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Short communication

Characterization of chemotactic ability of peptides containing *N*-formyl-methionyl residues in *Tetrahymena* fMLP as a targeting ligand

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Abstract

The chemotactic effects of six formylated, putatively bacterial peptides (fMLP, fMLPP, fMMM, fMP, fMV, and fMS) were studied. From the set of six peptides, only fMLP (one of the most effective chemoattractant peptides in mammals) elicited a significant positive chemotactic response in the eukaryotic ciliate *Tetrahymena pyriformis*, while the other formylated ligands, e.g. fMMM (which is also effective in mammals), had neutral or antagonistic effects in *Tetrahymena*. A study of their amino acid sequences points to an, as yet obscure, interaction between C-terminal f-Met and N-terminal aromatic Phe. Some optimal physicochemical characteristics (e.g. solvent exposed area, solubility) of the molecule may be responsible for this special feature of f-MLP at such a low level of phylogeny. This means that the unicellular *Tetrahymena* is able to select between related molecules, giving high priority to the molecule that is the most chemoattractive in mammals. The results call attention to the possible presence of f-Met receptors at a unicellular level and to the evolutionary conservation of chemotaxis-activating processes.

Keywords: Chemotaxis; Formylated peptides; Selection; Targeting; *Tetrahymena*; Phylogeny

1. Introduction

Chemotaxis is one of the most fundamental physiological responses that developed at prokaryotic and early eukaryotic levels of phylogeny. The two distinct levels possess diverse signalling mechanisms. Interaction of a well described receptor–intracellular signalling system (e.g. Trs, Trg receptors and the ChA–ChB–ChY–ChZ network) and several relatively simple ligands (amino acids, dipeptides, saccharides) are present in bacteria (Parkinson, 1993). In contrast, different membrane associated receptor complexes and their still poorly understood downstream signalling networks have been described in eukaryotic cells, e.g. chemokine receptors (Rossi and Zlotnik, 2000), G-protein linked signalling (Ridley, 2001). The receptor-mediated responsiveness of both unicellular levels is essential, as it allows detection of harmful and beneficial ligands (e.g. toxins and nutrients) alike.

Besides these independent prokaryotic or eukaryotic systems, some bacterial products have strong chemoattractant ability in multicellular organisms; several pathogenic bacteria (such as *Escherichia coli* or *B. substilis*) produce such substances. Pioneer work by the Freers group proved that several *N*-formyl methionyl peptides synthesized by bacteria possess chemoattractant abilities (Schiffmann et al., 1975). Modification of the C-terminal part of these peptides, especially with Met or Phe residues, could result in characteristic changes in chemotactic potency or release of lysosomal enzymes in neutrophil granulocytes (Freer et al., 1980). Among these products, short peptides with the *N*-formyl methionyl residue possess particularly strong chemo-

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attractant potency; *N*-For–Met–Leu–Phe (hereafter fMLP) is one of the strongest reference chemoattractant substances used in chemotaxis experiments with neutrophils (Showell et al., 1976). However, receptors of fMLP are also expressed in other white blood cells and another large group of mammalian cells, i.e. cells of the lamina propria of interstitial mucosa (Anton et al., 1998).

Investigations of this chemoattractant molecule show that chemotactic responsiveness to fMLP has a strong correlation with phosphoinositide 3-kinase signalling (Pellegatta et al., 2001), intracellular calcium release (Verploegen et al., 2002), adhesion (Willeke et al., 2001), and F-actin co-localization to the leading edge of polarized neutrophils (Howard and Oresajo, 1985). Further characterization of G protein-coupled fMLP receptors have revealed that, in addition to the above-mentioned components, all signal transduction networks (phospholipase A2, C, D; Ser/Thr or Tyr kinases; ion channels) and several chemotaxis related functions (degranulation, oxidant production, release of pro-inflammatory mediators) are activated (Murphy, 1996).

Results of other experiments have shown that there are significant homologies of chemotactic signalling in relatively simple eukaryotic members of phylogeny. The unicellular *Tetrahymena pyriformis* recognizes similar ligands, e.g. IL-8 (Kohidai and Csaba, 1998), endothelin-1 (Kohidai and Csaba, 1995) or insulin (Csaba et al., 1994), and responds in the same concentration range and direction as vertebrate model cells. Former results have shown that *Tetrahymena* membrane has a high affinity (K_D=4 × 10^{-9}–10^{-8} M) for fMLP (Leick, 1992), and other derivatives possessing the formyl group (For–Nle–Leu–Phe and BOC-f Nle–Leu–Phe) also have characteristic and competitive chemoattractant effects in this protozoa (Kohidai et al., 1994). Whilst the relationship of the molecular composition of these bacterial peptides with their chemotactic potency has been investigated in migratory cells of vertebrates (Schiffmann et al., 1975), data on eukaryotic protozoa is still lacking. The wide range of homologies and sensitivity of *Tetrahymena* to slight structural modifications in the relatively short and long chemoattractant peptide type ligands, e.g. proline-containing dipeptides (Kohidai et al., 1997) or insulin derivatives (Csaba et al., 1994), is another reason for using this unicellular organism in our experiments.

In the present study, our objectives were whether:

1. different bacterial formylated peptide derivatives are also chemotactic to eukaryotic ciliates (such as *T. pyriformis*)
2. there is any correlation between the composition of formylated peptides and their chemotactic potency
3. the chemotactic behavior and selectivity of *Tetrahymena* can be compared to that of mammalian granulocytes

For this purpose, six formyl-methionyl derivatives were assayed in *T. pyriformis* GL.

2. Materials and methods

2.1. Cell culture

Populations of *T. pyriformis* GL taxon in logarithmic phase of growth were cultured in 0.1% yeast extract containing 1% Bacto Tryptone (Difco, Michigan, USA) at 28 °C. Prior to the experiments, cells were transferred to Losina Losinsky (LL) medium (Losina-Losinsky, 1931) containing inorganic salts (see below), for 3 h. Cell density of LL cultures was 10^4 cell/ml.

2.2. Chemicals and buffers

Six *N*-formyl methionyl peptide derivatives were tested: For–Met–Leu–Phe (fMLP), For–Met–Leu–Phe–Phe (fMLPP), For–Met–Met–Met (fMMM), For–Met–Phe (fMP), For–Met–Val (fMV) and For–Met–Ser (fMS) were obtained from Sigma, St Louis, USA. Fmoc–Ser(tBu)–Wang resin, fluorenylmethyl-methionine (Fmoc-Met), dimethyl formamide (DMF), piperidine, methanol (MeOH), diisopropyl carbodiimide (DIC), 1-hydroxy-benzotriazole (HOBt), *p*-nitrophenyl formiate, triethyl silane (TES), trifluoroacetic acid (TFA) and ethyl-dimethylaminopropyl carbodiimide (EDCI) were products of Fluka Sigma–Aldrich Ltd, Budapest, Hungary. Effects of the amino acid residues of the formylated oligopeptides (For–Met, Met, Leu, Phe, Ser and Val) mentioned above were also assayed (all these amino acids were also obtained from Sigma, St Louis, USA). Composition of LL solution was 1% NaCl; 0.1% $MgCl_2$; 0.1% $CaCl_2$; 0.1% KCl and 0.2% $NaHCO_3$.

2.3. Synthesis of For–Met–Ser

Fmoc–Ser(tBu)–Wang resin (0.1 mmol, 200 mg) was swollen in DMF (3 × 3 ml, 3 × 1 min), incubated with piperidine/DMF (1:1 v/v, 3 × 3 ml, 2+10+5 min), washed with DMF, MeOH and DMF (3 × 3 ml and 1 min each). Reagents and solvents were removed by suction in every case. Fmoc–Met (0.3 mmol, 112 mg) was dissolved in DMF (2 ml) then DIC and HOBt (0.3 mmol each, 47 μl and 46 mg, respectively) were added. The solution was allowed to stand for 5 min and added to the resin. The slurry was shaken at RT for 2 h. The resin was washed with 4 × 3 ml DMF then deprotected with piperidine/DMF (see above). The peptide on the resin was then formylated with *p*-nitrophenyl formiate (Martinez and

Laur, 1982). The reagent (0.4 mmol, 80 mg) was dissolved in 0.6 ml DMF, for 3 h at RT. The resin was washed with DMF then MeOH, and dried over P_2O_5. The peptide was cleaved from the resin using a mixture of TFA, TES and water (90:5:5, v/v/v). The mixture (8 ml) and the dry resin were mixed in the cold (0 °C) and stirred at RT for 3 h, then filtered. The filtrate was evaporated in vacuo, the remainder was dissolved in water, freeze–dried and purified by HPLC, using a Phenomenex Jupiter C18 (100 A, 5 μm) column (250 × 10 mm). The structure was confirmed by ESI-MS with a Perkin–Elmer Sciex API2000 tandem mass spectrometer. M (theoretical)=264.3; M^+ (actual)= 265.0.

2.4. Chemotaxis assay

The chemotactic ability of *Tetrahymena* cells was evaluated using a modified two-chamber capillary chemotaxis assay (Kohidai et al., 1995). Tips of an eight-channel micropipette filled with the test substances served as the inner chamber of the system. The outer chamber consisted of a microtitration plate filled with the model cells. The incubation time was 20 min. This relatively short time facilitated the measuring of pure gradient, directed chemotactic responses and prevented contamination of the samples from randomly running chemokinetic responder cells (Kohidai et al., 2000). The concentration dependence of the chemotactic response was found to be in the range of 10^{-12}–10^{-6} M. In concurrent runs, pure LL medium served as the control substance. Control samples were evaluated in parallel in each case, to eliminate undesirable disturbances elicited by spontaneous mutations. After incubation, samples were fixed in 4% formaldehyde containing LL solution. The number of cells was determined oculometrically using a Neubauer haemocytometer.

2.5. Statistical evaluation

Origin 4.0 was used to analyze the data. Values of t-probe are shown in the figures: $x=P<0.05$; $y=P<0.01$; $z=P<0.001$.

3. Results and discussion

3.1. Concentration range of formyl–Met-X (-X-X) peptides

Out of the 6 peptides containing For–Met on the N terminus, only fMLP (the "classic" chemoattractant) could elicit dose-dependent chemotactic responses with a bell-shaped curve in *Tetrahymena*. Its most effective chemoattractant concentration range was 10^{-10}–10^{-8} M, although a slight chemoattractant activity was detected up to 10^{-6} M (Fig. 1a). Elongation of the C-terminus with an additional Phe residue (fMLPP) resulted in a diminished, neutral or slightly depressed chemotactic response over a wide (10^{-11}–10^{-6} M) concentration range, while the lowest concentration tested (10^{-12} M) was chemorepellent (Fig. 1b). In the case of the tripeptide composed of only methionines (fMMM), neutral or slightly depressed chemotactic activity was detected (Fig. 1c). The Leu-deletion variant dipeptide (fMP) showed that not only elongation, but also omission of fMLP, can result in the loss of chemoattractant ability, as fMP had a neutral character at all the tested concentrations (Fig. 1d).

The other two dipeptides (fMV and fMS) were assayed to test whether a combination of fMet with different amino acid residues on the C terminus could modify the chemotactic ability of the peptide. The chemoattractant character of fMV at 10^{-6} M (Fig. 1e) and the significant chemorepellent feature of fMS over a wide range (10^{-12}–10^{-8} M) (Fig. 1f) suggest that a low solvent exposed area (SEA) (Rose et al., 1985) in the C terminal amino acid (Val=23.5) is preferred over higher values (Ser=44.2 or Phe=28.7). We could conclude that both strong (Phe=2.87) and weak (Ser=0.07) hydrophobicity at the C terminus are disadvantageous with regards to chemoattractant ability, while a moderately hydrophobic (Val=1.87) C terminus results in a chemoattractant formylated dipeptide ligand (Jones, 1975).

The concentration range studies showed that slight modifications to the short formylated (putatively bacterial) peptides can alter their chemotactic character, however, a few peptides possess some chemotactic ability (e.g. fMV as a chemoattractant and fMS as a chemorepellent), hence the identity of fMLP seems to be the result of a sensible structure-function relationship.

The chemoattractant or repellent characteristics of the peptides described above may be explained by some of their physicochemical properties. As previous studies have also described, chemotactic characteristics of short peptides show good correlation with SEA values or solubility of terminal residues of short peptides (Illyés et al., 2002). Our current results indicate that optimal values of mass, SEA or ratio of SEA:solubility are advantageous with respect to the chemoattractant ability of the ligand. Table 1 shows that fMLP possesses intermediate values; the higher or lower values are not favored in chemotactic signalling.

Comparison of our results on fMLP in *Tetrahymena* with Freers data obtained in neutrophils (Showell et al., 1976) shows a good overlap of chemotactic responsiveness in protozoa and neutrophils (both 10^{-10}–10^{-8} M). The neutral character of dipeptide fMP also shows a good correlation at both levels of phylogeny. In the case of peptide fMMM, significant differences were observed, as this compound exhibits a slight chemorepellent effect over a wide concentration range in *Tetrahymena*, in contrast to its strong chemoattractant effect in vertebrate neutrophils. These data suggest that phylogeny of

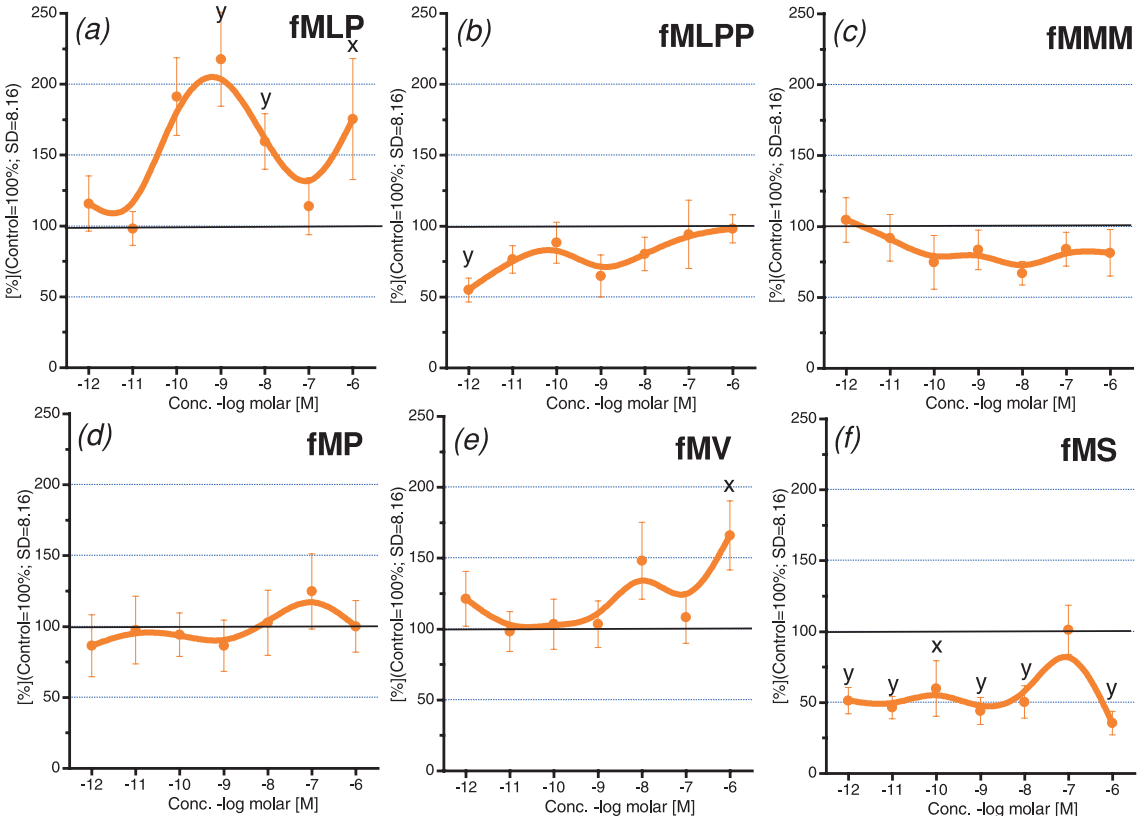

Fig. 1. Concentration dependence of chemotaxis elicited by oligopeptides containing *N*-formyl methyonyl residue in *Tetrahymena* (x=P<0.05; y=P<0.01; z=P<0.001).

Table 1
Physicochemical characterization of the tested oligopeptides (SEA-solvent exposed area). Calculation of values is based on reference data (Biemann, 1990; Rose et al., 1985; Budavari, 1989).

	Mass	SEA	SEA/Solubility of C-terminal residue
f-MV	230.33	54.0	6.10
f-MP	278.37	59.2	19.96
f-MS	218.27	74.7	14.87
f-MLP	391.53	88.2	29.74
f-MMM	393.59	91.5	27.06
f-MLPP	538.70	116.9	39.42

signalling in this family of chemoattractants includes continuity and divergence. Peptide fMLP is utilized in unicellular and multicellular organisms by similar effector mechanisms, while signalling of fMMM probably uses different effector mechanisms on the two levels. However, it must also be considered that fMLP is a real bacterial peptide (Broom et al., 1992; Fontan et al., 1992; Le et al., 2002; Mooney et al., 1991), while fMMM (and the others used) are synthetic peptides that may or may not be produced by bacteria. Therefore, *Tetrahymena* and neutrophils appear to react to the "natural" peptide and reject the synthetic ones.

3.2. Relationship between the chemotactic effect of amino acids and peptides

In the case of bacterial peptides, the expression of N terminal For–Met moiety is characteristic and functionally significant; in several cases (e.g. B. licheniformis), only the *N*-formylated precursor holoproteins can induce neutrophils, in contrast to the mature, *N*-formylated signal-lacking proteins (Bennett et al., 1980). Our first comparative study was to analyze the chemotactic ability of this structurally modified amino acid in parallel with methionine. Although both compounds were chemoattractant (Fig. 2), the presence of the formyl group itself did not improve chemotactic potential. Moreover, native methionine was more potent at 10^{-10} M in *Tetrahymena*.

Data in the literature document relationships between the chemotactic character of short peptides and their amino acid constituents (Almagor et al., 1981). Therefore, the chemotactic character of the remaining four amino acids of the tested peptides was also analyzed (Fig. 2). Our data show that Leu, Phe and Val have a wide-ranging (10^{-12}–10^{-6} M) chemorepellent effect. Only Ser displays a biphasic concentration dependence, being chemoattractant at low (10^{-12}–10^{-10} M) and chemorepellent at higher (10^{-6} M) concentrations.

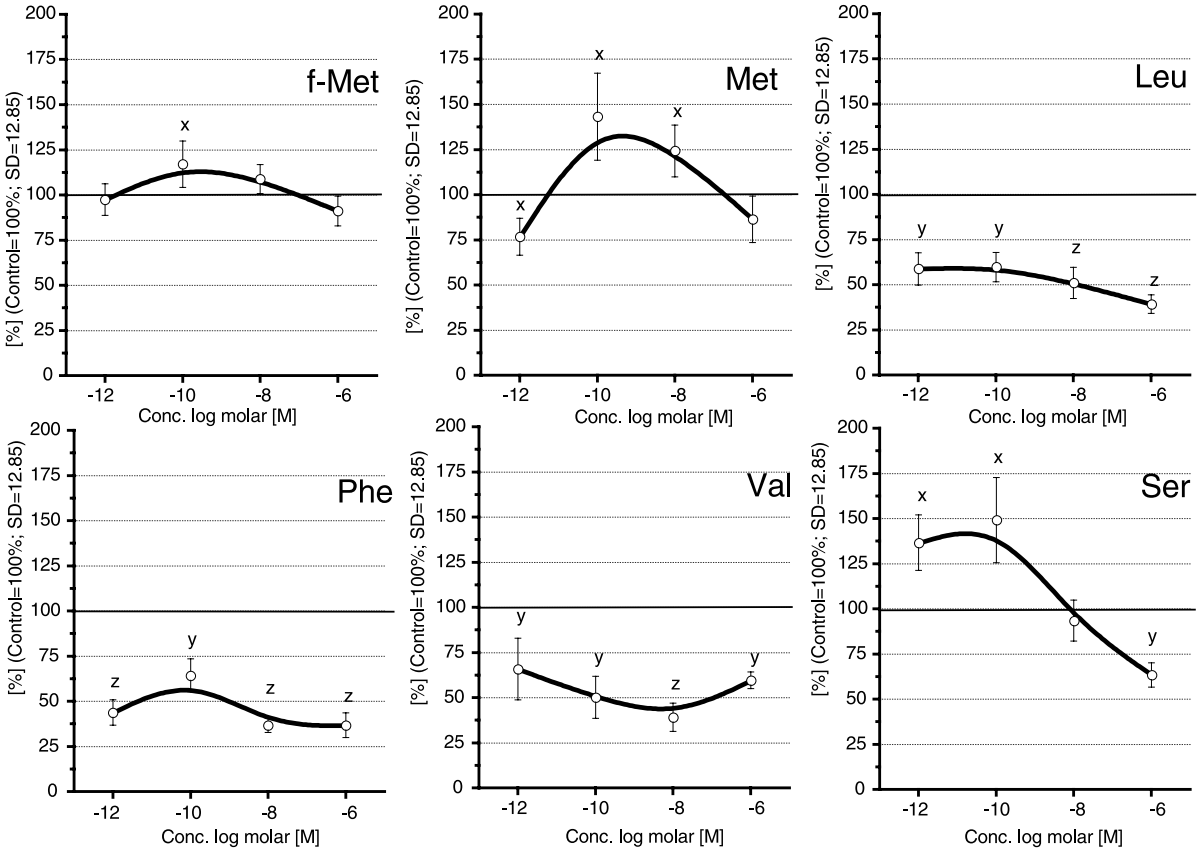

Fig. 2. Concentration dependence of chemotaxis elicited by amino acid components of the tested oligopeptides in *Tetrahymena* (x=P<0.05; y=P<0.01; z=P<0.001).

These results confirm other data in the literature that, even in short peptides (di- or tripeptides), the chemotactic character of the molecule cannot be derived from the features of the constituent amino acids. In spite of the fact that fMet is moderately chemoattractant in character and amino acids Leu, Phe and Val are strong repellents, peptides derived from these units form a diverse set of ligands. In peptides, their physicochemical characteristics (SEA, etc.) seem to be decisive.

In conclusion, our investigations on the chemotactic effects of N formyl-methionine-containing peptides suggest that fine, intramolecular physicochemical adjustments are required for the chemoattractant effect of the well-known reference peptide fMLP. Our results show that signalling of fMLP is a well-conserved mechanism in phylogeny, while other formylated ligands have neutral or antagonistic effects in low and higher levels of eukaryotes. The results clearly show that the chemotactic reaction and sensitivity of human granulocytes to chemoattractants can be traced back to the early stages of phylogeny, however, the repertoire of response-ability has been modified over time. The experiments also demonstrate that *Tetrahymena* can very keenly select between related peptides, which results in a preference for fMet receptors even at this low level of phylogeny.

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References

Almagor M, Ron A, Bar-Tana J. Chemotaxis in *Tetrahymena thermophila*. Cell Motility 1981;1:261–8.

Anton P, O'Connell J, O'Connell D, Whitaker L, O'Sullivan GC, Collins JK et al. Mucosal subepithelial binding sites for the bacterial chemotactic peptide, formyl-methionyl-leucyl-phenylalanine (FMLP). Gut 1998;42:374–9.

Bennett JP, Hirth KP, Fuchs E, Sarvas M, Warren GB. The bacterial factors that stimulate neutrophils may be derived from prokaryote signal peptides. FEBS Lett 1980;116:57.

Biemann K. Mass values for amino acid residues in peptides. Methods Enzymol 1990;193:888.

Broom MF, Sheriff RM, Munster D, Chadwick VS. Identification of formyl Met-Leu-Phe in culture filtrates of *Helicobacter pylori*. Microbios 1992;72:239–45.

Budavari S. The Merck Index. Nahway: Merck Co, 1989;11:MISC-60.

Csaba G, Kovács P, Kohidai L. *Tetrahymena* cells distinguish insulins according to their amorphous and crystalline condition or their bovine and porcine origin. Study of imprinting in aspects of hormone binding and chemotaxis. Microbios 1994;80:215–21.

Fontan PA, Amura CR, Garcia VE, Cerquetti MC, Sordelli DO. Preliminary characterization of *Pseudomonas aeruginosa* peptide chemotactins for polymorphonuclear leukocytes. Infect Immun 1992;60:2465–9.

Freer RJ, Day AR, Radding JA, Schiffmann E, Aswanikumar S, Showell HJ. Further studies on the structural requirements for synthetic peptide chemoattractants. Biochemistry 1980;19:2404–10.

Howard TH, Oresajo CO. The kinetics of chemotactic peptide-induced change in F-actin content, F-actin distribution, and the shape of neutrophils. J Cell Biol 1985;101:1078–85.

Illyés E, Hudecz F, Kohidai L, Láng O, Szabó P, Sebestyén F. Synthesis of oligopeptides with the sequence SXWS and their chemotactic effects on a ciliated protozoan *Tetrahymena pyriformis*. J Peptide Sci 2002;8:13–22.

Jones DD. Amino acid properties and side-chain orientation in proteins: a cross correlation approach. J Theor Biol 1975; 50:167–83.

Kohidai L, Csaba G. Effects of the mammalian vasoconstrictor peptide, endothelin-1, on *Tetrahymena pyriformis* GL, and the immunocytological detection of endogenous endothelin-like activity. Comp Biochem Physiol 1995;111C:311–6.

Kohidai L, Csaba G. Chemotaxis and chemotactic selection induced with cytokines (IL-8, RANTES and TNF*a*) in the unicellular *Tetrahymena pyriformis*. Cytokine 1998;10:481–6.

Kohidai L, Kovács P, Csaba G. Chemotactic response of unicellular *Tetrahymena* to leukocyte attractant peptide and its repellent derivative. Evolutionary conclusions. Cell Biol Internat 1994; 18:119–21.

Kohidai L, Lemberkovits É, Csaba G. Molecule dependent chemotactic responses of *Tetrahymena pyriformis* elicited by volatile oils. Acta Protozool 1995;34:181–5.

Kohidai L, Schiess N, Csaba G. Chemotactic selection of *Tetrahymena pyriformis GL* induced with histamine, di-iodotyrosine or insulin. Comp Biochem Physiol 2000;126C:1–9.

Kohidai L, Soós P, Csaba G. Effects of dipeptides containing the amino acid, proline on the chemotaxis of *Tetrahymena pyriformis*. Evolutionary conclusions on the formation of hormone receptors and hormones. Cell Biol Internat 1997;21:341–5.

Le Y, Yang Y, Cui Y, Yazawa K, Gong W, Qiu C et al. Receptors for chemotactic formyl peptides as pharmacological targets. Int Immunopharmacol 2002;2:1–13.

Leick V. Chemotactic properties, cellular binding and uptake of peptides and peptide derivatives: studies with *Tetrahymena thermophila*. J Cell Sci 1992;103:565–70.

Losina-Losinsky LK. Zur Ernährungsphysiologie der Infusorien. Untersuchungen über die Nahrungsmittel und Vermehrung bei *Paramecium caudatum*. Arch Protistenk 1931;74:18–20.

Martinez J, Laur J. Active esters of formic acid as useful formylating agents: Improvements in the synthesis of formyl-amino acid esters, N-alpha-formyl-Met-Leu-Phe-OH, and formyl-Met-Lys-Pro-Arg, a phagocytosis stimulating peptide. Synthesis 1982;11:979–81.

Mooney C, Keenan J, Munster D, Wilson I, Allerdyce R, Badshaw P et al. Neutrophil activation by Helicobacter pylori. Gut 1991; 32:853–7.

Murphy PM. The N-formylpeptide chemotactic receptor. In: Horuk R, editor. Chemoattractant ligands and their receptors. New York: CRC Press, 1996 269pp.

Parkinson JS. Signal transduction schemes of bacteria. Cell 1993; 73:857–71.

Pellegatta F, Radaelli A, Heltai S, Yan L, Chierchia SL, Folli F. Evidence for the involvement of phosphatidylinositol 3-kinase in fMLP-stimulated neutrophil adhesion to ICAM-1-transfected cells. J Cardiovasc Pharmacol 2001;37:751–61.

Ridley AJ. Rho GTPases and cell migration. J Cell Science 2001; 114:2713–22.

Rose GD, Geselowitz AR, Lesser GJ, Lee RH, Zehfus MH. Hydrophobicity of amino acid residues in globular proteins. Science 1985; 229:834–8.

Rossi D, Zlotnik A. The biology of chemokines and their receptors. Ann Rev Immunol 2000;18:217–42.

Schiffmann E, Showell HJ, Corcoran BA, Ward PA, Smith E, Becker EL. The isolation and partial characterization of neutrophil chemotactic factors from *Escherichia coli*. J Immunology 1975; 114:1831–7.

Showell HJ, Freer RJ, Zigmond SH, Schiffmann E, Aswanikumar S, Corcoran B et al. The structure-activity relations of synthetic peptides as chemotactic factors and inducers of lysosomal enzyme secretion for neutrophils. J Exp Med 1976;143:1154–69.

Verploegen S, van Leeuwen CM, van Deutekom HW, Lammers JW, Koenderman L, Coffer PJ. Role of Ca^{2+}/calmodulin regulated signaling pathways in chemoattractant induced neutrophil effector functions. Comparison with the role of phosphotidylinositol-3 kinase. Eur J Biochem 2002;269:4625–34.

Willeke T, Scharffetter-Kochanek K, Gaehtgens P, Walzog B. A role for beta2 integrin (CD11/CD18)-mediated tyrosine signaling in extravasation of human polymorphonuclear neutrophils. Biorheology 2001;38:89–100.