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Communications to the Editor

N-Terminus Urea-Substituted Chemotactic Peptides: New Potent Agonists and Antagonists toward the Neutrophil fMLF Receptor

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The directed migration of cells along a chemical concentration gradient (chemotaxis) is observed in both eukaryotic and prokaryotic cells. This process is triggered in human neutrophils by a variety of endogenous and exogenous chemotactic factors. In 1975, the bacterial isolate tripeptide *N*-formyl-Met-Leu-Phe (fMLF) was identified as a potent neutrophil chemoattractant,[1] or more specifically, a chemotactic peptide (cp). It is well established that cps bind to neutrophils via specific plasma-membrane receptors and that it is this interaction that initiates chemotaxis, which directs the cells to sites of infection and inflammation.[2] The receptor binding event also initiates the neutrophils' "metabolic burst", which includes the production of cytotoxic superoxide radicals and the release of proteolytic enzymes. These cellular processes comprise the immune system's first line of defense against invading prokaryotic organisms.[3]

fMLF receptor antagonists are of interest for use in diagnostic and therapeutic applications in the areas of inflammatory and infectious diseases.[4] Several laboratories have investigated the structural features required for cp agonist/antagonist activity, and a variety of natural and unnatural oligopeptide sequences have been prepared and screened.[5] Relatively little work has been done, however, on elaboration of the N-terminus of the wide range of cps reported. This is due in part to the fact that with typical oligopeptide cps N-formylation is generally thought to be required for maximum chemotactic activity, and significant N-terminus alterations result in decreased receptor binding.[5a] Indeed, the presence of a free amino group and acetylation at the N-terminus of MLF both result in drastic losses in agonist activity as compared to fMLF.[6] *N*-Carbamoyl-MLF (**1**, Figure 1, Table 1), which also displayed decreased agonist activity, has been reported as well.[5d] Incorporation of a *t*-Boc group onto the N-termini of cps (i.e., *t*-Boc-FDLFDLF[7,8]), however, imparts antagonist activity, albeit with a significant loss in binding potency. Alterations at the C-termini of cps appear to have less significant effects on binding affinity, with only the carbonyl functional group being required for potent activity.[5] Although the exact structure of an fMLF receptor−ligand complex has not yet been characterized, observations such as these suggest that the N-terminus of a cp interacts with the fMLF receptor. It then follows that any N-terminal blocking group on a cp would be expected to interact directly with the receptor via a combination of van der Waal and electronic interactions, which are further influenced by the degree of conformational flexibility imparted by each particular functional group. At the current level of knowledge on the structure of the fMLF receptor, however, it is difficult to predict how a cp's overall fit into the receptor pocket will be effected upon N-terminal functionalization.

There are large number of derivatizations at the N-terminus of a peptide that one can envision.[9] As mentioned earlier, carbamate-derivatized cps have been reported (i.e., *t*-Boc-MLF, a modestly active antagonist[6]) and several other representative *N*-carbamates have been prepared recently in our labs which were found to possess modest agonist or antagonist activity depending on the type of carbamate.[4] During the course of this work, we synthesized a series of new *N*-ureido-MLF and *N*-ureido-FDLFDLF cps which displayed unexpectedly

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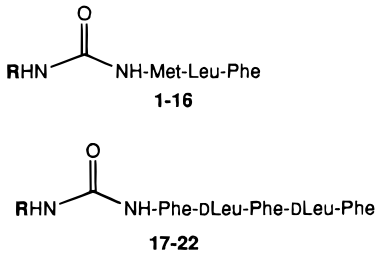


Figure 1. *N*-Ureido chemotactic peptides.

Table 1. fMLF-Receptor Binding and Biological Activity for Urea Tripeptides[a]

			superoxide production[a]	
compd	R	binding[a] (IC_{50}, μM)	agonist (EC_{50}, μM)	antagonist (IC_{50}, μM)
fMLF		0.02 ± 0.002	0.02 ± 0.002	
1	H	2 ± 1	3 ± 0.05	
2	methyl	3 ± 0.4	–	2 ± 1
3	ethyl	1 ± 0	–	3 ± 2
4	*n*-propyl	2 ± 1	–	3 ± 0.02
5	isopropyl	2 ± 0.08		5 ± 2
6	*n*-butyl	0.3 ± 0.09	0.3 ± 0.7	
7	isobutyl	1 ± 0.5	–	4 ± 0.5
8	*tert*-butyl	3 ± 2	–	2 ± 0
9	benzyl	0.8 ± 0.03		0.6 ± 0.2
10	phenyl	0.03 ± 0.007	0.04 ± 0.003	–
11	4-chlorophenyl	0.002 ± 0.001	0.001 ± 0	–
12	4-methoxyphenyl	0.002 ± 0.001	0.001 ± 0.001	–
13	*p*-tolyl	0.002 ± 0	0.002 ± 0.001	–
14	*m*-tolyl	0.1 ± 0.2	0.9 ± 0.02	1.6 (n = 1)
15	2-allyl	0.3 ± 0.03	0.4 ± 0.1	
16	1-adamantyl	0.9 ± 0.2		2 ± 0.7

[a] All values reported as mean ± SE for n = 2–3 independent experiments. Dashes indicate no activity at 30 μM.

potent fMLF-receptor agonist and antagonist activity. The results are reported herein.

Chemistry. All alkyl- and arylurea peptides (Figure 1) were prepared by reaction of the amino peptide with the appropriate isocyanate and 2 equiv of triethylamine in DMF.[10] The unsubstituted ureas **1** and **17** were prepared by reaction of the amino peptides with KNCO in glacial acetic acid. In most cases the crude urea product was recovered in 70–80% purity after acidic workup. The major impurity (typically ca. 15%) in this reaction was invariably the non-peptidyl symmetrical alkyl or aryl urea, which was separated by preparative HPLC and identified by ^{1}H NMR and mass spectral analysis. This byproduct most likely arises from hydrolytic decarboxylation of the isocyanate, followed by reaction of the resulting amine with another equivalent of isocyanate.[11] The symmetrical urea and other trace impurities were easily removed by simple recrystallization.

Biology. The urea cps were evaluated for fMLF-receptor binding and functional activity in two *in vitro* assays using human neutrophils, as described previously by Derian et al.[4] Firstly, the fMLF-receptor binding affinity of the cps was determined by measuring the extent to which they competed with tritiated fMLF. Agonist activity was determined by measuring a cp's ability to induce superoxide production (as measured by reduction of cytochrome C), while antagonist activity was determined by measuring a cp's ability to inhibit this process as stimulated by fMLF.

Results and Discussion: 1. *N*-Ureido-MLF derivatives. We first synthesized a number of *N*-ureido-MLF analogs (Figure 1), which encompassed a range of structural characteristics at the N-termini. All analogs were screened for fMLF-receptor binding affinity and agonist or antagonist activity in the two assays described above. For comparative purposes, fMLF was evaluated as the agonist standard. The results are tabulated in Table 1. In this series, modest *antagonist* behavior is seen when the urea is substituted with an aliphatic R group (compounds **2**–**8**). About a 100-fold decrease in receptor binding affinity was observed, however, as compared to fMLF. The chain length, degree of branching, or bulk of the alkyl tail appears to have little effect on the receptor binding or antagonist activity. Furthermore, it appears that the fMLF receptor may be larger (or less demanding) than previously thought, since even a bulky MLF urea such as adamantyl derivative **16** displayed modest receptor binding affinity. The fMLF receptor binding potencies observed here for the aliphatic urea cps were similar to those we reported earlier for the carbamate cps.[4] The smaller aliphatic urea cps (i.e., **2**), however, displayed antagonist activity, whereas the analogous carbamates (i.e., *N*-methoxycarbonyl-MLF), acted as agonists. It is unclear why in one isolated example in the aliphatic series, *n*-butylureido-MLF (**6**), more potent (ca. 10-fold) agonist behavior was observed.

If the R group was deleted, as in unsubstituted ureido-MLF **1**, agonist behavior was observed. This finding suggests that, in the aliphatic ureido-MLF series, the R group "tail" is required for antagonist activity and that the unsubstituted urea functionality may more closely resemble the smaller formyl group to the fMLF receptor, thus imparting agonist behavior. It is striking that replacement of the formyl hydrogen in fMLF with a simple NH_2 caused a 100-fold drop in binding potency.

The most potent activity in the ureido-MLF series was seen in compounds **10**–**13**, where several examples displayed receptor binding affinities greater than the fMLF control. The common structural feature among these is that they each contain a urea *directly* substituted with an aromatic R group. Notably, *para* substitution at the phenyl ring (i.e., **11**, **12**, and **13**) appears to lead to the highest potency. Compounds that exhibit binding affinities of this degree of potency (0.002 μM) are quite unexpected if one considers the well-accepted literature precedents which suggest that without N-formylation a loss in potency as compared to fMLF is unavoidable.[5a] Unlike the aforementioned aliphatic ureas, however, some examples (i.e., **10**–**13**) in this structural subclass act as potent *agonists*. We also found that subtle changes at the phenyl ring can produce a marked change in activity. Hence, while the *p*-tolylurea derivative, **13**, acted as a potent agonist, the *m*-tolylurea derivative **14**, displayed less potent mixed agonist/antagonist activity. Here, maximal agonist activity approximated only 70% of the fMLF response at 1 μM, above which antagonist activity was observed.

2. *N*-Ureido-FDLFDLF Derivatives. We next considered that N-terminus derivatization of the known cp, FDLFDLF, might produce compounds with higher binding affinities since a pentapeptide may have more points of contact with the receptor, thus rendering a tighter fit.[12] As an entry into this series, we prepared six ureido-FDLFDLF derivatives (Figure 1, Table 2). Indeed, all six analogs were found to be antagonists, and two examples, the isopropyl (**18**) and adamantyl (**21**) derivatives, had receptor binding affinities of about an

Table 2. fMLF-Receptor Binding and Biological Activity for Urea Pentapeptides[a]

			superoxide production[a]	
compd	R	binding[a] (IC_{50}, μM)	agonist (EC_{50}, μM)	antagonist (IC_{50}, μM)
17	H	0.8 ± 0.1	–	0.1 ± 0.01
18	isopropyl	0.2 ± 0.1	–	0.5 ± 0.3
19	*n*-butyl	0.3 ± 0.2	–	0.6 ± 1
20	phenyl	0.05 ± 0.01	–	0.5 ± 0.4
21	1-adamantyl	0.02 ± 0	–	0.3 ± 0.5
22	*m*-tolyl	0.2 ± 0.03	–	0.02 ± 0.0

[a] All values reported as mean ± SE for $n = 2-3$ independent experiments. Dashes indicate no activity at 30 μM.

order of magnitude higher than their corresponding MLF analogs, **5** and **16**, respectively. Perhaps most intriguing is the potent antagonist activity seen in urea cps **17**, **19**, and **20**. Since the analogous MLF analogs **1**, **6**, and **10** were agonists, it appears that switching from MLF to FDLFDLF while maintaining the same N-terminal urea modification produces a complete switch from agonist to antagonist activity. This reversal to antagonist behavior has been observed with all ureido-FDLFDLF analogs prepared to date.

Conclusion. There are some distinct differences at the N-terminus between the earlier reported carbamate cps and the urea cps described here. Hence, from our data it appears that changes produced by differences in hydrogen-bonding capability, conformation or the degree of rotational flexibility produce a distinct effect on the urea's fit into the fMLF receptor. Here we have shown that N-terminal urea cps display modest to potent fMLF receptor binding affinity and that agonist or antagonist behavior can be achieved depending on the nature of the R group of the urea. Perhaps the most important finding in this study, however, is that N-formylation of cps is not strictly required to attain potent fMLF receptor binding affinity and that the receptor is considerably more flexible than it was previously thought. Furthermore, by derivatizing FDLFDLF with the *N*-ureido functional group it appears that one can prepare fMLF-receptor antagonists exclusively, some with potencies comparable to fMLF.

Many additional ureas and other N-terminal functionalities can be introduced onto a cp which might also produce potent fMLF-receptor antagonism. It is also reasonable to consider that more potent antagonist behavior may be achieved by equipping any one of a variety of the reported unnatural oligopeptide cp sequences with a urea functionality. These two general approaches provide the framework for the preparation of many new and interesting cps.

References

(1) Schiffman, E.; Corcoran, B. A.; Wahl, S. M. N-formylated Peptides as Chemoattractants for Leukocytes. *Proc. Nat. Acad. Sci. U.S.A.* **1975**, *72*, 1059−1062.

(2) Becker, E. L. A Multifunctional Receptor on the Neutrophil for Synthetic Chemotactic Oligopeptides. *J. Reticuloendothel. Soc.* **1979**, *26*, 701−709.

(3) Harvarth, L. Neutrophil Chemotactic Factors. *In Cell Motility Factors*; Goldberg, I. D., Ed.; Birkhauser Verlag: Basel, Switzerland, 1991; pp 35−46.

(4) Derian, C. K.; Solomon, H. F.; Higgins, J. D. III; Beblavy, M. J.; Santulli, R. J.; Bridger, G. J.; Pike, M. C.; Kroon, D. J.; Fischman, A. J. Selective Inhibition of N-Formylpeptide-Induced Neutrophil Activation by Carbamate-Modified Peptide Analogues. *Biochemistry* **1996**, *35*, 1265−1269.

(5) (a) Freer, R. J.; Day, A. R.; Muthukumaraswamy, N.; Pinon, D.; Wu, A.; Showell, H.; Becker, E. Formyl Peptide Chemoattractants: A model of the Receptor on Rabbit. *Biochemistry* **1982**, *21*, 257−263. (b) Dentino, A. R.; Raj, P. A.; Bhandary, K. K.; Wilson, M. E. Role of Peptide Backbone Conformation on the Biological Activity of Chemotactic Peptides. *J. Biol. Chem.* **1991**, *266*, 18460−18468. (c) Torrini, I.; Paglialunga, P.; Zecchini, G.; and Lucente, G. New Amino Acids for Peptides of Biological Interest: 2[2′-(methylthio)ethyl] Methionine Derivatives. *Synth. Commun.* **1994**, *24* (2), 153−58. (d) Toniolo, C.; Crisma, M.; Becker, E. L. Replacement of the N-blocking Group in the Formyl-methionyl Tripeptide Chemoattractant: An Insight into the Mode of Binding at the Receptor on Rabbit Neutrophils. *Il Farmaco* **1990**, *45*, 921−925.

(6) Freer, R. J.; Day, A. R.; Radding, J. A.; Schiffman, E.; Aswanikumar, S.; Showell, H. J.; Becker, E. Further Studies on the Structural Requirements for Synthetic Peptide Chemoattractants. *Biochemistry* **1980**, *19*, 2404−2410.

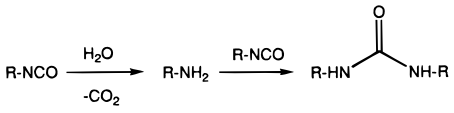
(7) Aswanikumar, S.; Corcoran, B.; Schiffman, E.; Pert, C. B.; Morell, J. L.; Gross, E. Peptides with Agonist and Antagonist Chemotactic Activity. In *Peptides*; Goodman, M., Meinhofer, J., Eds.; Wiley and Sons: New York, 1977; pp 141−145.

(8) Day, A. R.; Pinon, D.; Muthukumaarswamy, N.; Freer, R. J. Synthesis of Several Chemotactic Peptide Antagonists. *Peptides* **1980**, *1*, 289−291.

(9) Green, T. W. *Protective Groups in Organic Synthesis*; Wiley: New York, 1981; pp 223−249.

(10) As a general example, the synthesis of **18** is described as follows: 100 mg (0.12 mmol) of FDLFDLF TFA were dissolved in 5 mL of dry DMF, and 34 μL (0.25 mmol) Et_3N was added followed by 18 μL (0.19 mmol) of isopropyl isocyanate. The reaction mixture was stirred at room temperature for 16 h, after which the DMF was removed on a rotoevaporator. The resulting residue was dissolved in 1 mL of methanol, and 15 mL of 1 N HCl was added with rapid stirring. The crude urea was collected on a frit, washed with water, and recrystallized from $EtOH/H_2O$ (0.075 g, 81%): ^1H NMR (DMSO, d_6) δ 8.38 (d, $J = 8.8$ Hz, 1H), 8.20 (d, $J = 8.4$ Hz, 1H), 8.10 (d, $J = 9.2$ Hz, 1H), 8.02 (d, $J = 8.5$ Hz, 1H), 7.20 (m, 15H), 5.98 (d, $J = 7.3$ Hz, 1H urea NH), 5.98 (d, $J = 7.4$ Hz, 1H, urea NH), 4.40 (m, 5H), 3.37 (m, 1H, urea *N*-isopropyl CH), 2.80 (m, 6H), 1.06 (m, 6H), 0.94 (d, $J = 6.5$ Hz, 6H, urea *N*-isopropyl CH_3), 0.65 (m, 12H); FABMS *m/z* 771 (M^+); HPLC (C_{18}: CH_3CN/H_2O, 0.1% TFA) t_R 13.49 m, RPA 96%.

(11) Possible route to bisurea impurities:



(12) Sannomya, P.; Craig, R. A.; Clewell, D. B.; Suzuki, A.; Fujino, M.; Till, G. O.; Marasco, W. A. Characterization of a class of nonformylated *E. faecalis*-derived neutrophil chemotactic peptides: The sex pheromones. *Proc. Nat. Acad. Sci. U.S.A.* **1990**, *87*, 66−70.

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