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Bioorganic & Medicinal Chemistry Letters 17 (2007) 1943–1945
Bioorganic & Medicinal Chemistry Letters

Anti-formyl peptide antibodies

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Received 20 December 2006; accepted 9 January 2007
Available online 1 February 2007

Abstract—Antibodies that selectively bind to *N*-formylmethionyl leucyl phenylalanine (fMLF, also known as fMLP) have been generated. These antibodies bound to fMLF with higher affinity than to non-formylated peptide MLF: the differences in the binding energies between fMLF and MLF were 1.4–>2.1 kcal/mol.

N-Formyl peptides, such as *N*-formylmethionyl leucyl phenylalanine (fMLF, also known as fMLP), are derived from degradation of mitochondrial proteins or of bacterial proteins and are potent chemotactic factors for leukocytes.[1,2] In bacteria, protein synthesis starts with an *N*-formylmethionine and as a result all newly synthesized bacterial polypeptides possess a formylated N-terminus.[3] Although the majority of formylated peptides are deformylated before yielding mature functional proteins,[4,5] bacteria secrete *N*-formyl peptides.[1,2,5] In eukaryotes, such as humans, protein synthesis in organelles (mitochondria and plastids) initiates with *N*-formylmethionine whereas protein synthesis in the cytoplasm does not.[3] When microbial infection or tissue damage occurs, *N*-formyl peptides are released into affected area. *N*-Formyl peptides bind to formyl peptide receptors and initiate signals that result in defense against bacteria and wound healing.

Although binding of *N*-formyl peptides to formyl peptide receptors is important for the defensive responses and healing, excess *N*-formyl peptides cause inflammation.[2,6] Unwanted inflammation caused by *N*-formyl peptides may be suppressed by removing the *N*-formyl peptides. Diseases caused by a toxic molecule have been successfully treated by antibodies that bind to the toxic molecule.[7] Antibodies that selectively bind to *N*-formyl peptides should be useful for removing excess *N*-formyl peptides; these antibodies that neutralize unwanted *N*-formyl peptides should bind to *N*-formyl peptides but not to non-formylated peptides. Non-formylated peptides and proteins are abundant in biological systems; antibodies for neutralization of *N*-formyl peptides must bind to *N*-formyl peptides discriminating from non-formylated peptides and proteins. Here we report generation of antibodies that selectively bind to fMLF and analyses of binding features of these antibodies.

To generate antibodies that bind to *N*-formyl peptides, the formyl peptide derivative **1**-KLH[8] (KLH = keyhole limpet hemocyanin) shown in Figure 1 was used for immunization of mice. This conjugate included the *N*-formylmethionyl leucyl group and KLH was attached from the side chain of the third residue via a linker. Using typical hybridoma technology[9] and binding selection with **1**-BSA[10] (BSA = bovine serum albumin), 17 monoclonal antibody IgGs that bound to **1**-BSA in an enzyme-linked immunosorbent assay (ELISA) were obtained. These antibodies were evaluated for ability to bind to **2**[11] by inhibition ELISA (also known as competitive ELISA) using **1**-BSA and **2**. Eight antibodies bound to **2**; the binding of these antibodies to **1**-BSA was inhibited in the presence of **2**. These eight antibodies were next tested for their ability to bind to fMLF by inhibition ELISA using **1**-BSA and fMLF. Of these eight antibodies, four antibodies, FTD2F2, FTD4H10, FTD6C3, and FTD6G5, bound to fMLF. Although these four antibodies bound to both **2** and fMLF, they

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doi:10.1016/j.bmcl.2007.01.082

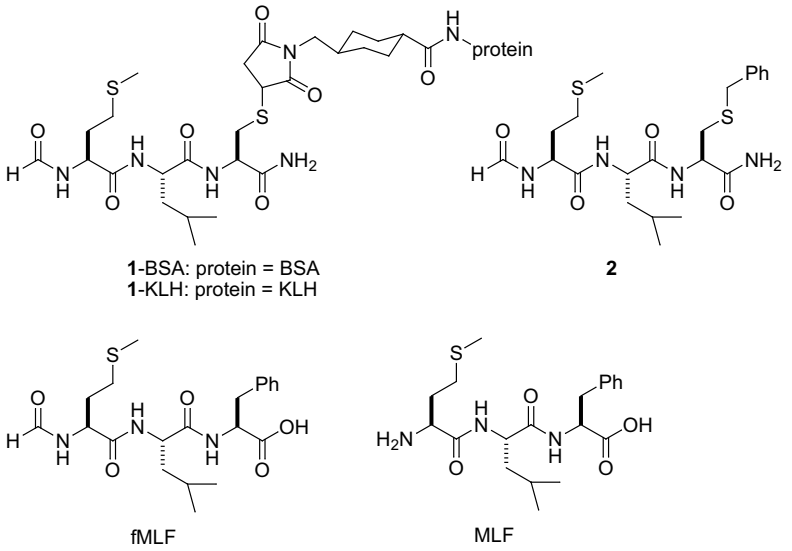

Figure 1. *N*-Formyl peptide conjugates **1**-BSA and **1**-KLH, *N*-formyl peptides **2** and fMLF, and non-formylated peptide MLF used for generation and characterization of antibodies that selectively bound to *N*-formyl peptides. BSA, bovine serum albumin. KLH, keyhole limpet hemocyanin.

did not efficiently bind to the non-formylated peptide methionyl leucyl phenylalanine (MLF) as evaluated by inhibition ELISA using **1**-BSA and MLF.

The apparent K_d values of the four antibodies that bound to fMLF and **1** were determined by inhibition ELISA and are shown in Table 1. Figure 2 shows a representative assay with antibody FTD6G5. Inhibition ELISA of an antibody IgG often gives lower affinity values (higher K_d values) than true binding because of the avidity of the bivalent IgG.[12] However, the ratio of the K_d values can be used to analyze the differences of the binding.[12] These antibodies bound to fMLF with $\geqslant$10-fold higher affinity than to MLF. Although these antibodies show better binding affinity to **2** than to fMLF, they efficiently bound to both **2** and fMLF. The differences in the binding energies between fMLF and MLF were 1.4–>2.1 kcal/mol for these antibodies. These results indicate that the formyl group of fMLF is recognized by the antibodies.

In summary, we have generated antibodies that selectively bind to *N*-formyl peptides. These antibodies will be useful for starting points to develop humanized versions of antibodies[13] that selectively bind to *N*-formyl peptides and that can be used as therapeutics to cure inflammation caused by *N*-formyl peptides. Crystal structure-based analysis of the mechanism of the selective recognition of the formyl group of fMLF by antibody FTD6G5 will be reported in the near future.

Table 1. Apparent dissociation constants of antibodies for **2**, fMLF, and MLF[a]

Antibody	K_d of **2** (μM)	K_d of fMLF (μM)	K_d of MLF	[1/(K_d of fMLF)]/[1/(K_d of MLF]	$\Delta\Delta G$[b] (kcal/mol)
FTD2F2	1	20	200 μM	10	1.4
FTD4H10	30	200	>2 mM	>10	>1.4
FTD6C3	5	30	>1 mM	>33	>2.1
FTD6G5	14	100	>2 mM	>20	>1.8

[a] Dissociation constants were determined by inhibition ELISA. K_d was determined as the concentration of compound required to inhibit 50% of the maximal binding in the inhibition ELISA.

[b] $\Delta\Delta G = -RT\ln[(K_d \text{ of fMLF})/(K_d \text{ of MLF})]$.

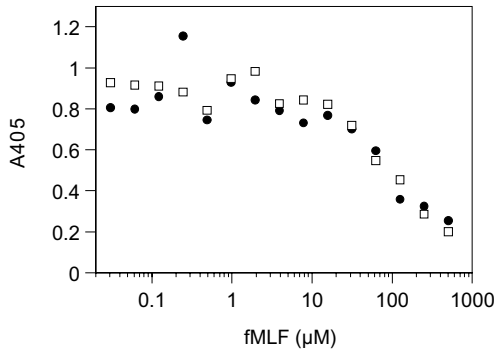

Figure 2. Inhibition ELISA of FTD6G5 with fMLF. Duplicated experiments are shown. Wells of a plate were coated with **1**-BSA and the ELISA of antibody FTD6G5 was performed in the presence of fMLF. Bound antibody FTD6G5 was detected by peroxidase-conjugated anti-mouse IgG and peroxidase substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). *Y*-axis is absorption at 405 nm without background correction.

Acknowledgment

This study was supported in part by The Skaggs Institute for Chemical Biology.

References and notes

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8. Preparation of **1**-KLH. To a solution of *N*-formylmethionyl leucyl cysteine amide (*N*-formyl-MLC-NH_2, 0.75 mg, 1.9 mmol) in DMSO (30 μL)-50 mM Na phosphate, pH 7.2 (30 μL), a solution of maleimide activated KLH (2.4 mg) in 50 mM Na phosphate-150 mM NaCl-100 mM EDTA, pH 7.2 (240 μL), was added at room temperature. After 1 day, the mixture was purified by Sephadex G-25M gel filtration column (PBS) to give **1**-KLH.
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10. Preparation of **1**-BSA. To a solution of *N*-formyl-MLC-NH_2 (1.0 mg, 2.6 mmol) in DMSO (40 μL)-50 mM Na phosphate, pH 7.2 (40 μL), a solution of maleimide activated BSA (2.0 mg) in 50 mM Na phosphate-150 mM NaCl-100 mM EDTA, pH 7.2 (200 μL), was added at room temperature. After 1 day, the mixture was purified by Sephadex G-25M gel filtration column (PBS) to give **1**-BSA.
11. Synthesis of **2.** A mixture of *N*-formyl-MLC-NH_2 (19.8 mg, 0.050 mmol), benzyl bromide (7.0 μL, 0.059 mmol), and Cs_2CO_3 (22.4 mg, 0.069 mmol) in DMF (0.5 mL) was stirred for 18 h at room temperature. The mixture was added to 0.5 N HCl and extracted with ethyl acetate. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. Generated solids were collected and washed with $CHCl_2$-MeOH to give **2** (9.2 mg, 38%) as a colorless solid. HRMS: calcd for $C_{22}H_{34}O_4N_4S_2Na$ (MNa^+) 505.1914, found 505.1896.
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