Peptide Derivatives as Agonists or Antagonists of Formylpeptide Receptors: Analysis of their Effects on Neutrophils

Alessandro Dalpiaz* and Angelo Scatturin

Dipartimento di Scienze Farmaceutiche, Via Fossato di Mortara 19, 44100 Ferrara, Italy

Abstract: The effects of peptide derivatives as agonists or antagonists of formylpeptide receptors are described, taking into account the related cellular responses by neutrophils. These effects are related to the structure of peptide derivatives, some of which are potent anti HIV-1 agents. Finally, formylpeptide receptor models are depicted.

Keywords: Formyl-peptide receptor; Neutrophil; Inflammation; Chemotaxis; G-protein coupled receptor; AIDS/HIV; Agonist; Antagonist.

INTRODUCTION

Polymorphonuclear neutrophils are phagocytic cells specialized in the inactivation of microorganisms, and consequently play a protective role against infections. This function is allowed by their migration from blood vessels to the site of infection along concentration gradients of chemoattractants [1-3]. It has been demonstrated that small formyl-peptide derivatives, obtained as bacterial metabolites [4,5] or derived from disrupted mitochondria [6], can be potent chemoattractants for phagocytes. fMLF, which shows this property, has been used as a model chemoattractant for the study of phagocyte functions. The results of these researches led to the identification, on neutrophil membrane, of a G-protein-coupled receptor (FPR) which has since been cloned [2, 7-8]. In addition to this receptor, which shows high affinity toward fMLF, human neutrophils express a low affinity variant (FPRL1) [9]. The binding of fMLF 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 signalling pathways are crucial for various neutrophil functions, such as adhesion, chemotaxis, free radical generation and the secretion of lysosomal enzymes, all of which constitute the physiological defense against bacterial infections and tissue damage [1]. On the other hand, although destruction of infectious agents occurs intracellularly, in several pathological conditions the inappropriate release of cytotoxic molecules into the extracellular milieu can damage body tissues [10].

In view of the phenomena outlined above, the development of FPR agonists and antagonists appears of considerable interest for the following reasons:

(i) Radiolabeled chemotactic peptide analogues able to act as FPR agonists can be effective agents for imaging sites of inflammation, and consequently useful for the identification of infection sites [11]. Moreover, it has recently been demonstrated that some synthetic peptides which are highly efficacious in inhibiting HIV infection are also FPR agonists [12,13].

(ii) It has been reported that peptide derivatives able to inhibit the neutrophil effects induced by fMLF can be excellent anti-inflammatory agents [14]. As a consequence, the development of FPR antagonists could prove of considerable interest as therapeutic agents in the treatment of anti-inflammation related disorders. For example, annexin I peptides have recently been reported as novel, endogenous FPR ligands able to induce antiinflammatory effects [15]. It must be emphasized that mice with a disrupted FPR gene display impaired antibacterial immunity [16], indicating that an inflammatory response of neutrophils towards chemotactic peptides also plays a role in immune responses. Nevertheless, the development of receptor antagonists of neutrophil stimulators – which are able to transiently inhibit cellular responses- should improve our knowledge about leukocyte chemoattractant functions, and could be of clinical relevance.

AIM OF THE REVIEW

This review will briefly describe the functional N-formyl peptide receptors which so far identified. Peptide derivatives able to act as FPR agonists or antagonists will be depicted. Their affinity and activity will be taken into account, evaluating the cellular responses of neutrophils obtained by receptor binding experiments, measurements of Ca^{2+} intracellular concentration (as a second messenger), chemotaxis, superoxide anion production and enzyme release.

*Address correspondence to this author at the Dipartimento di Scienze Farmaceutiche, Via Fossato di Mortara 19, 44100 Ferrara, Italy; Phone: +39-0532-291268 (*Angelo Scatturin*); +39-0532-291273 Fax: +39-0532-291296; Email: dla@dns.unife.it

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Finally, a description will be provided of FPR models obtained by structure-activity relationships, computational and site-directed mutagenesis studies.

FORMYL PEPTIDE RECEPTORS

The FPRs of human and rabbit neutrophils have been characterized biochemically as receptors that couple to pertussis toxin-sensitive G proteins [17,18]. Molecular cloning of the human FPR provided the first direct evidence that chemoattractant receptors share a seven-transmembrane domain structure characteristic of the G-protein coupled receptor superfamily. The sequence of a high affinity human FPR was deduced from cloned cDNAs in 1990 [9,19]. Subsequent cloning attempts resulted in the identification of a number of FPR orthologs and homologs in humans and other species. Two human genes (*FPRL1* and *FPRL2*) and their corresponding cDNAs have been cloned which encode proteins with 69% and 56% amino acid sequence identity to FPR [9,20-23]. FPRL1 is a low affinity receptor for fMLF; on the other hand, the putative FPRL2 product has no known ligand and its function is undefined [9,23]. Moreover, FPR, but not FPRL1, has been shown to be a chemotactic receptor [24]; and FPRL1, but not FPR, has been shown to be a functional lipoxin A4 receptor [25,26]. Neutrophils express the FPR and its homologue FPRL1, whereas monocytes express FPR, FPRL1 and FPRL2 types [27].

Rabbit FPR was cloned and found to bind fMLF with high affinity, similarly to human FPR [28]. On the other hand, cloned mFPR is a low affinity receptor for fMLF [29]. The FPR gene cluster has six members in the mouse, two of which, named mFPR and FPR2, appear to be functional counterparts of human FPR and FPRL1, respectively [29-31].

FPR AGONISTS

FPR N-formylated Peptide Agonists

Several *N*-formylated peptide chemoattractants have been purified from natural sources. As an example, the prototype *N*-formylated tripeptide, fMLF (Figure **1A**), is the major neutrophil chemotactic factor produced by *E. coli* [4]. Moreover, *N*-formylated peptides corresponding to the amino terminus of the murine mitochondrially encoded NADP dehydrogenase subunit 1 were found to trigger the chemotactic receptor [32]: this information suggested that the biologically relevant ligands for FPR were N- formylated peptides secreted by bacteria at sites of infection, or by mitochondria released from damaged tissues.

The interest in formyl peptides as chemoattractants and activators of leukocytes has led to several studies about the structural requirement for optimal ligand binding and cellular activation. A series of synthetic peptides related to fMLF has been systematically analysed on rabbit neutrophils [33,34]. The results of this analysis are the following:

(i) The formyl group of fMLF is essential for good biological activity. In fact, N-acetylation, the removal of the α-amino group or the replacement by an ethyl group results in a drastic loss of chemotactic potency.

(ii) The sulfur-containing side chain of methionine produces optimum activity of the tripeptide. Analogues containing other sulfur aminoacids (ethionine) were less active, as were a variety of analogues containing linear aliphatic, aromatic or branched aliphatic side chains at position 1.

(iii) Both linear aliphatic and branched aliphatic residues in postion 2 induce potent chemoattractants.

(iv) As far as position 3 is concerned, the Phe residue generates active chemoattractants, and the addition of a large, highly charged Lys residue allow the retention of a large degree of chemotactic activity.

(v) Tripeptide benzyl esters and the benzylamide derivative of fMLF have been found to be more active than their acid counterparts. Only peptide derivatives containing the C=O function of Phe have been found to display good biological activity.

It has subsequently been demonstrated that two N-formylated tetrapeptides with phenylalanine in position 3 (fMet-Ile-Phe-Leu and fMet-Leu-Phe-Ile) are full chemotactic agonists on human monocytes [5]. A similar behaviour toward human neutrophils has recently been confirmed for the free acid peptide derivative fMet-Ile-Phe-Leu (Figure **1B**) [35]. Moreover, it has been shown that the C-terminal methyl ester fMet-Ile-Phe-Leu homologues have an agonist power similar to that of the free acid derivative. The FPR affinity and activity values of these N-formyl-tetrapeptides are one order of magnitude higher than those of fMLF [35].

It has been demonstrated that synthetic pentapeptides Met-Nle-Leu-Phe-Phe, either N-formylated (Figure **1C**) or N-acetylated, are at least one order of magnitude more potent than fMLF, in evoking a transient alteration of Ca^{2+} concentration in human neutrophils [36]. The unacetylated form is also a good activator of neutrophil functions, even if it is two orders of magnitude less active than the acetylated homologue [36]. Similarly, the pentapeptide Met-Met-Trp-Leu-Leu has been identified as a quite active FPR agonist and its formylated form, f- Met-Met-Trp-Leu-Leu, is more potent than the classical FPR agonist fMLF [37].

Non N-formylated FPR Peptide Agonists

A series of amino-terminal carbamate analogues of fMLF- in particular unbranched carbamates such as methoxycarbonyl, ethoxycarbonyl and *n*-butyloxycarbonyl - have been demonstrated to be FPR agonists on human neutrophils. The agonist power of these fMLF homologues has however been found to be one or two orders of magnitude lower than that of the parent compound [38].

Aminoterminal urea-substituted modified MLF peptides have been shown to be FPR agonists on human neutrophils. It has been demonstrated that some of these (4-chloro phenyl-, 4-methoxyphenyl-, p-tolyl-ureido derivatives, Figure **1D**) are more potent agonists than fMLF [39].

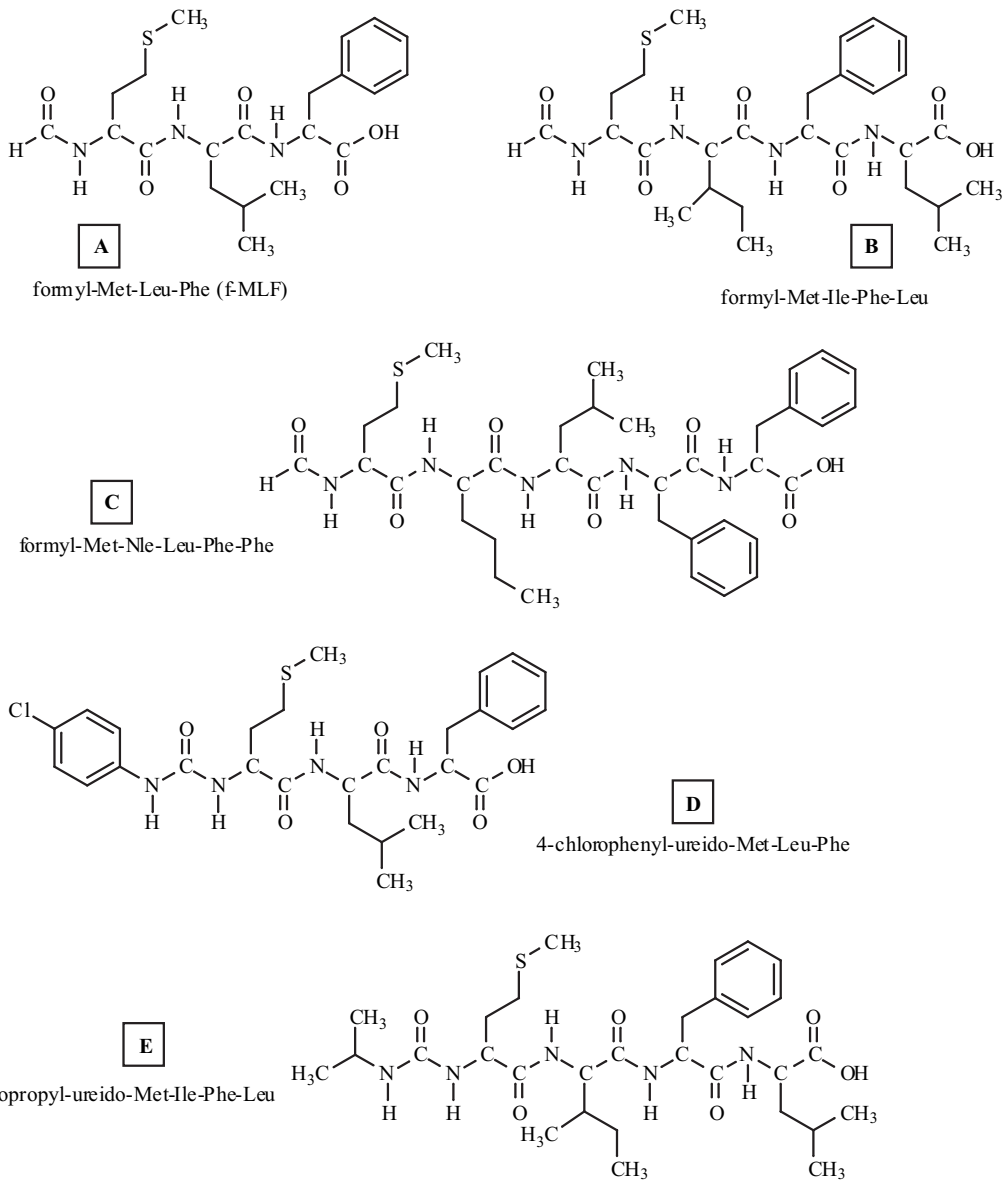


Fig. (1). FPR agonists.

N-ureido isopropyl-Met-Ile-Phe-Leu derivatives (Figure **1E)** have been shown to be weak partial agonists toward FPR on human neutrophils [35]. It has moreover been demonstrated that the agonist properties of these tetrapeptide derivatives are not noticeably influenced by C-terminal methyl esterification or by conversion to the corresponding amide [35].

Recently, the hexapeptide Trp-Lys-Tyr-Met-Val-D-Met has been reported to be a very potent stimulant of several human leukocytic cell lines, as well as of peripheral blood neutrophils [40-42]. It has been demonstrated that this hexapeptide exhibits an extraordinarily high efficacy on FPRL1 [43], and that it can act as an agonist toward mouse mFPR [44].

These results suggest that FPR interacts with a broad spectrum of agonists, and that the *N*-formyl group is not necessary for high-affinity interaction with FPR. In this context, peptide sequences derived from the HIV-1 protein gp41 have been shown to bind human FPR and FPRL1 [12,13,45].

FPR Peptide Agonists and Anti-HIV Drugs

A precursor of the envelope proteins of HIV-1 (gp160) is cleaved by proteinases to yield mature proteins gp120 and gp41 [46]. The viral envelope gp41 appears to be essential in the fusion of HIV-1 and host cell membranes [47]. It is known that the gp41 ectodomain contains two segments: one termed T21/DP107, in the NH_2 terminus, and the other termed T20/DP178, in the carboxyl terminus [48]. T21/DP107 and T20/DP178 correspond to the amino acid sequence 558-595 and 643-678, respectively, of gp41 [48,49]. Synthetic analogues of both TP21/DP107 (Ac-NNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQ-NH_2) and TP20/DP178 (Ac-YTSLIHSLIEESQNQQEKNE QELLELDKWASLWNWF-NH_2) have been shown to inhibit virus-mediated cell-cell fusion, and to reduce the

infectious titer of cell-free virus [49,50]. In particular, it has been reported that TP20/DP178 inhibits fusion completely at low nanomolar concentrations [49-51]. Because of its exceptionally efficacious anti-HIV-1 activity *in vitro*, T20/DP178 has been proposed for clinical trials, and is currently being tested as a novel type of anti-retroviral drug [52,53].

It has been observed that preexposure of human monocytes to HIV-1 envelope protein gp41 inhibits their chemotactic responses to the bacterial chemotactic peptide fMLF [54]. Such a phenomenon has been hypothesized to cause the reduced migratory response of monocytes from AIDS patients to a variety of chemoattractants [55,56]. The effects on human immune cells of the selected peptide segments of gp41, T21/DP107 and T20/DP178 have therefore been evaluated with the aim of further defining the structural basis for the capacity of HIV-1 envelope proteins to desensitize host cells [12,13,45]. The following results have been obtained:

(i) The synthetic T21/DP107 segment of protein gp41 has been shown to be a potent stimulant of migration and Ca^{2+} mobilization in human monocytes and neutrophils. This activity appeared pertussis toxin-sensitive, suggesting the involvement of G_i-coupled receptors. It has subsequently been demonstrated that T21/DP107 activates both human FPR and FPRL1, showing a much higher affinity for FPRL1. T21/DP107 has therefore been identified as a selective FPRL1 agonist, and it has been suggested that this peptide domain of the HIV-1 gp41 could be able to activate innate host immune responses interacting with FPR and FPRL1 on phagocytes [45].

(ii) It has been demonstrated that the synthetic T20/DP178 segment of protein gp41 is a potent FPR activator on human phagocytes [12]. Moreover, T20/DP178 has been shown to also be a potent chemotactic agonist at the human low affinity FPRL1, even if it has been concluded that FPR is the major phagocyte T20/DP178 receptor *in vivo* [13]. Moreover, it has been proposed that a mouse model could make it possible to study the effects of this gp41 protein fragment on immunity and, in particular, to define the cause of the local inflammation subsequent to T20/DP178 injection reported in Phase I clinical trials [13,53].

FPR ANTAGONISTS

The structural requirements for FPR antagonists do not appear extensively described. It has been reported that the *t*-Boc peptide derivative *t*-Boc-Phe-D-Leu-Phe-D-Leu-Phe (Figure **2A**) displays FPR antagonist activity on rabbit neutrophils [57]. In this context it has been suggested that the *t*-Boc group on peptide derivatives is essential for imparting FPR antagonist activity to rabbit neutrophils, even if it causes a loss in binding potency [33]. The ability to antagonize rabbit neutrophil functions has not been reported to be greatly dependent on the primary sequence (from tri- to penta- peptides) or chirality of peptide derivatives. On the other hand, the antagonist's capacity to interact with FPR has been shown to be much more influenced by such structural characteristics [58-61]. In particular, it has been demonstrated that the derivative *t*-Boc-Phe-D-Leu-Phe-D-Leu-Phe has FPR affinity one order of magnitude higher than that of *t*-Boc-Phe-Leu-Phe-Leu-Phe [61]. Results derived from further, more detailed studies on rabbit neutrophils indicate that the *t*-Boc-Phe-Leu-Phe-Leu-Phe-OMe peptide derivative can show a definite agonist activity, while the homologous *t*-Boc-Phe-D-Leu-Phe-D-Leu-Phe-OMe is a full antagonist [62]. Moreover, it has been demonstrated that the C-terminal methyl esterification reduces the ability of the penta peptide derivative to inhibit the release of glucosaminidase [24].

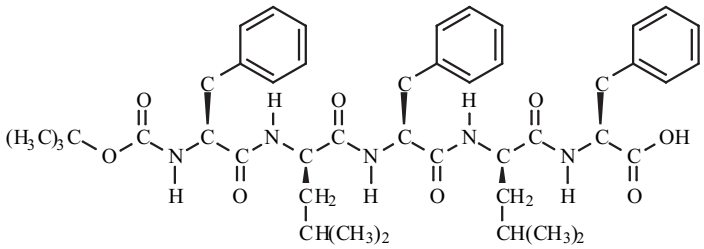
A series of amino-terminal carbamate analogues of fMLF, in particular branched carbamates such as *i*-Boc (Figure **2B**), *t-Boc* and bezyloxycarbonyl, have been demonstrated to be FPR antagonists on human neutrophils. The peptide antagonists were found to be more potent inhibitors of superoxide anion release than cell adhesion [38].

Aminoterminal urea-substituted modified MLF peptides have been shown to be FPR antagonists on human neutrophils. This is true for *N*-ureido substituents such as methyl-, ethyl-, *n*-propyl-, *iso*-butyl-, *tert*-butyl- and benzyl-ureido [39]. Moreover, it has been reported that *N*-ureido-Phe-D-Leu-Phe-D-Leu-Phe peptide derivatives (Figure **2C**) show an enhanced FPR affinity and antagonist power on human neutrophils, with respect to the tripeptide MLF homologues [39].

It has been investigated if *t*-Boc or *N*-ureido–aliphatic substituents in the Met-Ile-Phe-Leu chain (CHO-Met-Ile-Phe-Leu is a potent FPR full agonist) can induce an antagonist behaviour on human neutrophils [35]. In this context, the presence of *N*-isopropylureido substituent in the tetrapeptide chain has been found to impart weak partial agonist properties, whereas the *t*-Boc-Met-Ile-Phe-Leu derivative does not appear able to interact with FPR [35].

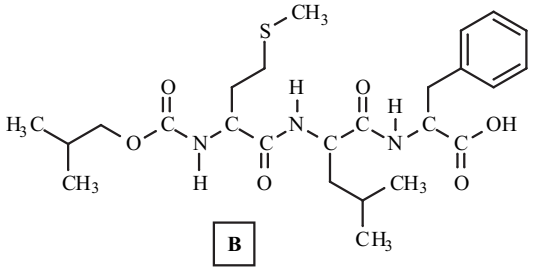
A series of free acid and methyl-ester Phe-D-Leu-Phe-D-Leu-Phe analogues, including either *N*-*t*-Boc or four different *N*-ureido substituents (for example see Figure **2D**), were analysed in detail on human neutrophils [63,64]. It has been demonstrated that these peptide derivatives are able to antagonise the multiple neutrophil functions evoked by fMLF, i.e. chemotaxis, O_2^- production and secretagogue activity. Also in this case, these peptide antagonists were found to be more potent inhibitors of superoxide anion release than of cell adhesion. Moreover, it has been shown that C-terminal methyl-esterification is detrimental to the FPR affinity and antagonist activity of these pentapeptide derivatives [63,64].

Annexin I peptides have recently been reported as novel, endogenous FPR ligands able to induce antiinflammatory effects [15]. The immunomodulatory activity of cyclosporins, proposed as cancer chemotherapeutic drugs, has been related to the inhibition of FPR functions [65].



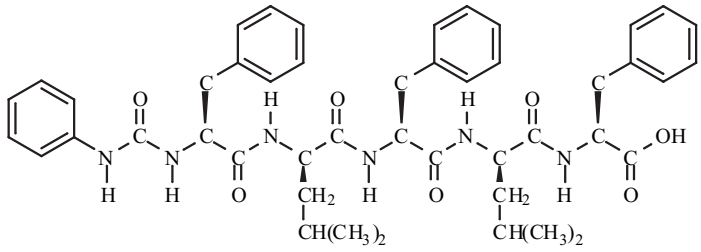
A

t-Boc-Phe-$_D$Leu-Phe-$_D$Leu-Phe



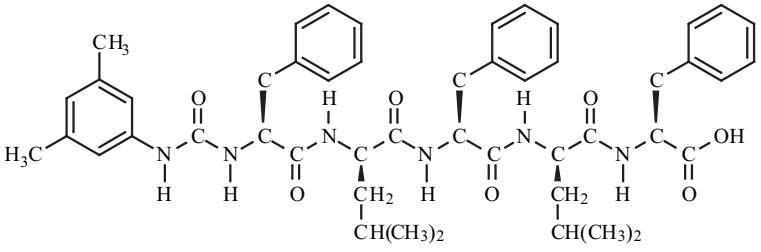
B

i-Boc-Met-Leu-Phe



C

phenyl-ureido-Phe-$_D$Leu-Phe-$_D$Leu-Phe



D

xilen-ureido-Phe-$_D$Leu-Phe-$_D$Leu-Phe

Fig. (2). FPR antagonists.

It should be stressed that analogues of the anti HIV peptides can act as FPR antagonists [12]. In particular, four synthetic T20/DP178 analogues which lack 3, 5, 7 and 12 amino acids at the N-terminus of the peptide have been demonstrated progressively reduce anti-HIV-1 efficacy *in vitro* and to act as FPR antagonists [12].

FORMYL PEPTIDE RECEPTOR MODELS

A first hypothetical model of the chemotactic peptide receptor of rabbit neutrophils was proposed in 1982 by Freer *et. al.*, on the basis of affinity and activity data obtained for fMLF and a series of related derivatives [34]. Five critical areas of drug-receptor interaction were proposed.

(i) The participation of the formyl group of peptide derivatives in H bonding to an H-bond acceptor in the receptor area.

(ii) The occupancy of the methionine side chain of position 1 of hydrophobic pocket, limited in depth and area, in the receptor.

(iii) The occupancy of the leucine side chain with a large hydrophobic area of the receptor.

(iv) The location of the phenylyalanine side chain in a hydrophobic area of limited depth.

(v) A critical interaction of the carbonyl group of the phenylalanine residue with the receptor, possibly via hydrogen bonding.

It was demonstrated in 1990 that FPR belongs to the family of G-protein coupled receptors, which are built around a common motif made up of seven transmembrane α-helices (domains), linked by intra and extracellular loops, with an extracellular N-terminus and an intracellular C-terminus [9,19]. Such a structure and its requirements for the binding to FPR have been analysed by using C5a-FPR chimeras [66]. On the basis of the results obtained by this study, a FPR model was proposed where domains and loops show the following organisation. The first, second, and third domains should be in the same plane as the membrane. The second extracellular loop contains a Cys^{98} that could form a disulfide bond with Cys^{176} of the third extracellular loop. Portions of the third and fourth extracellular domains would be posterior to the second extracellular loop, and should form a ligand pocket. The amino-terminal domain would provide a lid to the pocket [66].

A speculative model of FPR, using the crystallographic coordinates of bacteriorhodopsine as a template [67], has been proposed [68]. The presence of a disulphide bond between Cys^{98} and Cys^{176} was hypothesised. It has been suggested that the formyl group of fMLF makes important hydrogen bonding contacts with Thr^{103} and Asp^{106}; in addition, it has been proposed that the C-terminal carboxyl group interacts with the positively charged Lys^{170} on the fourth domain. This model is consistent with the structure-activity relationships previously described. As an example, the phenyl side chain of the ligand protrudes into a large hydrophobic pocket at the helix-loop interfaces, where large substituents are tolerated. Moreover, mutagenesis data with FPR chimeras supports the hypothesis that Thr^{103} and Lys^{170} are involved in ligand binding [69].

Other mutagenesis studies performed on FPR have indicated that Leu^{78} (domain II), Asp^{106}, Leu^{109} (domain III), Thr^{157} (domain IV), Arg^{201}, Ile^{204}, Arg^{205} (domain V), Trp^{254}, Tyr^{257} (domain VI) and Phe^{291} (domain VII) contribute to the receptor affinity toward f-Nle-Phe-Nle-Tyr-Lys-fluoroscein [70]. Of the above aminoacids, Asp^{106}, Arg^{201} and Arg^{205} have been shown to be involved in receptor G-protein coupling, suggesting that these residues may also contribute to signal transduction [70]. A 3D FPR model which takes this information into account has recently been proposed [71]. It has furthermore been demonstrated that the FPR selectivity for the binding of different NH_2-terminal analogs of Met-Met-Trp-Leu-Leu or MLF (as an example, N-formylated or unformylated homologues) can be altered by mutating Asp^{106} to asparagine or Arg^{201} to alanine. These mutations have been shown to also induce an enhanced ability of the receptor to bind the HIV-1 peptide T20/DP178 [72]. According to these overall data, it has been proposed that the most likely positioning of fMLF in the binding pocket of FPR is approximately parallel to the fifth transmembrane domain, with the formamide group of fMLF hydrogen-bonded to both Asp^{106} (as previously proposed [68]) and Arg^{201}. It has also been hypothesized that the leucine side chain of fMLF points toward the second transmembrane region, whereas the COOH-terminal carboxyl group is ion-paired with Arg^{205} [72].

ABBREVIATIONS

FPR = Formyl peptide receptor

FPRL1 = Formyl peptide receptor-like 1

FPRL2 = Formyl peptide receptor-like 2

mFPR = Mouse formyl peptide receptor

fMLF = Formyl-Met-Leu-Phe

i-Boc = *Iso*-butyloxycarbonyl

t-Boc = *Tert*-butyloxycarbonyl.

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