REVIEW

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The main functions and structural modifications of tripeptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) as a chemotactic factor

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Gram negative bacteria-derived and synthetic *N*-formyl peptides play a key role in host defense as chemotactic factors for phagocytic leukocytes. The first compound to be identified was *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) which contains highly potent leukocyte chemoattractant. Natural fMLP was subsequently purified and identified in supernatants of gram negative bacteria. Recently, much more attention has been focused on the human formyl peptide receptor (FPR) and its variant formyl peptide receptor-like 1 (FPRL1) and formyl peptide receptor-like 2 (FPRL2). Chemotactic factors such as fMLP interact with their specific cell surface receptors, which results in multiple biological responses through a G protein-coupled signal pathway. In this review, the functions and structural modifications of fMLP are discussed in view of future drug development.

1. Introduction

Over the last three decades, a large number of chemoattractants have been identified. These chemoattractants include *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (Fig. 1), the activated complement fragment C5a, leukotriene B4 (LTB4), and a superfamily of chemokines that have characteristic cysteine residues (Zlotnik et al. 1999). Among all these chemoattractants discovered so far, fMLP is the major chemotactic factor derived from bacterial sources or disrupted mitochondria. Moreover, fMLP is the smallest chemotactic peptide, which contains only three amino acids. Due to its important (various) biological functions and the easy synthetic way of preparation, fMLP has become a model chemotactic factor for the study of peptide-receptor interactions and phagocyte functions.

Currently, scientists have identified three fMLP receptors, formyl peptide receptor (FPR), formyl peptide receptor-like 1 (FPRL1) and formyl peptide receptor-like 2 (FPRL2). All the fMLP receptors have been considered as novel pharmacological targets. FPR is a chemoattractant G protein-coupled receptor (GPCR) which shows high affinity toward fMLP ($K_d < 1$ nM). Firstly, it was found in the surface of phagocytes, and further studies showed that it was also expressed in astrocytes, dendritic cells (DCs), microglia cells, hepatocytes, the tunica media of coronary arteries and non-leukocytic cells (Kim et al. 2007). FPRL1 and the related putative receptor FPRL2 were identified in human cells. Although FPRL1 possesses 69% identity to FPR at the amino acid level, it shows nearly 500-fold lower affinity to fMLP (estimated $K_d = 430$ nM vs.

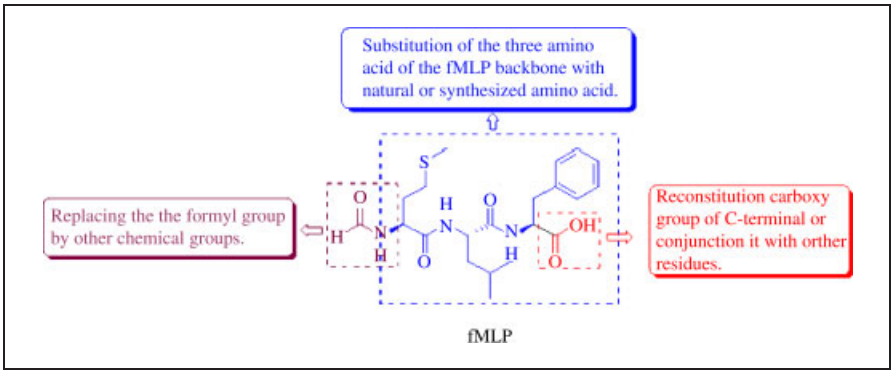

Fig. 1:
Structure and structure-activity relationships (SAR) of fMLP

$K_d < 1$ nM). FPRL2 shows 56% identity in the sequence to FPR, but it could not be activated by fMLP. The receptor sequence analysis as well as chimeric receptor construction revealed several key residues in the first and the third extracellular loops of the FPR, and their adjacent transmembrane domains may be essential for high-affinity binding of fMLP (Quehenberger et al. 1993). Further studies proposed that this binding was not dependent on a defined structure, and these charged moieties may function as important "contacts" in receptor-ligand interactions (Radel et al. 1994).

There are many studies discussing fMLP and its chemotactic activity. However, the detailed mechanism of its interaction with specific cell surface receptors, and some biological responses, were rarely reported. This review will put emphasis on the signaling pathways and the functions of fMLP. Further structural modifications in view of future drug research will also be discussed.

2. Signaling cascade triggered by fMLP

fMLP-mediated responses are well characterized and have provided deep insights into phagocytes function. Studies on leukocytes and receptor transfected cell lines indicated that most responses were mediated by FPR. The human FPR belongs to the seven transmembrane domains GPCR family and is functionally coupled with PTX-sensitive G protein Giα1, Giα2 and Giα3 (Wenzel-Seifert et al. 1999). After binding to fMLP, FPR undergoes a conformational change that transmits signals to heterotrimeric G proteins, which rapidly dissociate into α and $\beta\gamma$ subunits. Subsequently the $\beta\gamma$ subunits could stimulate phospholipase C (PLC) and phosphoinositide 3-kinase (PI3K). The activation of PI3K results in conversion of the membrane phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 3,4,5-trisphosphate (PIP3). The breakdown of PIP3 by PLC leads to generate the secondary messengers: inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). The former regulates mobilization of Ca^{2+} from intracellular stores (Revankar et al. 2006), and the latter activates protein kinase C (PKC) isoforms. PKC isoforms could rapidly stimulate mitogen-activated protein kinase (MAPK) (Torres et al. 1993) and tyrosine kinase (TK) (Ptasznik et al. 1995). Together with Ca^{2+} they could also activate other intracellular effectors such as phospholipases A and D (PLA, PLD). Studies on targeted gene disruption revealed that PI3Kγ is the sole PI3K isoform coupled with receptors for several chemoattractants including fMLP (Li et al. 2000). In short, the binding of fMLP to FPR triggers a highly complex signal transduction network, involving the activation of multiple effector enzymes and the production of arrays of second messengers. These signaling pathways are crucial for various neutrophil functions, including adhesion, chemotaxis and superoxide anion release (Lavigne et al. 2002), which contribute to the physiological defense against bacterial infections and cell disruption. And other cell responses such as actin polymerization, shape change, mediator release, gene transcription, cytokine release are of unknown biological significance.

Due to the low-affinity with fMLP, the signal transduction pathways mediated by FPRL1 have not been extensively studied. It is suggested that FPRL1 may share many signaling characteristics observed with FPR based on their high level of homology. Further studies need to be conducted to demonstrate the detail cell responses to the activation of FPRL1 by fMLP. Thus, fMLP may play important roles in proinflammatory, immunological diseases, HIV-1 and some other physiological functions by interacting with its receptors.

3. Main functions of fMLP

3.1. Anti-bacterial properties

As we all know, neutrophils are specialized in the inactivation of microorganisms, and consequently play a protective role against infections. Gene targeting studies convincingly supported that mice with a disrupted FPR gene displayed impaired anti-bacterial immunity (Gao et al. 1999). fMLP was identified as the most potent FPR agonist. In addition to neutrophils, fMLP also markedly activates monocytes and macrophages. By reacting with its high affinity receptor FPR on phagocytic leukocytes, fMLP induces chemotaxis along concentration gradients of chemoattractants, driving the phagocytic cells toward bacteria. Besides chemotaxis, fMLP also induces phagocytosis, the secretion of lysosomal enzymes and free radical generation, all of which constitute the physiological defense against invading microorganisms when phagocytic cells arrived at the site of infection. Soehnlein et al. (2007) presented that neutrophil-derived granule proteins which was obtained by treatment with fMLP induced a several-fold increase in bacterial phagocytosis by monocytes and macrophages. The combination of tumor necrosis factor α (TNF-α) and fMLP was investigated by Ogle et al. The synergistic and additive effects of this combination could be of great importance in host defense against bacterial infections (Ogle et al. 1992).

3.2. Anti-cancer activity

Binding of fMLP to its receptor also activates phagocytic leukocytes to elaborate effector functions necessary to fight malignancies. In an original study, fMLP was conjugated with two monoclonal antibodies (OC125 and OC133) which could react with human ovarian carcinomas. Conjugates retained the ability to bind to a human ovarian carcinoma line (OVCA433) and chemotaxis for human blood monocytes. As monocytes could be im-

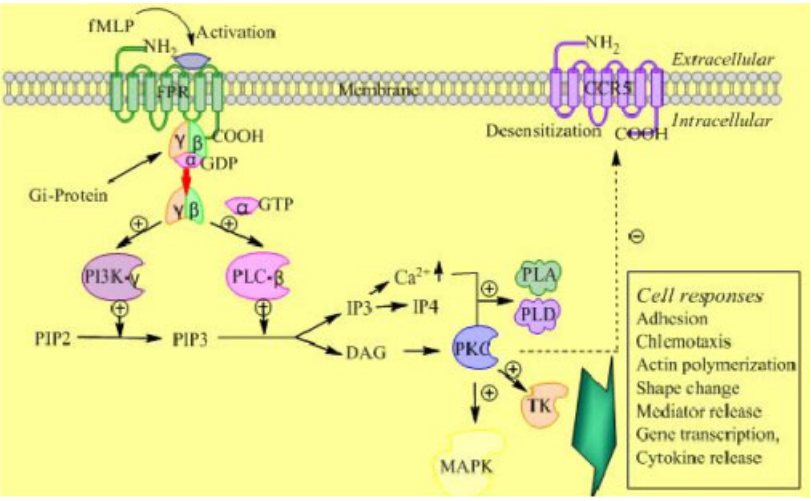

Fig. 2:
Schematic signaling pathways of activated FPR. Upon fMLP binding

portant effectors of antibody dependent cell mediated cytotoxicity, fMLP conjugates might increase monocyte concentrations at tumor sites and potentiate serotherapy for certain human neoplasms (Obrist et al. 1983a). The fact that IgG-fMLP conjugates enhance macrophage invasion of tumors to improve the curative effect was reported in another two articles (Obrist et al. 1983b; Obrist et al. 1991).

Previous studies demonstrated that liposome-incorporated synthetic chemotactic peptide fMLP generated tumoricidal properties in mouse macrophages (Morikawa et al. 1988). *In vivo* and *in vitro* studies showed that fMLP could activate macrophages to induce lysozyme production, superoxide anion formation, proinflammatory cytokines release and NO production, thus resulting in the attainment of tumoricidal properties (Sodhi and Biswas 2002). Shrivastava found that fMLP-induced tumoricidal activity could be downregulated by the protein tyrosine kinase (PTK) inhibitors egenestein and lavendustin A (Shrivastava 2007). In addition, fMLP could also activate and attract leukocytes and macrophages which may interfere with the process of tumor growth, invasion, and metastasis.

Boanmycin (BAM) is effective against a panel of cancers in clinical trials. Recently, Li's group investigated the anti-tumor activity of BAM in combination with fMLP. They found that fMLP may enhance the anti-tumor effects of BAM, but the enhancement may need the participation of macrophages (Li et al. 2002).

In certain instances, fMLP are initially cleaved by CD10/NEP (neutral endopeptidase) and further hydrolyzed by CD13/APN (aminopeptidase N) in a variety of tissues (Shipp and Look 1993). Especially APN is a useful clinical anti-tumor target. So the strategic substrate reconstitutions based on fMLP for the APN inhibitors have also been studied recently.

3.3. Anti-HIV activity

HIV-1 enters its target cells by fusion at the plasma membrane. The primary cellular receptor for HIV is CD4, but this molecule is insufficient to permit viral fusion. In 1996, the necessary entry co-factors were identified as being members of the seven-transmembrane-spanning receptor family fusin: chemokine receptors CXCR4 and CCR5, which are for T-tropic strains and M-tropic strains, respectively. According to the process of heterologous desensitization, the activation of fMLP receptors could affect the expression and function of these two major HIV-1 co-receptors (Le et al. 1999). Shen's group tested the capacity of activated fMLP receptors to affect signaling and phosphorylation of HIV-1 chemokine co-receptors and found that fMLP could rapidly induce serine-phosphorylation and down-regulation of CCR5 in monocytes. These changes could result in significant attenuation of cell responses to CCR5 ligands, inhibition of HIV-1 enveloped glycoprotein-mediated fusion and infection of cells expressing CD4, CCR5, and FPR (Shen et al. 2000).

Recently, it has been demonstrated that some synthetic FPR agonistic peptides based on the structure of fMLP are highly efficacious in inhibiting HIV infections. In addition, Trp-Lys-Tyr-Met-Val-D-Met-$CONH_2$ (WKYMVm), a synthetic peptide which is a highly potent agonist for fMLP receptors, attenuates the function of CCR5 in immature dendritic cells (DCs) in association with a PKC-dependent serine phosphorylation of CCR5 (Le et al. 1999).

Peptide analogues which are obtained by modifications of fMLP, may be more resistant to peptidase degradation and maintain a longer half-life *in vivo*. In contrast, fMLP itself and small synthetic peptide analogues may not be antigenic *in vivo*. Therefore fMLP may represent an additional approach to the design of anti-HIV-1 agents.

3.4. Anti-nociceptive effects

Although fMLP was always considered as a proinflammatory agent, researchers proposed that this chemoattractant could, under certain circumstances, act as an anti-inflammatory molecule (Isturiz et al. 2004). In the nociceptive threshold of mice experiment, Stefano and co-workers first found that fMLP induced anti-nociceptive effects in the formalin test both after the peripheral and central administration (Stefano et al. 2004). And another agonist, annexin I, which was postulated as a mediator of the anti-inflammatory activity of glucocorticoids displayed the same effects. Previous studies showed that agonists elicit different signaling pathways depended on the ligand concentration present in the reactions. The authors believed that probably the low concentrations of annexin I as well as fMLP inhibited the accumulation of neutrophils, release of interleukin-1β (IL-1β) and TNF-α (Vulcano et al. 1998), which could reduce the formalin-induced nociceptive. Interestingly, the fMLP receptor antagonist Boc-Met-Leu-Phe-OH (Boc-MLP) did not alter the response to formalin, but was able to block the anti-nociceptive effects of annexin I and fMLP.

3.5. Anti-alopecia effects

Tsuruki and colleagues found that intraperitoneally administered fMLP could prevent alopecia in neonatal rats induced by the anticancer agent etoposide. The anti-alopecia effect of fMLP cannot be inhibited by a selective antagonist of FPR, Boc-Phe-Leu-Phe-Leu-Phe-OH (Boc-FLFLF), but it could be partly inhibited by an antagonist of the FPRL1 receptor, Trp-Arg-Trp-Trp-Trp-Trp-$CONH_2$ (WRWWWW, WRW^4). The anti-alopecia effect of fMLP can also be inhibited by Lys-D-Pro-Thr (K(D)PT) and pyrrolidine dithiocarbamate, which are inhibitors of interleukin-1 (IL-1) and nuclear factor-κβ (NF-κβ), respectively. This phenomenon suggested that the anti-alopecia mechanisms of intraperitoneally administered fMLP include the activation of NF-κβ *via* IL-1 release downstream of the FPRL1 receptor homolog in rats (Tsuruki et al. 2007).

4. Structure modifications of fMLP

In view of the descriptions outlined above, fMLP has many useful physiological functions. Although these functions still to be further investigated and confirmed, fMLP represents the reference molecular model for modification on account of the following reasons:

(i) fMLP could induce a full responses by neutrophils ("full" agonist), which may cause a sequence of complicated physiological reactions. A research program based on the synthesis of new fMLP analogues should be carried out in order to obtain selective therapeutic agents ("pure" agonist) (Fabbri et al. 2000).

(ii) On the other hand, under several pathological conditions, the inappropriate release of cytotoxic molecules into the extracellular milieu could damage body tissues. So the development of strong FPR antagonists by fMLP is therefore of considerable interest, particularly for their potential use as therapeutic agents in the treatment of inflammation-related disorders (Higgins et al. 1996).

(iii) Moreover, many researchers worked with radiolabeled chemotactic analogues through the parent peptide fMLP. These analogues acting as FPR agonists could be effective agents for imaging sites of inflammation, and consequently be useful for the identification of infection sites (Stephenson et al. 2005).

(iv) What's more? The fMLP and its analogues could also be used as drug carriers, fMLP can conjugate to anti-HIV drugs. These conjugates could improve drug delivery and effects due to the feasibility for targeting macrophages, a primary HIV reservoir site (Wan et al. 2007). As to fMLP analogues, they may fail to stimulate chemotaxis, but could elicit killing mechanisms by activating the specific receptor conformation. These analogues exhibited a receptor affinity greater than fMLP, thereby rendering them suitable to be used as carriers for various drugs.

Numerous studies stressed the importance of each part of the fMLP molecule. A series of synthetic peptides related to fMLP have been systematically synthesized. The biological properties of the peptide analogues were determined on human neutrophils by means of several *in vitro* assays: receptor binding, chemotaxis, O^{2-} production and lysozyme release, and were compared with fMLP or fMLP-OMe which behaves identical to that of the prototype peptide. The structure-activity relationships (SAR) were discussed in detail on Giorgio's review (Giorgio et al. 2006). In this report, modification methods could mainly be grouped into four categories (Fig. 1). Some brief points on the activity and alteration at construction site are also be made herein.

(i) Replacing the formyl group by other chemical groups.

It was suggested that the presence of a formyl group is not a necessity for triggering the physiological functions of human neutrophils. Some N-terminal substitution represented high-affinity interaction with FPR, even obtained strong FPR antagonists (Higgins et al. 1996).

(ii) Substitution of the three amino acids of the fMLP backbone with natural or synthesized amino acids.

Both hydrophilic and hydrophobic residues are able to fit the specific receptor pockets. Methionine is essential for

the high efficacy at position 1, and its substitution would lead to more selective analogues (Freer et al. 1980; Torrini et al. 1994; Cavicchioni et al. 1998). As to position 2, both linear aliphatic and branched aliphatic residues were optimal for triggering killing mechanisms (Dugas et al. 1993; Spisani et al. 2002; Cavicchioni et al. 2001; Spisani et al. 2002; Pag et al. 2001; Giordano et al. 2003). With regard to position 3, phenylalanine was regarded as the best residue for both recognition and activation of the receptor needing to be re-hypothesized (Spisani et al. 2003). However, a hydrophilic side chain of the residue at position 3 is also available (Kraus and Attardo 1992). These analogues were potent antagonists of superoxide generation, and could be very promising in the field of anti-inflammatory drugs.

(iii) Reconstitution of the C-terminal carboxy group or conjunction it with other residues.

The carboxy group of the phenylalanine is important for a good biological activity. Methyl esterification of the carboxy group exhibited the same activity than its prototype, while benzyl esters and the benzylamide derivatives of fMLP were more active. The carboxy group were linked with other residues (natural or synthesized amino acids) to make a longer peptide chain and to show different biological effects. This site could also be linked with therapeutic drugs, making fMLP as useful drug carrier (Wan et al. 2007).

(iv) Other variation methods.

Cyclic analogues presented a good activity and a promising selectivity of action due to the folding of the peptide's backbone (Zecchini et al. 1993). The double-substituted peptide failed to stimulate chemotaxis, but could elicit killing mechanisms by activating the specific receptor conformation (Cavicchioni et al. 2005; Spisani et al. 2007). The dimeric analogues exhibited a receptor affinity greater than the parent fMLP-OMe, thereby rendering them suitable to be used as carriers for various drugs (Susanna et al. 2007).

5. Conclusions

Over the past two decades, the importance of fMLP was recognized in view of their multiple physiological functions. Research in this field expanded to multiple disciplines, including fMLP receptors and the signaling events. A major limitation in the use of fMLP as a therapeutic agent for the treatment of diseases is that this tripeptide was not selective. Therefore, a number of fMLP analogues were synthesized in view of better and/or selective biological activities. Meanwhile, the development of new FPR antagonists based on fMLP is also highly desirable. Additional studies would help to further understand the chemical mechanisms involved in the binding of fMLP to its receptors and explore fMLP analogues from a therapeutic viewpoint.

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