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SMALL PEPTIDE INHIBITORS OF $\alpha_4\beta_7$ MEDIATED MAdCAM-1 ADHESION TO LYMPHOCYTES

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Abstract

Specificity of leukocyte trafficking to intestinal mucosal tissues is largely mediated by MAdCAM-1/$\alpha_4\beta_7$ interaction. A series of small peptides was prepared to establish L-D-T-S-L as a recognition motif involved in the $\alpha_4\beta_7$ interaction. N-Terminus acylation of the L-D-T-S-L motif has led to low micromolar inhibitors of leukocyte adhesion. Copyright © 1996 Elsevier Science Ltd

Inflammation is characterized by infiltration of the affected tissue by leukocytes, such as lymphocytes, lymphoblasts, and mononuclear phagocytes. The remarkable selectivity by which leukocytes preferentially migrate to various tissues during both normal circulation and inflammation results from a series of adhesive and activating events involving multiple receptor-ligand interactions.[1,2] Initially, there is a transient, rolling interaction between leukocytes and endothelium, resulting from the interaction of selectins with their carbohydrate ligands.[3] This is followed by activation mediated by chemokines and their receptors, which trigger the firm adhesion of leukocytes to endothelium via leukocyte integrins and their endothelial ligands, the Ig-like receptors. Subsequent transendothelial migration from the circulation across the vascular endothelium is mediated by chemokine concentration gradients to the site of the inflammation.

MAdCAM-1 (**M**ucosal **Add**ressin **C**ell **A**dhesion **M**olecule-**1**) an immunoglobulin superfamily adhesion molecule for lymphocytes, is preferentially expressed in the gastrointestinal tract, specifically binds the lymphocyte $\alpha_4\beta_7$ integrin and participates in the homing of these cells to these mucosal sites.[4,5] In a non human primate model of IBD, a blocking antibody to $\alpha_4\beta_7$ has demonstrated both a clinical and histologic improvement in inflammatory activity and disease.[6]

The N-terminal domain (domain 1) of MAdCAM-1 has homology to the N-terminal integrin-binding domains of ICAM-1 and VCAM-1.[4] Mutagenesis studies in domain 1 have shown that amino acid residues within a short motif with the consensus sequence, G-(I/L)-(D/E)-(T/S)-(P/S)-L, are essential for integrin binding in other Ig-like adhesion molecules including domain 1 of ICAM-1, ICAM-2, and ICAM-3, and domains 1 and 4 of VCAM-1. The conserved motif in VCAM-1, Q-I-D-S-P-L, appears to be highly exposed on the N-terminal portion

of the CD loop of the first Ig domain[7,8] in a position readily accessible to bind integrins. Incorporation of this VCAM recognition motif, Q-I-D-S-P, into a cyclic peptide mimicking the C-D loop in domain 1, led to a small peptide inhibitor of VCAM/ $\alpha_4\beta_1$ mediated leukocyte adhesion.[8] Based upon the importance of this short motif, its presence in domain 1 of murine MAdCAM-1, and the observation that a single point mutation of murine MAdCAM-1 (L/R61) abolished MAdCAM-1 interactions with resting lymphocytes expressing $\alpha_4\beta_7$, it has been suggested that murine MAdCAM-1 also requires this L-D-T-S-L motif for binding to the $\alpha_4\beta_7$ integrin.[9]

We wish to report sequential epitope studies that further support L-D-T-S-L as a recognition or binding motif required for MAdCAM-1/$\alpha_4\beta_7$ interactions. We have also synthesized modified peptide analogues that based upon this sequence inhibit lymphocyte cells from adhering to MAdCAM-1.

Peptide Synthesis

Peptides were synthesized utilizing solid-phase peptide synthesis methodology,[10,11] with Fmoc/*t*-Bu chemistry on a Gilson 422 automated multiple peptide synthesizer starting with Rink Amide Am resin (50 mg, 0.5 mmol/g). Acylations were carried out twice with 10 equivalents of Fmoc-(9-fluorenylmethoxycarbonyl) amino acid, 10 equiv of HBTU [2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate], and 20 equiv of 4-methyl-morpholine or alternatively 10 equiv of PyBOP (benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluoro-phosphate) and 20 equiv of 4-methyl-morpholine using 20-45 min per coupling. For the final acylation, the Fmoc-amino acid was substituted with an appropriate organic acid such as acetic acid, or pyrenebutyric acid. Fmoc deprotection was carried out with 2% DBU and 10% piperidine in DMF for 24 min. Treatment with Reagent-R (TFA-EDT:thioanisole:anisole, 90:5:3:2) for 2 h was used to deblock and remove the peptides from the resin. The peptides were then precipitated from ether and lyophilized from HOAc. Peptides synthesized were analyzed for purity by reverse phase HPLC (deltapak C18, 5 mm column, eluted with a linear gradient over 30 min period of 0.1% TFA in CH_3CN and 0.1% TFA in water from 1:0 to 0:1 with flow rate of 1 mL/min) and mass spectral analysis by matrix-assisted laser desorption ionization time of flight mass spectrophotometer (MALDI-1, TOF, Kratos, Inc.).

Cell Adhesion Assay.

A cell adhesion assay involving the T-cell lymphoma, HUT78 cells activated with Mn^{+2} and soluble murine MAdCAM-1, produced in a baculovirus expression system, was used in a 96-well format.[9,12,13] Hut 78

cells have previously been shown to bind murine MAdCAM-1 in the presence of Mn^{+2} as an integrin activator. The extracellular domain of MAdCAM-1 was fused to a constant region of the murine κ light chain and cloned into a baculovirus shuttle vector. Fusion proteins produced from these vectors constructs contained the integrin binding sequences of murine MAdCAM-1 at the N-terminus, and the mCκ sequence at the carboxy-terminus. Recombinant baculovirus was harvested from infected SF9 insect cells and the fusion protein was detected from supernatents of infected SF9 cells by ELISA using a horseradish peroxidase-linked polyclonal mCκ antibody and chromogenic substrate according to standard protocols. Recombinant protein was also verified by immunoprecipitation with antimurine MAdCAM-1 monoclonal antibody, MECA-367.

HUT78 cells were fluorescently labeled by preincubation with BCEF stain (Molecular Probes) washed, and resuspended in assay buffer containing 1% DMSO and 2 mM Mn^{+2}. Antibodies or compounds were tested in HBSS/2% FCS/25 mM HEPES buffer at 2.5 X 10^6 cells per mL. The typical assay consisted of a final volume of 200 μL containing 50 μL of cells at 1.25 X 10^5 cells per well. Adhesion assays for MAdCAM-1 were washed on an automatic plate washer using a wash buffer consisting of 50 mM Tris/2mM $MnCl_2$, pH 7.2, in a wash volume of 500 μL for 2 wash cycles. Assays were then read on an Idexx fluorescent plate reader at 485/535 ηm. Inhibition was determined by the number of cells adhering to the plates in the presence and absence of an inhibitor and IC_{50} values (the concentration of inhibitor required to prevent 50% of cells from adhering to MAdCAM-1 plates) were determined using Kaleidoscope (Adelbeck Software) and are reported as an average of multiple determinations.

Discussion

The sequence, G-L-D-T-S-L, has been suggested to be important for the MAdCAM-1/$\alpha_4\beta_7$ interaction based upon an analogy to other adhesion molecules and point mutation studies.[8] We first synthesized peptides **1-5** that possessed 5-15 residues corresponding to the murine MAdCAM sequence. Each of peptides containing L-D-T-S-L were inhibitors of MAdCAM-1/$\alpha_4\beta_7$ mediated leukocyte adhesion. We then synthesized a set of 43 overlapping octapeptides covering the region of residues 22-70 of murine MAdCAM-1,[4] which included the L-D-T-S-L motif. These peptides were evaluated as potential inhibitors of MAdCAM-1/$\alpha_4\beta_7$ mediated leukocyte adhesion. The L-D-T-S-L motif is the most significant inhibitory sequence within this region as only peptides **7-12** were inhibitors of cell adhesion. Individual residue replacement within Ac-LDTSL-NH_2, analogues

14-22, had a significant effect on inhibition confirming that these residues are important for MAdCAM/$\alpha_4\beta_7$ interactions.

Table 1. Inhibition of MAdCAM-1 / $\alpha_4\beta_7$ Mediated Cell Adhesion

Cpd	Peptides	% Inhibition at 500 μM[a]
1	Ac-R V H W R G **L D T S L** G S V Q-NH_2	88
2	Ac-H W R G **L D T S L** G S V-NH_2	71
3	Ac-W R G **L D T S L** G S-NH_2	71
4	Ac-R G **L D T S L** G-NH_2	75
5	Ac-**L D T S L**-NH_2	71
6	Ac-R V H W A G L **D**-NH_2	0
7	Ac-V H W R G L **D** T-NH_2	48
8	Ac-H W R G L **D** T S-NH_2	46
9	Ac-W R G L **D** T S L-NH_2	52
10	Ac-R G L **D** T S L G-NH_2	36
11	Ac-G L **D** T S L G S-NH_2	28
12	Ac-L **D** T S L G S V-NH_2	16
13	Ac-**D** T S L G S V Q-NH_2	0
14	Ac-**A** D T S L-NH_2	3
15	Ac-L **A** T S L-NH_2	0
16	Ac-L **N** T S L-NH_2	0
17	Ac-L **dD** T S L-NH_2	0
18	Ac-L **Y** T S L-NH_2	0
19	Ac-L D **S** S L-NH_2	38
20	Ac-L D **V** S L-NH_2	85
21	Ac-L D T **T** L-NH_2	100
22	Ac-L D T S **F**-NH_2	86
23	Ac-**A A A** S L-NH_2	0

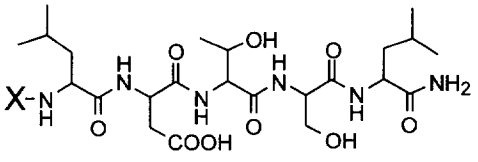
[a] Inhibition was determined by the number of cells adhering to the MAdCAM-1 plates in the presence and absence of an inhibitor. Values are reported as an average of multiple determinations.

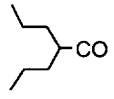
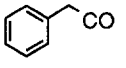
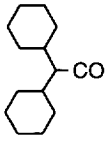
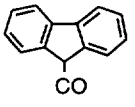
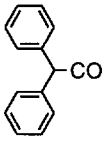
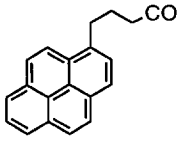
[b] dD represents D-aspartic acid.

Further modifications of Ac-LDTSL-NH_2, **5**, were made to identify possible accessory binding regions adjacent to the N-terminus region that could increase inhibitor potency. We have found that increasing size of the acyl group from acetyl (**5**) to diphenylacetyl (**26**) and 4-(1-pyrene)-butyryl (**29**) moieties improved inhibitor potency up to 50-fold suggesting that a hydrophobic binding pocket exists at the N-terminus region of the recognition motif. The change from diphenylacetyl (**26**) to fluorene carbonyl (**28**) did not improve activity. The aliphatic acyl analogues, **24** and **25**, were inactive. (Table 2)

Based upon our studies, it appears likely that finding small molecule inhibitors of rather large protein-protein interactions such as with MAdCAM-1/$\alpha_4\beta_7$ mediated leukocyte adhesion may be possible. Through structural modification of the recognition motif, L-D-T-S-L, low micromolar inhibitors of leukocyte adhesion have been identified.

Table 2. N-Acylated **X**-L D T S L Analogs



Cpd	X	IC_{50} (μM)	Cpd	X	IC_{50} (μM)
5	CH_3-CO	250			
24		>1000	27		>1000
25		>1000	28		18.6
26		13.4	29		5

IC_{50} is the μM concentration of inhibitor required to prevent 50% of cells from adhering to MAdCAM-1.

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