Selective α4β7 Integrin Antagonists and Their Potential as Antiinflammatory Agents

Nathan J. P. Dubree,[†] Dean R. Artis,[†] Georgette Castanedo,[†] James Marsters,[†] Daniel Sutherlin,[†] Lisa Caris,[‡] Kevin Clark,[‡] Susan M. Keating,[‡] Maureen H. Beresini,[‡] Henry Chiu,[§] Sherman Fong,[§] Henry B. Lowman,[||] Nicholas J. Skelton,[||] and David Y. Jackson*[,†]

Departments of Bioorganic Chemistry, Pharmacology, Immunology, and Protein Engineering, Genentech Inc., 1 DNA Way, South San Francisco, California 94080

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The accumulation of leukocytes in various tissues contributes to the pathogenesis of numerous human autoimmune diseases. The integrin α4β7, expressed on the surface of B and T lymphocytes, plays an essential role in lymphocyte trafficking throughout the gastrointestinal (GI) tract via interaction with its primary ligand, mucosal addressin cell adhesion molecule (MAdCAM). Elevated MAdCAM expression in the intestines and liver has been linked to GI-associated autoimmune disorders, including Crohn's disease, ulcerative colitis, and hepatitis C. Monoclonal antibodies that block the interaction of α4β7 with MAdCAM inhibit lymphocyte homing to murine intestines without effecting migration to peripheral organs; this suggests that α4β7-selective antagonists might be useful as GI specific antiinflammatory agents. Here, we report the discovery of highly potent and selective α4β7 antagonists affinity selected from a random peptide-phage library. Subsequent optimization of initial peptide leads afforded α4β7-selective heptapeptide inhibitors that competitively inhibit binding to MAdCAM in vitro and inhibit lymphocyte homing to murine intestines in vivo. Substitution of a single carboxylate moiety alters selectivity for α4β7 by more than 500-fold to afford a potent and selective α4β1 antagonist. The antagonists described here are the first peptides to demonstrate potency and selectivity for α4β7 compared to other integrins.

Introduction

Integrins are noncovalently associated α/β heterodimeric cell surface receptors involved in numerous cellular processes ranging from cell adhesion and migration to gene regulation. In the immune system, integrins play an essential role in leukocyte trafficking during inflammatory processes.[1−3] Through interactions with extracellular matrix proteins (EMPs) and cell adhesion molecules (CAMs), integrins function as tissue specific adhesion receptors. Differential expression of integrins can regulate a cell's adhesive properties, allowing different leukocyte populations to be recruited to specific organs in response to different inflammatory signals.[4] If left unchecked, integrin-mediated adhesion processes can lead to chronic inflammation and autoimmune disease.[5,6] Inhibitors of specific integrin−ligand interactions might, therefore, find utility as antiinflammatory agents for the treatment of autoimmune diseases.

The α4 integrins, α4β1 and α4β7, play essential roles in lymphocyte migration throughout the gastrointestinal tract.[3,7,8] They are expressed on most leukocytes, including B and T lymphocytes,[9,10] where they mediate cell adhesion via binding to their respective primary ligands, vascular cell adhesion molecule (VCAM)[11,12] and mucosal addressin cell adhesion molecule (MAdCAM).[8,13] Both VCAM and MAdCAM are multidomain transmembrane proteins, but their integrin binding sites are thought to reside primarily within their first N-terminal domain based on previously reported mutagenesis studies.[14−18] The results of these mutagenesis studies indicate that both VCAM and MAdCAM contain a critical aspartic acid located within highly conserved tripeptide sequences (IDS in VCAM and LDT in MAdCAM). The proteins differ in binding specificity, however, in that VCAM binds both α4β1 and α4β7, while MAdCAM is highly specific for α4β7.[19] Although the molecular basis for MAdCAM's specificity is unknown, a recent publication demonstrated that fusion of MAdCAM's second domain with the first domain of VCAM afforded a hybrid protein that was selective for α4β7. This result suggested that MAdCAM's specificity might be conferred primarily by its second domain.[20]

The X-ray structures of VCAM and MAdCAM indicate that there are significant differences in both the first and second domains.[21−23] A protruding loop (the DE loop) in the second domain of MAdCAM might help explain its effect on binding specificity. There are also notable differences in the first domains. For example, the aforementioned LDT binding sequence in MAdCAM lies in a region connecting two β strands known as the CD loop. The analogous loop in VCAM, which contains the IDS motif, is shifted by approximately 10 Å when the two protein backbones are superimposed. Because both VCAM and MAdCAM require aspartic acid in their respective CD loops for integrin binding, these structural differences in the first domain could also play a role in determining MAdCAM's specificity for α4β7.

* To whom correspondence should be addressed. Tel: (650) 225-6405. Fax: (650) 225-2061. E-mail: davej@gene.com.
[†] Department of Bioorganic Chemistry.
[‡] Department of Pharmacology.
[§] Department of Immunology.
[||] Department of Protein Engineering.

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Differences in the expression profiles of VCAM and MAdCAM provide the most convincing evidence for their role in inflammatory diseases. Both are constitutively expressed in the gut; however, VCAM expression extends into peripheral organs,[24,25] while MAdCAM expression is confined to organs of the gastrointestinal tract.[9,24,26] In addition, elevated MAdCAM expression in the gut has now been correlated with several gut-associated inflammatory diseases, including Crohn's disease, ulcerative colitis, and hepatitis C.[27,28] Current treatments for these diseases involve the use of nonselective immunosuppressive agents or steroids which are often ineffective or toxic when used for chronic indications.[29] Antibodies directed against α4β7 or MAdCAM ligands have proven to be effective modulators of inflammatory processes in animal models for inflammatory bowel disease, and a humanized α4β7 specific antibody (LDP-02) is being investigated in a phase II clinical trial for ulcerative colitis.[30] Small molecule α4β7 antagonists might, therefore, find utility as gut specific antiinflammatory agents. In addition, several recent studies suggest that selective inhibition of α4β7 versus α4β1 might be essential for safe treatment of gastrointestinal autoimmune diseases.[31,32]

We previously reported simple cyclic peptides containing the LDT motif which selectively inhibit MAdCAM-mediated adhesion to α4β7 positive JY cells with IC_{50} values of 150−300 μM.[18] More recently, Boer and co-workers reported similar inhibitors of α4β7 derived from a panel of cyclic peptide amides intended to constrain the LDT motif into different conformations. Although the peptides demonstrated structural similarity with MAdCAM's LDT epitope, they had low affinity for α4β7 (IC_{50} values ∼ 200−500 μM) and their selectivity versus α4β1 was not determined.[33] Here, we report the discovery of potent (sub-micromolar) α4β7 antagonists affinity selected from a random peptide library expressed on bacteriophage M13. Optimization of initial leads afforded novel heptapeptide antagonists that demonstrate selectivity for α4β7 compared to other closely related integrins, including α4β1. Relocation of a single carboxylate moiety alters the binding selectivity more than 500-fold and affords a highly potent α4β1-selective antagonist. Moreover, one peptide selectively inhibits lymphocyte homing to murine intestines in vivo without impacting peripheral sites. These results provide new insight into α4 integrin selectivity and validate α4β7 as a promising pharmaceutical target for treatment of gastrointestinal inflammation.

Results

To identify selective α4β7 antagonists, we immobilized α4β7 in 96-well plates and affinity selected peptide inhibitors from previously described random peptide-phage libraries.[34,35] The libraries contained bacteriophage M13 gene 8 fused peptides of the general sequence $X_LCX_MCX_N$ where X represents any of the 20 naturally occurring amino acids and $L + M + N = 18$. A linear octapeptide library was also screened (Table 1). Peptide-phage binding enrichment was measured as the ratio of phage recovered from an α4β7-coated plate versus a control plate after three rounds of affinity selection (see Experimental Methods). Phagemid DNAs from several enriched libraries (B, C, L, and M; Table 1) were sequenced, and individual clones were selected based on their frequency of occurrence. Several clones representing consensus amino acid sequences were further evaluated for binding α4β7 using a previously described phage ELISA.[36]

Table 1. Enrichment of Peptide-Phage Libraries after 3 Rounds of Affinity Selection against α4β7

library	general sequence	enrichment
A	$X_4CX_2GPX_4CX_4$	1
B	$X_7CX_4CX_7$	270
C	$X_7CX_5CX_6$	1100
D	$X_6CX_6CX_6$	1
E	$X_6CX_7CX_5$	1
F	$X_5CX_8CX_5$	1
G	$X_5CX_9CX_4$	1
H	$X_4CX_{10}CX_4$	3
I	X_8	60
J	$X_2CX_2CX_2$	1
K	$X_2CX_3CX_2$	1
L	$X_2CX_4CX_2$	140
M	$X_2CX_5CX_2$	180
N	$X_2CX_6CX_2$	1
O	$X_2CX_7CX_2$	1
P	$X_2CX_8CX_2$	1
Q	$X_2CX_9CX_2$	1
R	$X_2CX_{10}CX_2$	1

High-affinity peptide-phage were sequenced, and the corresponding peptides were synthesized using standard solid-phase methods. Purified peptides were then evaluated for their ability to inhibit binding of α4β7 and α4β1 to MAdCAM and VCAM, respectively, using standard ELISA methods. Several novel peptides were identified which selectively inhibit binding of α4β7 to MAdCAM at low micromolar concentrations. One peptide (**1**, Table 2), derived from library C (Table 1), contained a tripeptide sequence, SDT, and demonstrated 10-fold selectivity favoring α4β7 over α4β1. Its homology with MAdCAM's LDT motif in combination with its selectivity for α4β7 led us to choose this lead for further optimization studies.

Reduction of Peptide 1 to a Minimal Binding Epitope. To determine a minimal peptide binding motif for selective inhibition of α4β7, we synthesized analogues of peptide **1** (Table 2) in which the N- and C-termini were truncated incrementally (Table 2). All peptides were synthesized as C-terminal amides in order to mimic the affinity-selected peptide-phage which is lacking a carboxy terminus. The peptides were then cyclized via oxidation of the cysteine residues. The relative affinities of analogues **2**−**9** for α4β7 and α4β1 indicate that residues flanking the two cystienes can be removed without a significant loss of potency or selectivity for α4β7. In fact, complete removal of N- and C-terminal amino acids afforded **9** which demonstrated enhanced potency and selectivity for α4β7. Further deletion of intra-disulfide residues yielded peptides with no measurable affinity for either α4β1 or α4β7, indicating that the cyclic heptapeptide **9** likely contains a minimal binding epitope.

Alanine Scanning. The relative contributions of each of the remaining amino acid side chains were determined via synthesis of peptides in which each residue was substituted with alanine. The results indicate that all five intra-disulfide amino acid side chains in **9** contribute significantly to the overall binding affinity for α4β7 (Table 3). Substitution of the aspartic acid side chain had the greatest effect and resulted in

Table 2. Effect of N- and C-Terminal Truncations on the Affinity of Peptide **1** for α4β1 and α4β7

#	sequence	ELISA IC_{50} (μM) α4β1/VCAM	ELISA IC_{50} (μM) α4β7/MAdCAM
1	Ac-WERWLMD**C**RSDTL**C**GESHQE-NH_2	12.1	1.3
2	MD**C**RSDTL**C**GE-NH_2	4.2	0.19
3	MD**C**RSDTL**C**G-NH_2	8.8	0.34
4	MD**C**RSDTL**C**-NH_2	11.8	0.64
5	D**C**RSDTL**C**GE-NH_2	8.3	0.26
6	CRSDTL**C**GE-NH_2	1.1	0.03
7	CRSDTL**C**G-NH_2	3.8	0.11
8	D**C**RSDTL**C**-NH_2	8.6	0.52
9	CRSDTL**C**-NH_2	4.2	0.12

a 1000-fold loss of affinity. Interestingly, the effects of alanine substitution on α4β1 affinity were much smaller, and only three of the five side chains (Asp, Thr, and Leu) contribute significantly to the overall affinity of **9** for α4β1. Alanine substitution of arginine or serine in **9** yielded peptides with slightly better affinity for α4β1, indicating that optimal affinity for this receptor had not been achieved during the affinity selection process.

Table 3. Alanine Scan of the Minimal Integrin Binding Peptide **9**

#	sequence	IC_{50} (μM) α4β1/VCAM	IC_{50} (μM) α4β7/MAdCAM
9	CRSDTLC-NH_2	4.2	0.12
10	C**A**SDTLC-NH_2	2.8	1.1
11	CR**A**DTLC-NH_2	3.6	2.2
12	CRS**A**TLC-NH_2	>100	>100
13	CRSD**A**LC-NH_2	60	10
14	CRSDT**A**C-NH_2	40	23

Optimization of Affinity and Selectivity for α4β7. Although alanine replacement is useful for identifying important side chains, such analogues afford little information about the nature of the interactions or indirect effects due to changes in the peptide structure. To determine if affinity selection of the random peptide-phage libraries yielded optimal α4β7 peptide antagonists, we synthesized analogues in which each side chain in **9** was replaced with more subtle isosteric or isoelectronic amino acid substitutions (Table 4).

First, the arginine side chain was substituted with lysine or aromatic amino acids, resulting in little change in affinity for α4β7 (Table 4). In contrast, substitution of arginine with aromatic amino acids (**16**−**18**) significantly enhanced binding affinity and selectivity for α4β1. In fact, substitution of arginine with tyrosine afforded a highly potent (IC_{50} = 9 nM) α4β1 antagonist **16**, primarily due to a 450-fold improvement in affinity for α4β1. Interestingly, none of the arginine substitutions (**15**−**19**) demonstrated enhanced affinity for α4β7, yet all of the substitutions resulted in higher affinity for α4β1. These results suggest that selection of the randomized peptide-phage library also optimized arginine in this position for binding to α4β7 but not α4β1.

Substitutions of the second residue (serine) in **9** resulted in significantly lower α4β7 affinity for all five analogues tested (**20**−**24**, Table 4). Conversely, affinity for α4β1 was enhanced upon substitution with hydrophobic amino acids, such as Lue, Ile, or Met. Although most substitutions resulted in relatively moderate changes in binding affinity for each receptor, the effects were in opposite directions and resulted in significant changes in receptor selectivity. For example, analogue **22**, in which serine was substituted with isoleucine, demonstrated a 500-fold reversal in selectivity due to a reduction in affinity for α4β7 combined with enhanced affinity for α4β1. Analogue **24**, where L-serine was replaced by D-serine, had lower binding affinity for both α4β1 and α4β7, indicating a stereochemical preference for L-serine at this position. Again, these results suggested that serine in this position was affinity optimized for binding α4β7 but not α4β1.

The most dramatic effects on potency and selectivity were seen when aspartic acid was substituted with several other amino acids. Alanine substitution at this position indicated that the removal of the aspartate side chain was not well tolerated (**12**, Table 3). To better define the nature of this interaction, we synthesized peptides containing more subtle side chain alterations (**25**−**28**, Table 4). The results indicate a strict requirement for L-aspartic acid at this position. Replacement

Table 4. Effect of Individual Amino Acid Substitutions on the Potency and Selectivity of the Cyclic Peptide CRSDTLC (**9**) for α4β7 and α4β1

#	Sequence	ELISA IC_{50} (μM) α4β1/VC	α4β7/MC	β1/β7
9	**C**RSDTL**C**-NH_2	4.2	0.12	35
15	C**K**SDTLC-NH_2	1.5	0.25	6
16	C**Y**SDTLC-NH_2	0.009	0.13	0.07
17	C**F**SDTLC-NH_2	0.05	0.55	0.09
18	C**W**SDTLC-NH_2	0.16	0.43	0.37
19	C**H**SDTLC-NH_2	1.1	0.39	2.8
20	CR**L**DTLC-NH_2	0.8	1.9	0.42
21	CR**T**DTLC-NH_2	7.4	2.1	3.5
22	CR**I**DTLC-NH_2	1.2	20	0.06
23	CR**M**DTLC-NH_2	3.2	2.2	1.5
24	CR**s**DTLC-NH_2	>100	43	>2.3
25	CRS**N**TLC-NH_2	88	175	2
26	CRS**E**TLC-NH_2	3.0	30	0.1
27	CRS**Q**TLC-NH_2	>100	>100	?
28	CRS**d**TLC-NH_2	>100	>100	?
29	CRSD**S**LC-NH_2	14	0.29	48
30	CRSD**V**LC-NH_2	13	0.84	15.5
31	CRSD**t**LC-NH_2	20	7.9	2.5
32	CRSDT**P**C-NH_2	37	40	0.9
33	CRSDT**I**C-NH_2	25	1.8	14
34	CRSDT**V**C-NH_2	59	4.5	13.1
35	CRSDT**G**C-NH_2	>100	11	>9

Table 5. Effect of Carboxylate Substitutions on Potency and Selectivity for α4β7 versus α4β1

#	sequence	IC_{50} (μM) α4β1/VC	α4β7/MC	β1/β7
9	CRSDTLC-NH_2	4.2	0.13	32
25	CRS**N**TLC-NH_2	88	175	0.5
36	CRSDTLC-**OH**	0.4	0.13	3.1
37	CRS**N**TLC-**OH**	2.2	40	0.06

with isosteric asparagine, carboxylate extension with glutamic acid, or substitution with D-aspartic acid all resulted in a significant loss of affinity (>1000-fold) for α4β7. A moderate loss of affinity for α4β1 was also observed when aspartic acid was replaced with Asn or Gln. Conversely, analogue **26** (Asp to Glu) demonstrated enhanced affinity for α4β1 and a 300-fold reversal in β chain selectivity. Again, the fact that none of the analogues demonstrated enhanced affinity for α4β7 suggested that aspartic acid in this position was optimized during the affinity selection process.

Substitution of threonine in **9** resulted in a comparable loss of affinity for both receptors, but the effect was relatively small compared with other positions in the peptide. Again, none of the substitutions enhanced the potency of peptide **9** for α4β7 (Table 4). A significant decrease in potency was observed upon substitution with D-threonine, which may indicate a change in peptide structure rather than a loss of an important binding interaction. The fact that the relatively isosteric analogue **29** (Thr → Val) demonstrated only slightly reduced affinity for the receptor supports this notion.

Finally, analogues in which leucine was replaced by other L-amino acids also had significantly reduced potency for α4β7 (**32**−**35**, Table 4). Even relatively benign replacements, such as isoleucine and Val (**33** and **34**), resulted in greater than 10-fold losses in affinity. The impact on α4β7 binding affinity was equally deleterious for all analogues except **32** (Leu to Pro), leading us to speculate that Leu might interact primarily with the α4 subunit common to both receptors. Curiously, substitution of leucine with proline, which might be expected to affect the peptide structure, had little or no effect on affinity for α4β1, yet substantially reduced affinity for α4β7.

Carboxylate Substitutions. In a previous publication, we demonstrated that the C-terminal carboxylate of YCDPC, a potent α4β1 peptide antagonist, was essential for inhibition of the α4β1−VCAM binding interaction.[37] Because the peptide-phage libraries described here are linked to the viral surface via their C-terminus, they lack a C-terminal carboxylate anion. Substitution of the aspartic acid in **9** with asparagine, however, resulted in a significant loss of affinity for both α4β1 and α4β7. These results prompted us to investigate the contribution of the C-terminus in **9** to the overall binding affinity for α4β7. Amide to carboxylate substitution at the C-terminus of **9** afforded **36** (Table 5) and had little effect on its affinity for α4β7. Potency for α4β1, however, was enhanced by approximately 10-fold. Subsequent replacement of the aspartate in **36** with asparagine afforded an α4β1-selective peptide **37**. Overall, switching the Asp carboxylate and the C-terminal amide in peptide **9** resulted in a 500-fold net reversal in selectivity from α4β7-selective **9** to an α4β1-selective peptide **37**.

Table 6. Selectivity of Antagonist **6** for α4β7 versus Other Integrins[38−41]

assay	IC_{50} (μM)
α4β7/MAdCAM	0.03
α4β1/VCAM	1.1
α5β1/fibronectin	6.1
αIIbβIIIa/fibrinogen	>25
αvβ3/vitronectin	1.0
αLβ2/ICAM-1	>100

Selectivity for α4β7. The results presented in the preceding sections demonstrate that the affinity-selected inhibitors preferentially bind α4β7 over α4β1. To determine the binding selectivity versus other integrin receptors, a representative antagonist **6** was chosen for evaluation in several different integrin binding assays. The relative binding selectivity of cyclic peptide **6** (CRSDTLCGE-amide) for α4β7 was determined using previously described binding assays for the inhibition of α5β1/fibronectin,[38] αIIbβIIIa/fibrinogen,[39] αvβ3/vitronectin,[40] and αLβ2/ICAM-1[41] (Table 6). Although moderate inhibition of both α5β1 and αvβ3 was observed, cyclic peptide **6** demonstrated good selectivity for inhibition of α4β7/MAdCAM versus other integrin receptors tested.

Inhibition of Lymphocyte Homing. Lymphocyte homing to the intestines is known to be dependent upon the interaction of α4β7 with MAdCAM. The accumulation of radiolabeled lymphocytes in the intestines of mice with chemically induced or spontaneous colitis can be inhibited by pretreatment with anti-α4β7 or anti-MAdCAM antibodies.[42−45] To evaluate the antiinflammatory activity of our peptide inhibitors in vivo, CRF2.4 (IL10r-KO) mice with spontaneous colitis were treated intravenously with analogue **6** (50 mg/kg) prior to injection with ^{51}Cr-labeled mesenteric lymph node cells (see Experimental Methods). Control mice were treated with an equal amount of either a linear peptide YSAPC (negative control), which does not bind α4β7, or an anti-α4 antibody (PS/2) as a positive control. The data shown in Figure 1 demonstrate that pretreatment of colitic CRF2.4 mice with **6** (50 mg/kg) inhibits lymphocyte accumulation in the large intestine roughly equivalent to the inhibition seen in mice treated with the PS/2 (anti-α4) antibody. Importantly, identical treatment did not inhibit lymphocyte migration to peripheral inguinal lymph nodes where MAdCAM is not overexpressed. These results support the notion that α4β7-selective antagonists inhibit lymphocyte accumulation in the GI tract without impacting peripheral organs and suggest that similar antagonists may find utility as gut specific antiinflammatory agents.

Conclusions

We have identified potent and selective α4β7 antagonists from a random naive cyclic peptide library and demonstrated that affinity selection afforded inhibitors that are also affinity optimized for the target receptor α4β7 but not the closely related receptor α4β1. The antagonists inhibit binding of α4β7 to MAdCAM in vitro and lymphocyte accumulation in vivo. Further optimization afforded peptides that bind α4β7 with affinities

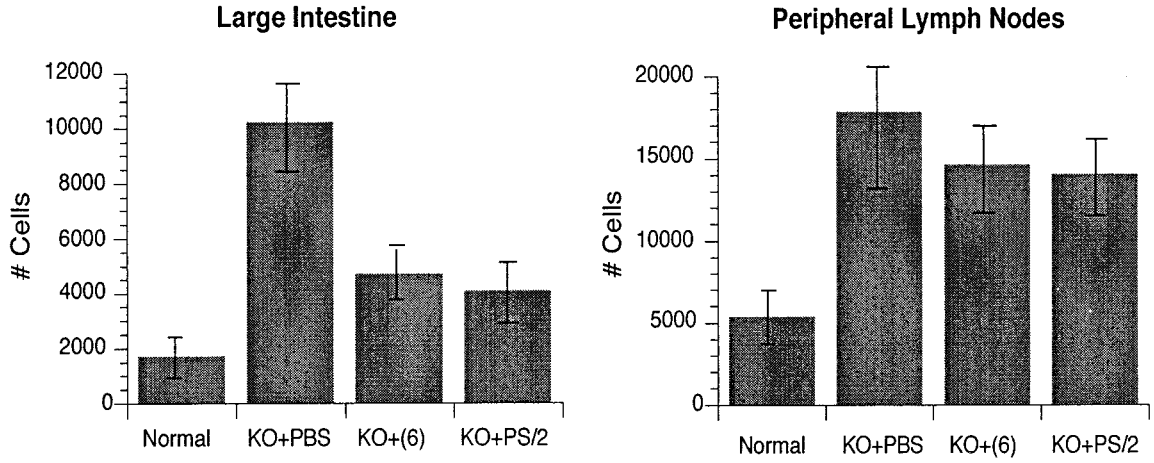

Figure 1. Inhibition of Cr^{51}-labeled lymphocyte homing to the large intestines and peripheral lymph nodes in CRF2-4 (IL-10 receptor −/−) mice with spontaneous colitis.[49]

approaching that of the native protein ligand MAdCAM (K_d = 14 nM) and are selective for α4β7 versus several other integrins involved in cell adhesion. The antagonists share important functional groups with a known MAdCAM binding epitope, yet show little direct structural similarity to the protein. Binding specificity for α4β7 appears to be dependent upon the location of a single carboxylate anion. Relocation of this carboxylate moiety from the aspartate side chain to the C-terminus affords a highly potent and selective α4β1 antagonist.

Although therapeutic strategies using integrin antagonists are relatively new, antibodies, peptides, and small molecules that block integrin interactions are currently under clinical investigation for a variety of indications. With new integrin targets emerging every year, it seems likely that interest in integrin antagonists will continue to expand. The results presented here provide new insight into the mechanisms involved in α4 integrin selectivity and provide further evidence that blocking integrin-mediated cell adhesion interactions, specifically α4β7−MAdCAM, may be useful for the treatment of gastrointestinal inflammatory disorders and potentially lead to new classes of organ-selective antiinflammatory agents.

Experimental Methods

Peptide-Phage Binding Selections and Assays. Phagemid library preparations, selections, and amplifications in *Escherichia coli* strain XL-1 Blue (Stratagene) were performed with modifications of previously described procedures.[36] Nunc 96-well maxisorb plates were coated with purified a4b7 (100 μL/well) diluted to 2 μg/mL in coating buffer (1 mM $MgCl_2$, 1 mM $CaCl_2$, 150 mM NaCl, 10 mM Tris; pH 7.5). Thereafter, the coating solution was removed, and the plates were blocked with a 0.5% solution of bovine serum albumin (BSA) in coating buffer. BSA control plates (containing no α4β7) were also coated with this solution. A phage stock from each peptide-phage library was precipitated twice with a salt/PEG-8000 solution, and the precipitate was resuspended in 150 mM NaCl. This stock of phagemid particles was then diluted to about 1×10^{11} phage/mL ($OD_{268} = 1.1 \times 10^{13}$ phage/mL) in binding buffer (coating buffer with 0.5% BSA and 0.05% Tween-20). For each library, 8 aliquots (0.1 mL) were dispensed to separate wells of the coated plates, and incubation was carried out at room temperature for 1.5 h. The binding solution was then removed, and each plate was washed 10 times with wash buffer (coating buffer containing 0.05% Tween-20). To elute bound peptide phagemids, 0.1 mL of HCl (100 mM) was added per well followed by incubation for 10 min at room temperature. Eluted phage were transferred to a nonadsorbent plate (Nunc F96) containing 0.05 mL/well 1 M Tris 8 to neutralize the solution. After the first round of selection, $^1/_2$ of the eluted phage volume was used to infect XL-1 *E. coli* (1:10 dilution) for overnight amplification in liquid culture. A second round of selection was carried out by a PEG-precipitating phage and proceeding as described above. After elution, a third round of selection was carried out without amplification by diluting the neutralized phage to a 1:10 ratio in binding buffer and proceeding as described above. Eluted phage from α4β7 and BSA control plates were neutralized, transfected into XL-1 Blue *E. coli*, and plated for isolation of clones. Phagemid preparations from isolated clones were used either for further screening (below) or to prepare ss DNA for Sequenase* (Amersham) sequencing. Phage clones were isolated, propagated as above, and analyzed in a standard phage-ELISA assay for binding to α4β7.

Peptide Synthesis. All peptides were synthesized manually using standard solid-phase peptide chemistry with FMOC-protected amino acids[46] on *p*-alkoxybenzylamine resin[47] to afford C-terminal amides. Amino acids were purchased from BACHEM, Advanced ChemTech, or Calbiochem Corp. Couplings were performed for 1 h with 3 equiv of HBTU-activated amino acid and 6 equiv of *N*-methylmorpholine and washed 4 times alternating with CH_2Cl_2 and DMA. FMOC groups were removed with 20% piperidine in DMA. Cleavage with TFA containing 5% triethylsilane and 1% water, evaporation of the TFA, and washing the resin with 50 mL of diethyl ether afforded crude linear peptides. The crude peptides were then extracted from the resin with 2 × 50 mL of 1:1 a H_2O/CH_3CN mixture. Disulfide oxidation was carried out on the crude extracts at 25° C by dropwise addition of a saturated solution of iodine in acetic acid with stirring until a slight yellow color persisted. The crude oxidized peptides were lyophilized and purified on a 2 in. diameter preparative reverse-phase C18 HPLC (CH_3CN/H_2O gradient containing 0.1% TFA, 0−60% CH_3CN in 60 min, at 20 mL/min) to afford the purified peptide. Fractions >95% pure by analytical C18 HPLC were pooled and lyophilized to dryness. The pure lyophilized peptides were then analyzed by electrospray LC/MS (Sciex, API2000); the data are listed in Supporting Information with retention times and the calculated and observed mass for each peptide. The analytical data are summarized in the Supporting Information. The actual HPLC/MS data are also included as Supporting Information. The lyophilized cyclic peptides were dissolved in DMSO at a concentration of 10 mM just prior to evaluation by enzyme-linked immunosorbent assay (ELISA).

Receptor Binding ELISA for α4β7. Compounds were assayed for their ability to block the α4β7−MAdCAM-1 binding interaction using a protein ELISA format. Nunc ELISA plates were coated overnight at 4 °C with 100 μL of 9F10 antibody (antihuman α4 Ig, #31470D, PharMingen, San Diego, CA) at 2 μg/mL PBS. The plates were decanted and blocked with

assay buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $MnCl_2$, 0.05% Tween-20, and 0.5% BSA) at room temperature for 1 h, with gentle shaking. After the plates were washed 3 times with wash buffer (in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM $MnCl_2$, and 0.05% Tween-20), purified $\alpha4\beta7$ receptor (2 μg/mL) in assay buffer was added. The receptor was isolated from transfected JY cells as described previously.[18] The plates were incubated at room temperature for 2 h with gentle shaking and washed 3 times with wash buffer. Serial dilutions containing 50 μL of test compounds diluted in assay buffer (1 nM → 200 μM final concentration) were added to the plates in triplicate followed by the addition of 50 μL of MAdCAM-1−Ig−HRP conjugate (1 μg/mL) in assay buffer. The plates were incubated for 2 h at room temperature, with gentle shaking, followed by washing three times with wash buffer. The bound MAdCAM-1−Ig−HRP conjugate is detected by the addition of the peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (TMB, Kirkegaard & Perry, Gaithersberg, MD) for 10 min, followed by addition of 1 M phosphoric acid to stop the reaction. The absorbance of the solutions is read at 450 nm on a 96-well plate reader. Results were plotted as absorbance (determined by calculating the average of the triplicate wells) versus concentration. The standard deviations never exceeded 10% of the averaged triplicate value and, therefore, were not listed in the data tables. The concentration of peptide at the half-maximal absorbance value was reported as the IC_{50}.

Receptor Binding ELISA for $\alpha4\beta1$. Inhibitor concentrations affording 50% inhibition (IC_{50}) of $\alpha4\beta1$ binding to VCAM-1 were determined by a previously described protein-based receptor binding ELISA.[37] The $\alpha4\beta1$ receptor was extracted from Ramos cell membranes using wheat germ lectin Sepharose chromatography followed by gel filtration in 1% octylglucoside. $MnCl_2$ (1 mM) was included in all buffers during purification procedures and assay to maintain the active binding state. Recombinant soluble human VCAM-1 (55 kDa fragment composed of the first 5 N-terminal Ig-like domains) was purified from Chinese hamster ovary (CHO) cell culture media. Nunc Maxisorp 96-well plates were coated with 4 μg/mL VCAM-1 in phosphate-buffered saline. The wells were blocked with 1% BSA in phosphate-buffered saline (PBS). The assay buffer contained 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM $MnCl_2$, and 0.05% Tween-20. The inhibitor sample and $\alpha4\beta1$ were allowed to incubate in the wells for 2 h at 37 °C. The bound $\alpha4\beta1$ was detected with a nonblocking mouse anti-human b1 integrin monoclonal antibody (clone 2D4.6, Genentech, Inc.) followed by the addition of goat anti-mouse horseradish peroxidase (BioSource International, Camarillo, CA). Peroxidase activity was detected with TMB Microwell Peroxidase substrate (Kirkegaard and Perry Laboratories, Inc.). Reactions were stopped with 1 M phosphoric acid, and absorbance was measured at 450 nm. Results were plotted as absorbance versus concentration, and the concentration of peptide at the half-maximal absorbance value was reported as the IC_{50}.

Lymphocyte Homing Assay. The in vivo activity of was evaluated in a lymphocyte homing assay using ^{51}Cr-labeled syngeneic mesenteric lymph node cells administered to CRF2-4 mice (IL-10rec KO) with spontaneous colitis.[48,49] Cells were labeled with 150 Ci ^{51}of Cr (Amersham, Arlington Heights, IL) for 25 min at 37 °C. Mice were injected intravenously with 4×10^6 radiolabeled cells mixed with 1 mg of test peptide in PBS at time 0. The animals were sacrificed 1 h after receiving the radiolabeled cells. The intestines and inguinal lymph nodes were collected and counted for accumulated radioactivity. A monoclonal antibody (PS/2) which is specific for the $\alpha4$ subunit (American Type Culture Collection) was injected intravenously at time 0 as a positive control. The number of labeled cells was estimated based upon accumulated radioactivity in the various tissues.

Supporting Information Available: LC/MS data. This material is available free of charge via the Internet at http://pubs.acs.org.

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