Selective Inhibition of *N*-Formylpeptide-Induced Neutrophil Activation by Carbamate-Modified Peptide Analogues[†]

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ABSTRACT: Stimulation of the leukocyte *N*-formylpeptide receptor (FPR) induces chemotaxis, cell adhesion, free radical release, and degranulation, responses associated with infection and inflammation. Under conditions where continuous activation of the receptor prevails, neutrophil-dependent tissue damage ensues. Antagonists of the FPR have potential for use as diagnostic and therapeutic agents. Hence, we have synthesized and evaluated a series of amino-terminal carbamate analogues of the peptide Met-Leu-Phe (MLF) in order to determine the structural requirements for imparting agonist or antagonist activity at the human neutrophil FPR. Peptides were evaluated in three *in vitro* assays: receptor binding, superoxide anion release, and cell adhesion. Unbranched carbamates (methoxycarbonyl, ethoxycarbonyl, and *n*-butyloxycarbonyl) resulted in agonist activity, whereas branched carbamates (*iso*-butyloxycarbonyl, *tert*-butyloxycarbonyl, and benzyloxycarbonyl) were antagonists. The peptide antagonists were more potent inhibitors of superoxide anion release than cell adhesion by 4−7-fold. When *iso*-butyloxycarbonyl-MLF (*i*-Boc-MLF) was further modified at the carboxy terminus with Lys, antagonist potency was retained but without functional selectivity. Further C-terminal modification with the radionuclide linker diethylenetriaminepentaacetic acid did not alter the potency of *i*-Boc-MLFK. These results indicate that the switch from agonist to antagonist activity can be achieved by modifying the overall size and shape of the amino-terminal group; that modifications at both the amino and carboxy termini can alter the functional selectivity of the peptide; and that modifications can be tolerated at the carboxy terminus to allow for development of an antagonist for diagnostic applications.

Leukocytes are key cellular constituents identified at both inflammatory and infectious sites. The discovery that the tripeptide *N*-formyl-Met-Leu-Phe (fMLF),[1] isolated from bacterial extracts, was a potent chemoattractant for rabbit neutrophils (Schiffmann et al., 1975) led to the identification and subsequent cloning of the human (Williams et al., 1977; Boulay et al., 1990) and rabbit (Aswanikumar et al., 1977a; Ye et al., 1993) G-protein coupled formylpeptide receptor (FPR). Stimulation of leukocytes through engagement of the FPR is considered one of the primary physiological responses to bacterial infection. Chemotactic peptide agonists have been shown to selectively image sites of infection in various animal models (Fischman et al., 1991; Babich et al., 1993a,b; Fischman et al., 1993) supporting a role for the FPR in infection. However, the role of FPR activation may not be limited to combating infection. Formylpeptides are not only derived from bacterial sources but also can be synthesized by mammalian cells. Disrupted mitochondria release formylpeptides that interact with the FPR, suggesting that these peptides play a role in the leukocyte response to tissue injury (Carp, 1982). Increased leukocyte expression of the FPR occurs during sepsis and trauma as well as during acute inflammatory responses (Tschaikowsky et al., 1993; Mori et al., 1994). The development of potent and selective FPR agonists and antagonists is therefore of considerable interest due to their potential use both for diagnostic imaging agents for localization of sites of infection and/or inflammation and for therapeutic agents to treat inflammation-related disorders.

The specificity of interaction between peptide ligands and the rabbit FPR has been studied extensively for agonist peptides (Showell et al., 1976; Freer et al., 1980, 1982). Key interactions between agonist peptides and the FPR involve both hydrogen bonding of the N-terminal formyl group as well as specific hydrophobic interactions between amino acid side chains and the FPR (Freer et al., 1982; Fay et al., 1993). The structural requirements for antagonist peptides are not well characterized, however. With the discovery of the FPR antagonist *t*-Boc-Phe-(D)-Leu-Phe-(D)-Leu-Phe (Aswanikumar et al., 1977b), it became apparent that antagonist activity did not depend greatly on primary sequence, chirality, or C-terminal esterification (Day et al., 1980; Freer et al., 1980; Toniolo et al., 1990). Furthermore, the carbamate linkage of the *t*-Boc-protected peptide was found not to be the key element for determining antagonist activity since the *t*-Boc but not the methoxycarbonyl or benzyloxycarbonyl derivatives of MLF possessed antagonist properties (Freer et al., 1980).

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[1] Abbreviations: f, *N*-formyl; M or Met, methionine; L or Leu, leucine; F or Phe, phenylalanine; Me, methoxycarbonyl; Et, ethoxycarbonyl; *n*-Boc, *n*-butyloxycarbonyl; *i*-Boc, *iso*-butyloxycarbonyl; *t*-Boc, *tert*-butyloxycarbonyl; Cbz, benzyloxycarbonyl; DTPA, diethylenetriaminepentaacetic acid.

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The effect of amino-terminal modification on peptide binding to the human FPR and the subsequent relationship of bindng to functional activity is not well characterized. In this report, we explored modifications of both the N-terminus and C-terminus of the tripeptide MLF to better understand the structure−activity modifications required to impart potent receptor binding and antagonist activity.

MATERIALS AND METHODS

Peptide Synthesis and Characterization. All carbamate-MLF derivatives were synthesized as their methyl esters by conventional solution-phase methods (Bodansky & Bodansky, 1984) using *t*-Boc N-terminus protection and carbodiimide coupling. Methionine was protected as the desired carbamate and introduced in the final coupling step. Free C-terminal acids were then obtained by treatment of the carbamate tripeptide methyl esters with 1 N LiOH followed by acidification with 1 N HCl to pH 3 and filtration of the resulting solid. *t*-Boc-MLF was purchased from Sigma (St. Louis, MO). fMLF was purchased from Calbiochem (San Diego, CA).

The tetrapeptide MLFK-NH_2 was synthesized by the solid-phase method (Atherton & Shepperd, 1989) using *t*-Boc amino acid derivatives, 2-chlorobenzyloxycarbonyl side chain protection on the Lys, and dicyclohexylcarbodiimide/hydroxybenzotriazole coupling on a benzhydrylamine resin. Isobutyloxycarbonyl-MLFK-NH_2 (*i*-Boc-MLFK) was synthesized as follows: After deprotection of the amino-terminal Met, the peptide was acylated with excess isobutylchloroformate and 2 equiv of diisopropylethylamine (DIEA) in methylene chloride. The resin was washed and dried, and then the peptide was cleaved from the resin with HF. The peptide was extracted from the resin with 10% acetic acid and recovered by lyophilization. The crude products were purified by reverse-phase HPLC (C18) with a gradient of acetonitrile in 0.1% trifluoroacetic acid. Acetylation of the carboxy terminus with DTPA was performed as previously described (Fischman et al., 1991). The structure of the purified peptides was confirmed by amino acid analysis, mass spectrometry, and NMR spectrometry.

Cell Preparations. Human neutrophils were isolated from venous blood obtained from healthy adult volunteers. Neutrophils were separated on a Ficoll gradient (lymphocyte separation media, Organon Teknika, Durham, NC) followed by dextran sedimentation (Pharmacia LKB, Uppsala, Sweden) (Boyum, 1964). Contaminating red blood cells were lysed in 0.15 M ammonium chloride solution. The entire isolation procedure was carried out at 4 °C. This method resulted in preparations containing ≥95% viable neutrophils as assessed by differential staining and trypan blue dye exclusion.

Cryopreserved first-passage human umbilical vein endothelial cells (HUVECS) were purchased from Clonetics Corporation (San Diego, CA). HUVECS were grown in endothelial growth medium (Clonetics) at 37 °C in a humidified 5% CO_2 atmosphere. Cells were plated on 48-well plates (Costar, Cambridge, MA) precoated with a sterile solution of 1.5% gelatin (Difco Laboratories, Detroit, MI) and grown to confluence. Only HUVECS at passages 1−3 were used in the adhesion assay.

Formyl-ML[^{3}H]F Receptor Binding Assay. Neutrophils (8×10^5) resuspended in buffer containing 1.7 mM KH_2PO_4, 8.0 mM Na_2HPO_4, 0.117 M NaCl, 0.15 mM $CaCl_2$, and 0.5 mM $MgCl_2$ were incubated with or without test peptides and 10 nM fML[^{3}H]F at 24 °C for 60 min in the absence (total binding) and presence (nonspecific binding) of 10 μM unlabeled fMLF (Pike & Snyderman, 1988). Cell-bound radioactivity was determined by liquid scintillation counting. Specific binding was defined as the difference between total binding and nonspecific binding.

Assay of Superoxide Anion Production. Superoxide anion release was assayed by monitoring the superoxide dismutase-inhibitable reduction of cytochrome *c*. Neutrophils resuspended in Hanks' balanced salt solution were incubated with 10 μM cytochalasin B, 40 μM cytochrome *c*, and 30 nM fMLF in the absence or presence of 50 μg/mL superoxide dismutase. Potential inhibitory peptides were added 10 min prior to fMLF stimulation. The reduction of cytochrome *c* was measured spectrophotometrically at an absorbance of 550 nm (Pike et al., 1986).

Neutrophil−Endothelial Cell Adhesion Assay. The adhesion assay was performed as previously described by Derian et al. (1995). Briefly, neutrophils (5×10^5 cells) labeled with 5-carboxyfluorescein diacetate (Molecular Probes, Inc., Eugene, OR) were added to each well of the confluent 48-well plates and a minimum of 6 wells on an additional plate without cells to determine maximal fluorescence. Maximal fluorescence was between 3000 and 4000 arbitrary fluorescence units for all experiments. Cells were preincubated with potential inhibitors for 10 min at 37 °C followed by the addition of fMLF for an additional 30 min. Adherent cells were lysed for 15 min in 0.1 N NaOH at 37 °C, and fluorescent intensity was determined using a Cytofluor 2300 plate reader (Millipore Corp., Bedford, MA) configured with a 485-nm excitation filter and a 530-nm emission filter.

Data Analysis. For determination of EC_{50} values, data were expressed as a percentage of the maximal response obtained with each individual peptide. Maximal responses were assigned the value of 100%. In each experiment, samples were run in duplicate or triplicate to account for intraassay variability. Typically, the standard errors were within 10−15% of the mean. IC_{50} values were obtained by comparing the percent response in the absence (100%) or presence of inhibitor. Both EC_{50} and IC_{50} values were calculated from the mean of individual dose−response curves using the curve-fitting program Data Squeezer for Lotus. Statistical analysis was performed using either Student's *t*-test or analysis of variance followed by the Scheffé test where appropriate. A p value less than 0.05 was considered significant.

RESULTS

Receptor Binding. All 11 carbamate-modified peptides exhibited dose-dependent competition in the receptor binding assay. For comparative purposes, the peptides fMLF-OH and *t*-Boc-MLF-OH were evaluated as representative agonist and antagonist standards, respectively. Dose−response curves for the series with C-terminal acids are shown in Figure 1. The IC_{50} values calculated from these curves are summarized in Table 1. Although none of the peptides exhibited the potency observed with fMLF-OH, both the *n*-Boc (**6**) and *i*-Boc (**8**) free carboxylate derivatives were approximately 10-fold more potent than the other carboxylate peptides studied. Since C-terminal modification with benzyl

Table 1: Effects of Peptides on Receptor Binding, Superoxide Anion Release, and Cell Adhesion[a]

peptide	sequence	receptor binding IC_{50} (μM)	superoxide anion release EC_{50} (μM)	superoxide anion release IC_{50} (μM)	cell adhesion EC_{50} (μM)	cell adhesion IC_{50} (μM)
1	fMLF-OH	0.033 ± 0.006 (16)	0.044 ± 0.001 (6)	NT[b]	0.013 ± 0.003 (3)	NT
2	Me-MLF-OH	3.2 ± 0.2 (2)	0.98 ± 0.14 (3)		0.47 ± 0.10 (3)	
3	Me-MLF-OMe	2.4 ± 1.2(2)	3.8 ± 2.3 (2)	20.0 ± 4.7 (3)	+[c]	8.3 ± 1.5 (4)
4	Et-MLF-OH	2.6 ± 0.9 (3)	1.22 ± 0.33 (3)		0.14 ± 0.01 (2)	
5	Et-MLF-OMe	0.20 ± 0.01 (3)	0.55 ± 0.25 (3)		0.26 ± 0.06 (3)	
6	*n*-Boc-MLF-OH	0.28 ± 0.07 (3)	0.17 ± 0.04 (3)		0.06 ± 0.01 (3)	
7	*n*-Boc-MLF-OMe	2.2 ± 1.2 (6)	1.03 ± 0.16 (4)	10.7 ± 1.9 (3)	0.36 ± 0.18 (2)	4.2 ± 0.9 (4)
8	*i*-Boc-MLF-OH	0.57 ± 0.25 (6)		0.25 ± 0.09 (2)		6.9 ± 1.4 (9)
9	*i*-Boc-MLF-OMe	2.7 ± 0.8 (6)		1.74 ± 0.81 (7)		6.9 ± 1.8 (7)
10	*t*-Boc-MLF-OH	4.5 ± 1.0 (4)		1.19 ± 0.32 (3)		4.0 ± 1.0 (3)
11	Cbz-MLF-OH	2.7 ± 0.8 (3)		0.42 ± 0.09 (3)		38% (3)[d]
12	Cbz-MLF-OMe	5.7 ± 2.1 (4)		18.3 ± 7.1 (3)		

[a] Blank spaces indicate no activity observed. Data are given as mean ± SE with the number of individual experiments indicated in parentheses. [b] NT, not tested. [c] Agonist activity noted (see text). [d] Percent inhibition at 30 μM.

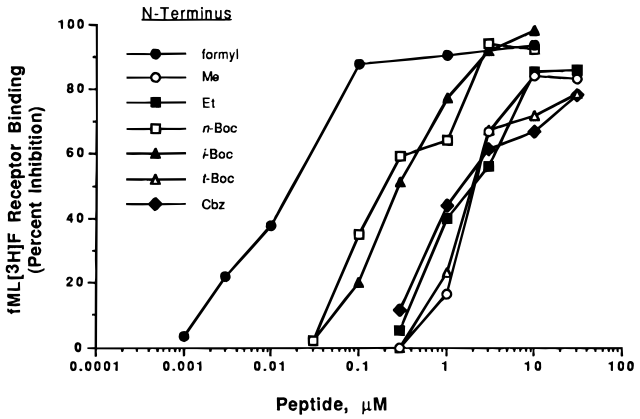

FIGURE 1: Receptor binding curves for carbamate-modified MLF-OH.

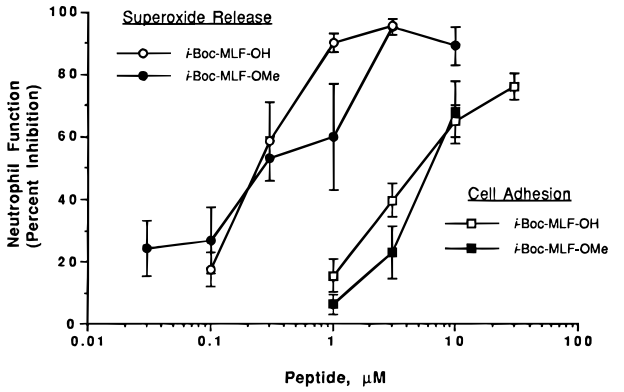

FIGURE 2: Inhibition of neutrophil superoxide anion release (circles) and cell adhesion (squares) by *i*-Boc-modified MLF.

esters has been shown to improve the potency of chemotactic tripeptides (Freer et al., 1982), corresponding C-terminal methyl esters were also synthesized and evaluated. The relative potency of C-terminally modified acids compared to their corresponding methyl esters appeared to be specific for each pair of N-terminally modified peptides. For example, for ethyl-MLF, the methyl ester (**5**) was 10-fold more potent than the free acid (**4**), whereas for *i*-Boc-MLF, the reverse was true (**8** and **9**).

Effect of Peptides on Superoxide Anion Release and Cell Adhesion. Two cell function assays were used to determine whether the peptides that competed in the binding assay possessed either agonist or antagonist activity:superoxide anion release and neutrophil adhesion to vascular endothelium. Peptides were evaluated for both agonist and antagonist activity. Peptides modified at the N-terminus with either methyl, ethyl or *n*-butyl carbamates (**2**−**7**) exhibited agonist activity in both assays when examined in the absence of fMLF (Table 1). In the presence of fMLF (studies for antagonist activity), the methyl ester derivatives of both methyl-MLF (**3**) and *n*-Boc-MLF (**7**) enhanced the effect of fMLF stimulation at concentrations below 1 μM, whereas at higher doses, the compounds antagonized the fMLF response. The latter response may be a result of agonist-mediated receptor downregulation by higher concentrations of the peptide or partial agonist activity.

Antagonist activity was observed with *i*-Boc- and Cbz-modified MLF. Both *i*-Boc derivatives **8** and **9** inhibited fMLF-induced activity in the functional assays (Table 1). No agonist activity was detected. Dose−response curves for these peptides are shown in Figure 2. Both peptides were significantly more potent in inhibiting superoxide anion release than neutrophil adhesion ($p < 0.05$). The potencies observed in the receptor binding assay more closely correlated with those observed for superoxide anion release than cell adhesion. The carbobenzoxy derivatives **11** and **12** had a more distinct profile. Both the free acid and methyl ester derivatives inhibited superoxide anion release, but minimal inhibitory activity was observed in the neutrophil adhesion assay.

Effect of C-Terminal Derivatization of i-Boc-MLF on Antagonist Properties. To determine the potential for developing an antagonist peptide for diagnostic imaging, *i*-Boc-MLFK-NH_2 (**14**) was synthesized. A C-terminal Lys amide allows for conjugation with the linker diethylenetriaminepentaacetic acid (DTPA), which can subsequently be radiolabeled with indium chloride. The tetrapeptide **14** exhibited weaker antagonist activity in all three assay systems compared to the corresponding tripeptides **8** and **9** (Table 2). No selectivity between superoxide anion release and cell adhesion was detected with the tetrapeptide, as was previously noted for the tripeptide. Acetylation of *i*-Boc-MLFK-NH_2 with DTPA (**15**) did not significantly alter the biological activity of the peptide (Table 2).

DISCUSSION

Previous structure−activity studies have demonstrated that N-terminal formylation of the chemotactic peptide MLF is required for agonist activity (Freer et al., 1980). Substitution of the formyl group of agonist peptides with *t*-Boc transforms

Table 2: Effect of Amino- and Carboxy-Terminal Modifications of the Tetrapeptide MLFK[a]

peptide	sequence	receptor binding IC_{50} (μM)	superoxide anion release EC_{50} (μM)	superoxide anion release IC_{50} (μM)	cell adhesion EC_{50} (μM)	cell adhesion IC_{50} (μM)
13	fMLFK-NH_2	0.007 ± 0.001 (2)	0.12 ± 0.02 (2)	NT[b]	0.016 ± 0.005 (3)	NT
14	*i*-Boc-MLFK-NH_2	9.3 ± 3.2 (8)		12.2 ± 3.3 (5)		16.1 ± 4.0 (3)
15	*i*-Boc-MLFK-DTPA	4.5 ± 0.5 (2)		10.3 ± 5.5 (3)		23.7 ± 3.3 (6)

[a] Blank spaces indicate no activity observed. Data are given as mean ± SE with the number of individual experiments indicated in parentheses. [b] NT, not tested.

agonist peptides such as fMLF and f-Phe-(D)-Leu-Phe-(D)-Leu-Phe to antagonist peptides (Aswanikumar et al., 1977b; Freer et al., 1980; Toniolo et al., 1990). We have synthesized and evaluated a series of N-terminal carbamate tripeptides based on the MLF sequence to study the N-terminal structural requirements for antagonists to the human FPR. This series of carbamate derivatives exhibited distinct agonist and antagonist profiles. Peptides functionalized with small unbranched carbamates, *i.e.*, methyl or *n*-Boc, were agonists. In contrast to *n*-Boc, *i*-Boc conferred antagonist activity similar to modification of peptides with *t*-Boc, the latter having increased bulk through branching. We found that the Cbz derivative exhibited antagonist activity in contrast to Freer et al. (1980). There is no obvious reason for this discrepancy other than species differences (rabbit versus human). Our results indicate that the overall size and shape of the N-terminal modifying group may be key to agonist versus antagonist activity. On the basis of our data, a carbamate with the appropriate size N-terminal extension can modify a peptide for antagonist activity.

The N-terminal blocking group of the chemotactic peptide MLF interacts with the receptor via both steric and electrostatic interactions. Hence, it follows that the overall composition of the blocking group, both the size and shape of its aliphatic or aromatic appendages, and the nature of the functional group at the N-terminus nitrogen may play major roles in the peptide's activity. Both the formyl and carbamate functionalities have relative nonbasic nitrogens and are capable of being hydrogen-bond acceptors via carbonyl oxygen or donors via the N−H. The most obvious difference between the two is the added bulk incurred with the carbamates, which may play the major role in the steric fit into the receptor. This, in turn, appears to impart a change in functionality. The weak agonist activity observed with unbranched carbamate-modified peptides supports a role for both N-terminal modification and peptide sequence in conferring potent agonist activity. Moreover, recent studies by Gao et al. (1994) have shown that N-terminal formylation is not an absolute requirement for potent agonist activity since nonformylated derivatives of the peptide MNleLFF maintained high agonist potency for the FPR.

Several mechanistic approaches have been used to describe the binding pocket for the FPR as well as the orientation of formyl peptides in the pocket. Studies employing peptide structure−activity (Freer et al., 1982), crystal structures (Edmundson & Ely, 1985; Wang et al., 1995), fluorescently tagged peptides (Sklar et al., 1990), and receptor chimeras (Quehenberger et al., 1993) have all indicated that the binding pocket is of limited depth, likely to accommodate four to five residues with the N-terminal formyl group oriented at the bottom of the binding pocket. Modifications of the N-terminus could therefore affect the way in which a peptide orients itself within the binding pocket and also have significant effects in imparting specific functional activity. As the nature of the binding pocket becomes more refined through mutagenesis and molecular modeling studies, our understanding of the interactions by which these carbamate-modified peptide confer agonist versus antagonist activity will be greatly enhanced.

The ability of fMLF to stimulate free radical release and degranulation (required for neutrophil adhesion via CD11b/CD18) requires different extents of receptor occupancy and intracellular signaling (Korchak et al., 1984). It is intriguing to speculate that this dissociation of functional responses involves interaction between the N-terminus of the peptide with the receptor followed by engagement of distinct signal transduction pathways. Whether or not an antagonist would have a similar effect on modulation of receptor activation is unknown. However, the enhanced potency we observed for inhibition of free radical release compared to adhesion by *i*-Boc, *t*-Boc, and Cbz derivatives of MLF would be consistent with the idea that the N-terminus can interact with the receptor in such a manner as to affect specific functional responses. In addition to the role of the N-terminus in imparting agonist versus antagonist activity to MLF, C-terminal extension also affected antagonist activity. Since the FPR binding pocket is capable of accommodating a tetrapeptide, it is logical to propose a role for such an interaction in imparting functional activity. Freer et al. (1982) proposed that C-terminal benzyl esters of tripeptides were interacting with the FPR to enhance agonist peptide activity by mimicking a fourth amino acid residue, a response not observed with tetrapeptide benzyl esters. When the C-terminus of *i*-Boc-MLF was extended by Lys, a significant loss in potency as well as functional selectivity was observed. These results suggest that occupancy of the fourth position of the ligand amino acid sequence, or lack thereof, is a key factor in determining modulation of select FPR-mediated responses.

The agonist and antagonist modifications seen in this series of carbamate-modified peptides suggest that subtle changes in the N-terminus can be used to impart antagonist behavior to chemotactic peptides. Further modifications of the N-terminus with additional functionalities may improve antagonist potency. In addition, the ability to maintain antagonist activity after C-terminal extension with Lys-DTPA suggests that the C-terminus is amenable to further modifications to improve diagnostic and therapeutic activities.

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