the maximum fluorescence, and the minimum fluorescence was then determined by addition of ethylene glycol tetraacetic acid. The concentration of intracellular calcium concentration was calculated using a K_d value of 224 nM for calcium ion. Data are reported as the mean value ± standard error for three independent experiments.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.11.046.

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