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NOVEL MODIFIED TRIPEPTIDE INHIBITORS OF $\alpha_4\beta_7$ MEDIATED LYMPHOID CELL ADHESION TO MAdCAM-1

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Abstract: MAdCAM-1 specifically binds the lymphocyte integrin $\alpha_4\beta_7$ and participates in the homing of leukocytes to intestinal mucosal sites. The LDT sequence located on the CD loop of MAdCAM-1 is an important binding site for MAdCAM-1/$\alpha_4\beta_7$ interactions. N-Terminus acylation of the LDT motif and modification of the C-terminus carboxamide with amines led to low micromolar MAdCAM-1 inhibitors.

The infiltration of leukocytes to the site of inflammation results from a series of adhesive and activating events involving multiple receptor-ligand interactions. The initial step is a transient, rolling of leukocytes mediated by selectin/carbohydrate interactions. This is followed by activation mediated by chemoattractants or chemokines resulting in the firm adhesion of leukocytes to endothelium and finally migration towards the site of the inflammation due to a chemokine gradient.

The gut specific trafficking of lymphocytes from the vascular to normal gastrointestinal mucosa and lymphoid tissues is mediated by adhesive interactions with the homing receptor, $\alpha_4\beta_7$, and Mucosal Addressin Cell Adhesion Molecule-1[1-4] (MAdCAM-1). MAdCAM-1 specifically binds both human and mouse lymphocytes that express the homing receptor, $\alpha_4\beta_7$, and participates in the homing of these cells to the mucosal endothelium.[5,6]

The sequence LDTSL from the CD loop of MAdCAM-1 has been suggested as a recognition motif for MAdCAM-1/$\alpha_4\beta_7$ interactions based upon mutagenesis of murine MAdCAM-1 and a single point mutation of MAdCAM-1 (L-R61), which abolished $\alpha_4\beta_7$ binding.[6] Previously, we reported that binding studies of overlapping small peptides derived from MAdCAM-1 identified the sequence LDTSL from the CD loop of the murine MAdCAM-1 as an important recognition motif for the murine MAdCAM-1/$\alpha_4\beta_7$ interactions.[7] Recently, human MAdCAM-1 was cloned and the CD loop containing the LDTSL sequence was found to be conserved.[8] We wish to report further studies that support LDTSL and more simply LDT as a binding motif required for the human MAdCAM-1/$\alpha_4\beta_7$ interactions. A peptide based structure-activity study was carried out leading to a number of potent MAdCAM-1 inhibitors.

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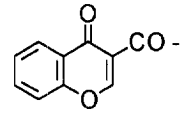
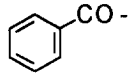
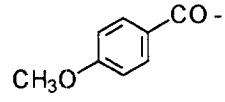
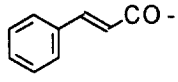
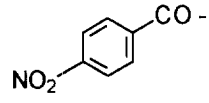
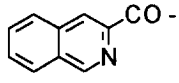
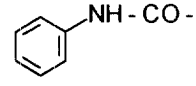
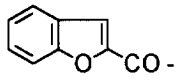
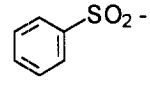
Discussion

Previously, the sequence, LDTSL, was shown to be an important recognition motif for murine MAdCAM-1/$\alpha_4\beta_7$ interactions.[7] This sequence is conserved in human MAdCAM-1 and is an important recognition motif there as well. In addition, we have found that LDTSL and LDT are equipotent inhibitors (IC_{50} = 250 μM) of MAdCAM-mediated leukocyte adhesion. To improve potency of the parent tripeptide Ac-LDT-NH_2, the tripeptide lead was modified at the N-terminal, C-terminal, and each amino acid residue of LDT peptide was replaced with unusual amino acids. We found that modifications of N-terminal amine with a variety of aryl and heterocyclic acyl groups enhanced the inhibitor potency significantly. By increasing the size of the acyl group from acetyl (**1**) to isoquinolyl (**4**) and 3-chromonyl (**6**) moieties, inhibitor potency was increased up to 200-fold.

Table 1. N-Acylated X-LDT-NH_2 Analogs

comp	X	IC_{50} (μM)	comp	X	IC_{50} (μM)
1	CH_3CO -	250	**6**		1.3
2		8.2	**7**		>25
3		4.9	**8**		9
4		3.1	**9**		>25
5		2.3	**10**		>25

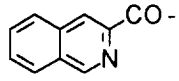
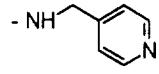
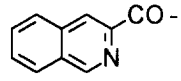
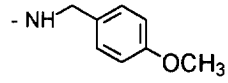
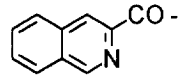
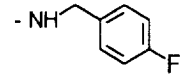
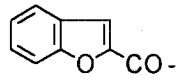
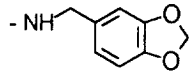
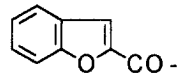
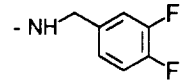
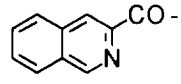
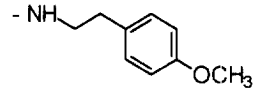
IC_{50} is the μM concentration of inhibitor required to prevent 50% of cells from adhering to MAdCAM-1.

The effect of various electron donating substituents including alkyl, alkoxy groups, and electron withdrawing substituents including halides, cyano, and nitro groups on the benzoyl moiety was examined. Generally electron donating groups such as methoxy (**7**) diminished inhibitor activity whereas electron

withdrawing groups such as nitro (**8**) had no negative effect on inhibitor potency. Modification of the N-terminus of LDT with a phenyl urea (**9**) and phenyl sulfonamide made peptide inhibitors ineffective (**10**).

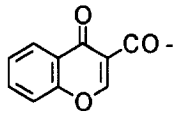
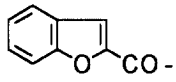
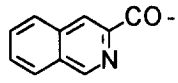
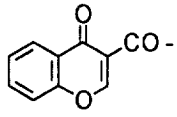
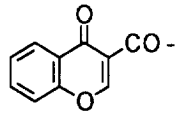
In order to examine the effects of the modifications of the C-terminus of LDT, the carboxamide was modified with a wide variety of alkyl, aryl and heterocyclic groups while N-terminus was acylated with a isoquinolyl or benzofuranyl group. Among all the C-terminal modifications, substituted benzyl amines were found to be the best modifications. Surprisingly, there was no effect of substituents on the benzyl group on the inhibitor potency. For example, 4-methoxybenzyl (**12**) and 4-fluorobenzyl (**13**) had similar inhibitor activity. However, the 4-methoxy-phenyl ethyl amine (**16**) was inactive suggesting that there may be only a small hydrophobic binding pocket.

Table 2. N- and C- Terminal Modified X-LDT-X' analogs

compd	X	X'	IC_{50} (μM)
11			1.1
12			3
13			5.2
14			4.4
15			5.2
16			>25

Unusual amino acids such as D-amino acids, β-amino acids etc.[9-12] when used in place of natural amino acids are known to improve potency and bioavailability of peptides. Each residue of LDT peptide was replaced with the corresponding D-amino acid, β-amino acid, N-methyl amino acid and α-methyl amino acid. While Leu and Asp could not be replaced with any unusual amino acids, Thr could be substituted with β-Thr (**17-18**), D-Thr (**19-20**) and β-PhSer (**21**) without a significant loss of inhibitor potency.

Table 3. N-Acylated X-Leu-Asp-AAA-NH_2 Analogs Containing Unusual Amino Acids

compd	X	AAA	IC_{50} (μM)
17		β-Thr	1.1
18		β-Thr	2.3
19		d-Thr	3.9
20		d-Thr	4.7
21		β-PhSer	6.4

Cell Adhesion Assay

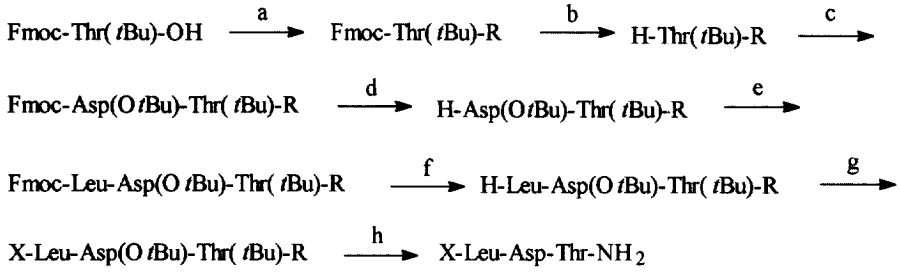
Cell adhesion assays were performed in a 96-well format utilizing a soluble human MAdCAM-1 IgG chimera immobilized to the plate in carbonate buffer, pH 9. RPMI 8866 cells a B cell lymphoma that expresses high levels of $\alpha_4\beta_7$ were fluorescently labeled by preincubation with BCEF-AM stain, washed, resuspended in assay buffer containing HBSS/2%FCS/25 mM HEPES pH 7.2 buffer at $2.5x10^6$ cells per mL. A typical assay consisted of a final volume of 200μL of which 50 μL are cells ($1.25x10^5$ cells) and the remaining volume consisted of inhibitor, control antibody such that the final DMSO concentration is 1%. Cell adhesion occurred in 30 min at ambient temperature after which plates were washed with 50 mM Tris/2 mM $MnCl_2$/0.14 M NaCl, pH 7.2 using an automatic plate washer in a wash volume of 500 μL for 2 wash cycle. Assays were then read on an Idexx fluorescent plate reader at 485/535 nm. Inhibition was determined by the number of cells adhering to the plates in the presence and absence of an inhibitor. The IC_{50} values (the concentration of inhibitor resulting in 50% of maximum binding) were determined using Kaleidograph and are reported as an average of multiple

determinations with a mean standard error of <25% of the IC_{50} values reported. Inhibition of cell adhesion response by "antibody" served as a positive control.

Peptide Synthesis

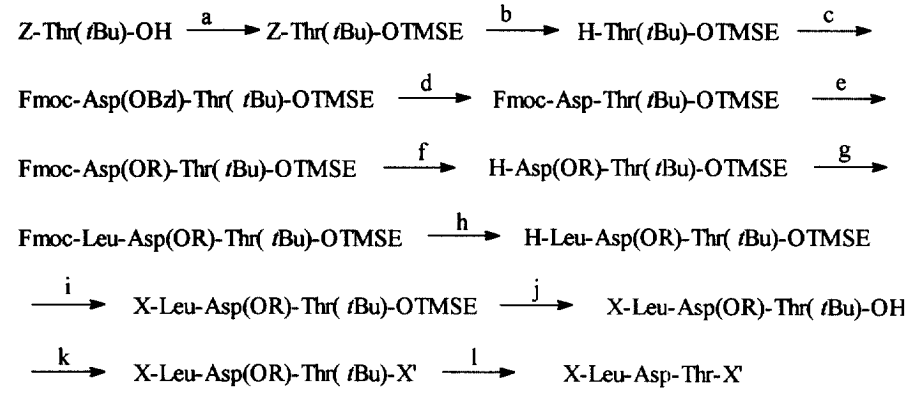
Peptides were synthesized via Fmoc/*t*-Bu chemistry[13] using solid-phase peptide synthesis methodology with Rink Amide Am resin (Scheme 1) and 2-chlorotrityl chloride resin (Scheme 2).

Scheme 1.



R = Rink amide MBHA resin, X = chromone-3-carbonyl. (a) Rink amide MBHA resin, TBTU, HOBt, DIEA, DMF, 2 h. (b) 20% PIP, DMF, 30 min. (c) Fmoc-Asp(O*t*Bu)-OH, TBTU, HOBt, DIEA, DMF, 2 h. (d) 20% PIP, DMF, 30 min. (e) Fmoc-Leu-OH, TBTU, HOBt, DIEA, DMF, 4 h. (f) 20% PIP, DMF, 30 min. (g) chromone-3-carboxylic acid, TBTU, HOBt, DIEA, DMF, 12 h. (h) 95%TFA, H_2O, 2 h.

Scheme 2.



X = 3-isoquinoline carbonyl, X' = 4-methoxyenzylamine, R = 2-chlorotrityl resin. (a) $(CH_3)_3SiCH_2CH_2OH$, DCC, CH_2Cl_2, 1.5 h. (b) 10%Pd/C/H_2, 30 min. (c) Fmoc-Asp(Obzl)-OH, HOBt, CH_2Cl_2, 20 h. (d) 10%Pd/C/H_2, 10 min. (e) 2-chlorotrityl chloride resin, DIEA, CH_2Cl_2, 40°C, 4 h. (f) 20% PIP, DMF, 30 min. (g) Fmoc-Leu-OH, TBTU, HOBt, DIEA, DMF, 3 h. (h) 20% PIP, DMF, 30 min. (i) 3-isoquinoline carboxylic acid, TBTU, HOBt, DIEA, DMF, 3 h. (j) TBAF, DMF, 5 min. (k) PYAOP, 4-methoxybenzyl-amine, collidine, 16 h. (l) 95% TFA, H_2O, 1 h.

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 spectrometer (MALDI-1, TOF, Kratos, Inc.).

In conclusion, a significant binding site of human MAdCAM-1 /$\alpha_4\beta_7$ was found to be in the CD loop of domain-1 of human MAdCAM-1. Peptides containing the LDT sequence can inhibit adhesion of human MAdCAM-1 to lymphocyte integrin $\alpha_4\beta_7$. Modifications of the N-terminal of LDT with isoquinolyl, benzofuranyl, chromonyl and substituted benzoyl groups were most effective while modifications of the carboxamide of X-LDT did not further enhance the MAdCAM-1 inhibitor potency. Within the peptide sequence LDT, replacement of the Thr residue with unusual amino acids such as d-Thr, β-Thr and β-PhSer gave potent inhibitors while replacement of Leu and Asp residues resulted in loss of inhibitory activity. Based on these studies, potent small molecule inhibitors such as the X-LDT-X' type of human MAdCAM-1/$\alpha_4\beta_7$ mediated leukocyte adhesion have been identified.

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