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Aza-bicyclic amino acid carboxamides as $\alpha_4\beta_1/\alpha_4\beta_7$ integrin receptor antagonists

Alexey B. Dyatkin,* Yong Gong, Tamara A. Miskowski, Edward S. Kimball, Stephen M. Prouty, M. Carolyn Fisher, Rosemary J. Santulli, Craig R. Schneider, Nathaniel H. Wallace, Pamela J. Hornby, Craig Diamond, William A. Kinney, Bruce E. Maryanoff, Bruce P. Damiano and Wei He

Drug Discovery, Johnson & Johnson Pharmaceutical Research & Development, LLC., Spring House, PA 19477-0776, USA

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Abstract—A series of *N*-carboxy, *N*-alkyl, and *N*-carboxamido azabicyclo[2.2.2]octane carboxamides were prepared and assayed for inhibition of $\alpha_4\beta_1$-VCAM-1 and $\alpha_4\beta_7$-MAdCAM-1 interactions. Potency and $\alpha_4\beta_1/\alpha_4\beta_7$ selectivity were sensitive to the substituent R^1–R^3 in the structures **6**, **7**, and **8**. Several compounds demonstrated low nanomolar balanced $\alpha_4\beta_1/\alpha_4\beta_7$ in vitro activity. Two compounds were selected for in vivo leukocytosis studies and demonstrated increases in circulating lymphocytes up to 250% over control.

1. Introduction

Integrins are members of a widely expressed group of heterodimeric cell adhesion receptors, consisting of α and β subunits. The α_4 integrin family, in particular $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins, plays important roles in adhesion of lymphocytes to extracellular matrix.[1]

The $\alpha_4\beta_1$ integrins (very late antigen-4, VLA-4) bind to their counter-receptor vascular cell adhesion molecule-1 (VCAM-1), which is expressed on endothelial cell surfaces and mediates cell adhesion and infiltration. It was shown that blockage of leukocyte infiltration may be beneficial for therapeutic treatment of such inflammatory diseases as asthma, multiple sclerosis, and rheumatoid arthritis.[1]

The $\alpha_4\beta_7$ integrins are critical in lymphocyte homing to the intestinal mucosa through interaction with its principal counter-receptor mucosal addressin cell adhesion molecule-1 (MAdCAM-1), which is expressed on the gut mucosal endothelium. Inhibition of this interaction was found to be beneficial in the treatment of inflammatory bowel disease (Crohn's disease or ulcerative colitis).[1]

The therapeutic potential of α_4 integrin antagonists has recently been highlighted by FDA approval of Tysabri (natalizumab), a humanized monoclonal antibody that binds the integrin α_4 subunit, for treatment of multiple sclerosis.[2] However, serious complications resulted in the voluntarily withdrawn of Tysabri shortly after its introduction.[3] In this paper, we describe the design, synthesis, and in vivo properties of novel small-molecule $\alpha_4\beta_1/\alpha_4\beta_7$ integrin antagonists.

2. Chemistry

There are many reports in the scientific and patent literature of small-molecule α_4 integrin antagonists.[4]

A major structural class of α-4 integrin antagonists is the *N*-acylphenylalanines, with the *N*-acyl group frequently resembling proline derivatives.[5] Several representatives of this class (**1**,[6] **2**,[7] **3**,[8] and **4**[9]) that demonstrated good in vitro activity in cell adhesion and ELISA assays are shown in Figure 1. We recently discovered that sulfonamides of aza-bicyclic amino acid derivatives[10] (e.g., **5**) are very potent $\alpha_4\beta_1$ and $\alpha_4\beta_7$ antagonists.

Keywords: α4β1 integrin; α4β7 integrin; VLA-4; VCAM-1; MAdCAM-1; Crohn's disease; Inflammatory bowel disease; Asthma; Multiple sclerosis; Rheumatoid arthritis; *N*-Acylphenylalanine; Azabicyclo[2.2.2]octane; Leukocytosis.
* Corresponding author. Tel.: +1 215 628 5008; fax: +1 215 628 4985; e-mail: adyatkin@prdus.jnj.com

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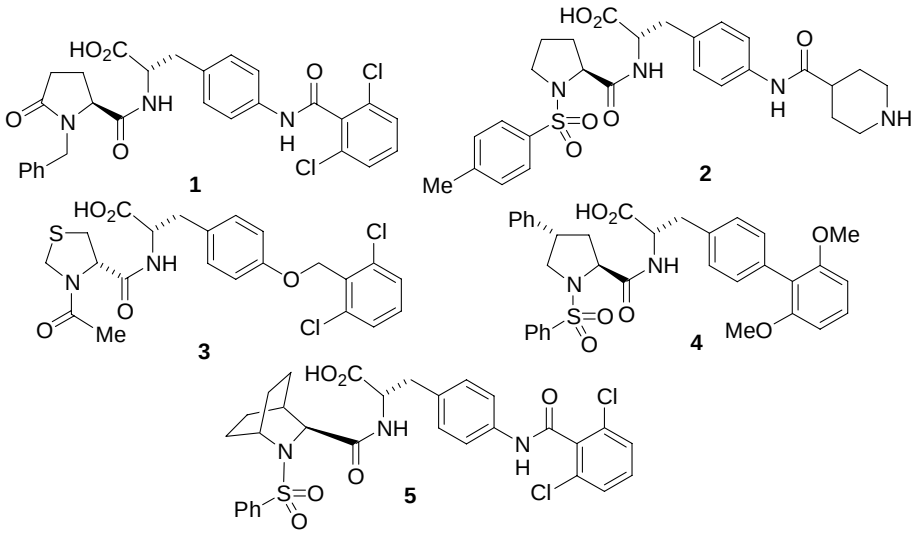


Figure 1. Nonpeptide $\alpha_4\beta_1/\alpha_4\beta_7$ integrin antagonists.

The sulfonamides of aza-bicyclic amino acids, such as **5**, have very high in vitro activity and in vivo efficacy, but are not orally bioavailable. We had chosen to investigate whether further manipulation of the structure, in particular replacement of the sulfonamide group, may lead to orally active dual $\alpha_4\beta_1/\alpha_4\beta_7$ integrin antagonists. We envisioned that by replacing the sulfonamide group with amides, ureas or amines, we may achieve greater compound diversity (prototype structures **6**–**8**), which may provide additional benefits in activity and selectivity (see Fig. 2).

Although the preparation of some amido derivatives of proline analogs, along with their $\alpha_4\beta_1$ activity, was described in the scientific[11] and patent[12] literature, very limited SAR data are available Our first goal was to investigate the $\alpha_4\beta_1$ and $\alpha_4\beta_7$ activity of non-sulfonamide analogs of aza-bicyclic amino acids.

A large number of 4-substituted phenylalanines were reported as components of the active α_4 integrin antagonists. We opted to retain the 2,6-dichloroisonicotinoyl amide derivatized analogs of phenylalanine[13] since these analogs demonstrated the highest activity with sulfonamides of aza-bicyclic amino acid derivatives.[10]

We used a convergent approach to the synthesis of the target compounds (Schemes 1–3). The methodology for preparation of the requisite bridged bicyclic amino acids **9** is well developed.[14] Acylation of **9** with benzyloxycarbonyl chloride followed by hydrolysis resulted in acid **11** (Scheme 1). The acid was coupled with amine **14**, prepared with near quantitative yield from Boc-4-nitrophenylalanine **12** (Scheme 2). Further deprotection and modification of the nitrogen atom of amine **15** followed by hydrolysis resulted in target compounds **6**, **7**, and **8** (Scheme 3). Final products

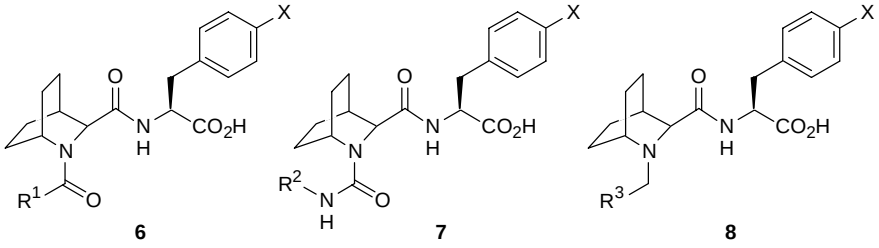
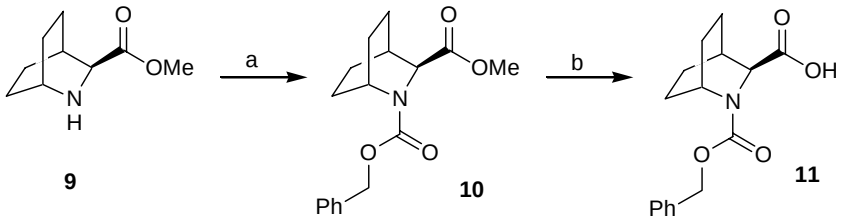
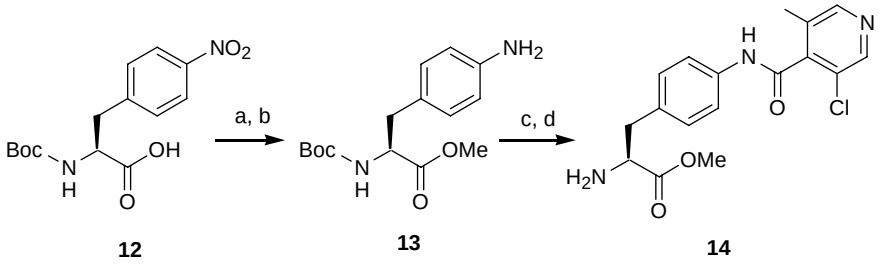


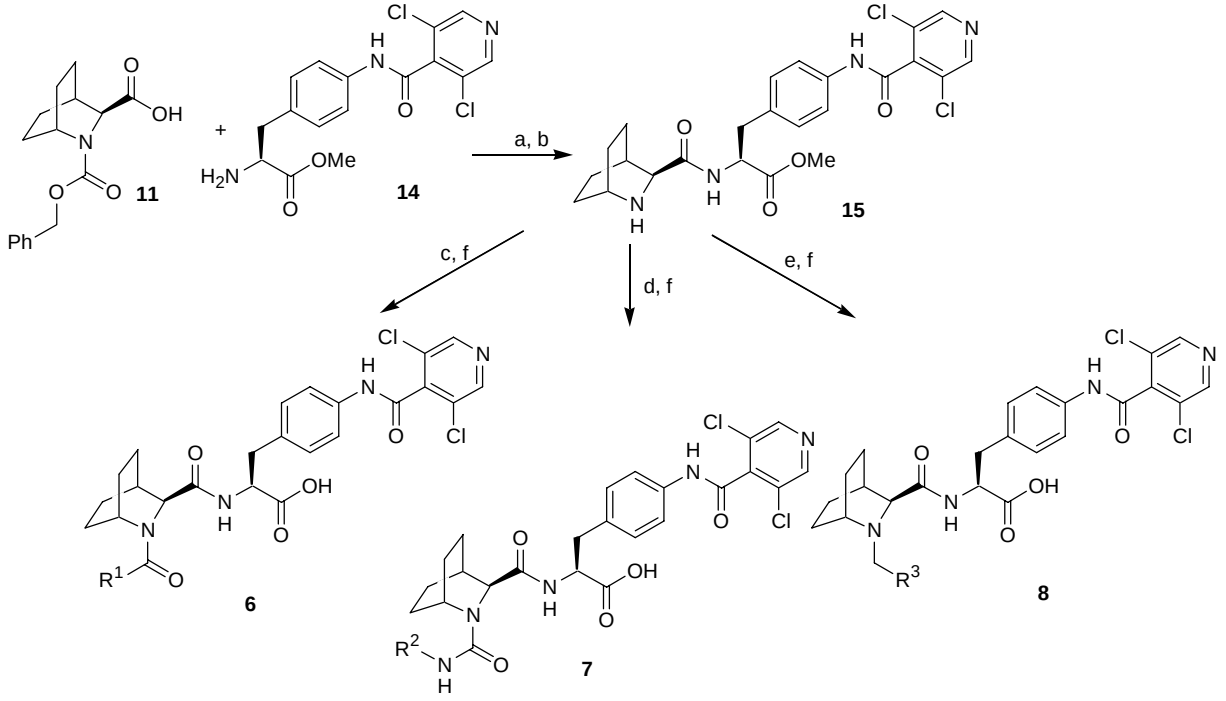
Figure 2. Prototype non-sulfonamide targets **6**, **7**, and **8**.



Scheme 1. Reagents and conditions: (a) CBZ-Cl, CH_2Cl_2, Et_3N, 23 °C (85%); (b) LiOH or NaOH, MeOH, water, 60 °C (70%).



Scheme 2. Reagents and conditions: (a) Me_3SiCHN_2, MeOH, $CHCl_3$, 0 °C; (b) H_2, Pd/C 10%, MeOH, 23 °C; (c) 2,6-dichloroisonicotinoyl chloride, CH_2Cl_2, Et_3N, 23 °C; (d) CF_3CO_2H, CH_2Cl_2, 23 °C (90% total yield).



Scheme 3. Reagents and conditions: (a) bis(2-oxo-3-oxazolidinyl)phosphinic chloride, *i*-Pr_2NEt/CH_2Cl_2, 23 °C; (b) HBr in AcOH, 23 °C; (c) RCO_2H, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride/1-hydroxybenzotriazole hydrate, *i*-Pr_2NEt/CH_2Cl_2, 23 °C; (d) $R_2N{=}C{=}O/CH_2Cl_2$, 23 °C; (e) RCHO, $Na(OAc)_3BH/ClCH_2CH_2Cl$; (f) LiOH or NaOH, MeOH, water, 60 °C (85%).

were isolated by HPLC and characterized by spectroscopic methods.

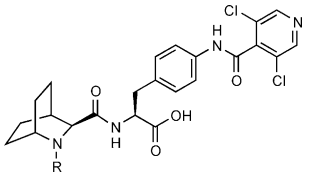
3. In vitro biological results

The aza-bicyclic target compounds were tested for inhibiting cell adhesions mediated by $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins, and the results are presented in the Table 1. All compounds were prepared and tested as mixtures of (*R*,*S*) and (*S*,*S*) diastereomers.[17] We found that the size and the type of R group have decisive effect on $\alpha_4\beta_1$ and $\alpha_4\beta_7$ activity with several trends being observed. The best substituents have a more or less bulky group (i.e., *t*-Bu, *i*-Pr, cycloalkyl or aryl) attached to the C_0–C_3 linker. The alternative to the bulky groups are smaller polar functional groups such as substituted or non-substituted amino (**22**–**24**), hydroxy (**25**) or alkoxy (**26**). These polar groups attached to C_2–C_3 linker also yielded potent integrin antagonists with dual activity. Retention of activity in this case may be explained by additional hydrogen bonding of polar functional groups with substrate. The length of the linker is very important, methylene and ethylene were found to be optimal (**18** is more potent than **16**). Further elongation of the linker diminished the activity, especially for $\alpha_4\beta_7$ (the activity of **20** was lower than **19**, for $\alpha_4\beta_7$). Carbon atoms in the linker may be replaced by O or S atoms with retention of activity (**41**–**43** vs **28**). The aromatic or heteroaromatic group attached to the linker may be substituted with acceptor groups without decreasing activity (**38**, **39**). Electron-donating groups slightly increased $\alpha_4\beta_1$ as well as $\alpha_4\beta_7$ activity (**40** vs **38** and **39**).

Ureas were less potent in vitro than the amides and sulfonamides. Compound **46** is the only example with $\alpha_4\beta_1$ activity below 100 nM. The activity against $\alpha_4\beta_7$ integrins of all synthesized ureas was modest.

N-Alkylated analogs (**47**–**50**) were found to be less potent than the N-acylated derivatives. The best compound, **47**, demonstrated balanced nanomolar

Table 1. Inhibition of cell adhesion mediated via $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins by azabicyclo[2.2.2]octane derivatives (IC_{50})



Compound	R	$\alpha_4\beta_1$/VCAM-1 (nM)	$\alpha_4\beta_7$/MAdCAM-1 (nM)	$\alpha_4\beta_1/\alpha_4\beta_7$ selectivity ratio
16	*t*-BuCO	390	830	0.5
17	*t*-BuCH_2CO	89 ± 1	150 ± 6	0.6
18	*i*-Pr$(CH_2)_2$CO	5 ± 1	15 ± 1	0.3
19	2-Cyclopentyl$(CH_2)_2$CO	28 ± 5	27 ± 9	1.0
20	3-Cyclohexyl$(CH_2)_3$CO	41 ± 20	250 ± 50	0.2
21	Boc$NH(CH_2)_2$CO	9 ± 8	30 ± 14	0.3
22	$NH_2(CH_2)_2$CO	28 ± 12	60 ± 21	0.5
23	$Me_2N(CH_2)_2$CO	24 ± 13	180 ± 16	0.1
24	$HO(CH_2)_2$CO	4 ± 1	0.6 ± 0.3	6.7
25	$HOC(Me)_2CH_2$CO	14 ± 4	47 ± 10	0.3
26	$MeO(CH_2)_2$CO	8 ± 1	110 ± 11	0.1
27	2-F–C_6H_4CO	440	150	2.9
28	$Ph(CH_2)_2$CO	1 ± 0.5	7 ± 2	0.1
29	2-ThienylCH_2CO	12 ± 4	30 ± 2	0.4
30	3-ThienylCH_2CO	4 ± 1	41 ± 14	0.1
31	4-MorpholinoCH_2CO	10 ± 6	30 ± 10	0.3
32	4-(MeO)$C_6H_4CH_2$CO	8 ± 3	110 ± 50	0.1
33	3,5-$(MeO)_2C_6H_3CH_2$CO	23 ± 5	140 ± 70	0.2
34	3,6-$(MeO)_2C_6H_3CH_2$CO	120	130	0.9
35	2-Pyridyl-CH_2CO	25 ± 8	32 ± 4	0.8
36	3-Pyridyl-CH_2CO	20 ± 7	62 ± 8	0.3
37	4-Pyridyl-CH_2CO	3 ± 1	32 ± 15	0.1
38	2-F–C_6H_4 $(CH_2)_2$CO	4 ± 1	46 ± 20	0.1
39	4-Cl–C_6H_4 $(CH_2)_2$CO	31 ± 4	24 ± 14	1.3
40	4-(MeO)C_6H_4 $(CH_2)_2$CO	2 ± 1	7 ± 1	0.3
41	$PhCH_2OCO$	9 ± 1	24 ± 4	0.4
42	2-Pyridyl-SCH_2CO	9 ± 1	10 ± 4	0.9
43	4-Pyridyl-SCH_2CO	6 ± 2	6 ± 2	1.0
44	PhNHCO	260	290	0.9
45	4-MeC_6H_4NHCO	580	120 ± 20	4.7
46	$PhCH_2$NHCO	72 ± 16	38 ± 5	0.2
47	*t*-Bu$(CH_2)_2$	47 ± 5	94 ± 21	0.5
48	$Ph(CH_2)_3$	62 ± 6	260 ± 90	0.2
49	4-$MeC_6H_4(CH_2)_2$	500	260	2.0
50	$PhCH_2$	540	310	1.8
51	H	45 ± 12	23 ± 13	2.0
5		19 ± 3	94 ± 25	0.2

Data represent inhibition of binding of $\alpha_4\beta_1^+$ cells to immobilized VCAM-1 or $\alpha_4\beta_7^+$ cells to immobilized MAdCAM-1. Confidence intervals were usually calculated with $N = 3$. Assay conditions were developed based on Ref. 15 and 16.

$\alpha_4\beta_1/\alpha_4\beta_7$ activity. A free amine **51** (R_1 = H) was very active, probably due to additional hydrogen bonding.

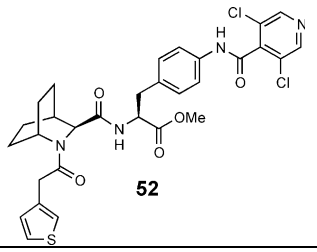
4. In vivo PK studies

The pharmacokinetic profiles in Sprague–Dawley rats of several *N*-amido and *N*-alkyl bicyclic derivatives were determined. Unfortunately, oral bioavailability of all tested compounds, (**30**, **47**, and **51**) was below 5%. These compounds also showed $t_{1/2}$ values below 30 min upon intravenous administration while being stable in vitro in the presence of human or rat liver microsomes. This is an evidence of primarily non-first pass in vivo metabolism for this class of compounds. To overcome low oral bioavailability, we prepared the Me ester of **30**—prodrug **52**. Results of PK studies are presented in the Table 2.

5. In vivo leukocytosis studies

Two compounds, **5** and **28**, representing the sulfonamides and amides, were selected for evaluation for effects on in vivo leukocytosis. Leukocytosis is the increase in circulating white blood cells (leukocytes) that can be brought about by preventing leukocyte binding to leukocyte adhesion molecule counter-receptors expressed on endothelium. This cell–cell adhesion occurs between immunoglobulin superfamily molecules and

Table 2. Oral PK data of **52** in Sprague–Dawley rats



52

C_{max}, μM	$t_{1/2}$, h (i.v.)	AUC μM-h	Clearance (dose/AUC), mg/kg/μM-h	F%
0.533	0.238	1.047	28.7	22

Mature male rats (250–300 g) were used. Each compound was administered at a dose of 30 mg/kg p.o. ($N = 3$) and 3 mg/kg i.v. ($N = 3$). The plasma levels for the compounds were determined by LC–MS. All parameters were measured for the acid **30**. Pro-drug **52** was not detected in plasma.

integrins. Relevant examples of these paired interactions include vascular cell adhesion molecule-1 with $\alpha_4\beta_1$ integrin, and mucosal addressin cell adhesion molecule-1 with $\alpha_4\beta_7$ integrin, respectively.

In this model, a compound that antagonizes these leukocyte–endothelial interactions will cause an increase in circulating leukocytes, defined as leukocytosis, as measured at 1 h post-administration, a time when drug plasma levels were allowed to achieve a maximum and have had sufficient time to manifest a sufficient biological effect. This leukocytosis is indicative that normal lymphocyte or leukocyte emigration from the peripheral circulation was prevented. Similar emigration of cells out of the circulation into inflamed tissues is responsible for the progression and maintenance of the inflammatory state. Leukocytosis is an indication that lymphocyte and leukocyte extravasation is prevented, and may be predictive of general anti-inflammatory activity. In vivo administration of the two compounds produced significant elevations in circulating lymphocytes and total leukocytes. Among the latter, circulating counts for cells of various granulocytic lineages remained unchanged.

Dose–responsive increases in lymphocyte counts were observed 1 h after subcutaneous administration of Compound **5** and Compound **28** to naïve animals. The lymphocytosis responses are shown in Figure 3. Compound **5** induced an increase in lymphocyte counts to 177% of vehicle control, and compound **28** induced lymphocyte counts to rise to 253% of vehicle control. Nearly, identical increases in total leukocyte counts were found to occur. Granulocyte counts remained unchanged (data not shown).

The increased numbers of circulating leukocytes were dose-dependent and indicate that blockade of integrin receptor-mediated binding to endothelial counter-receptors ($\alpha_4\beta_1$ to VCAM-1 and $\alpha_4\beta_7$ to MAdCAM-1) results in an inability of the cells to extravasate as part of a normal trafficking function. This further supports the utility of this method as a possible clinical surrogate and for evaluating dosing regimens, as discussed previously in the same context in studies with natalizumab, an antibody to α_4 integrin,[18] and with the synthetic integrin antagonist BIO5192.[19]

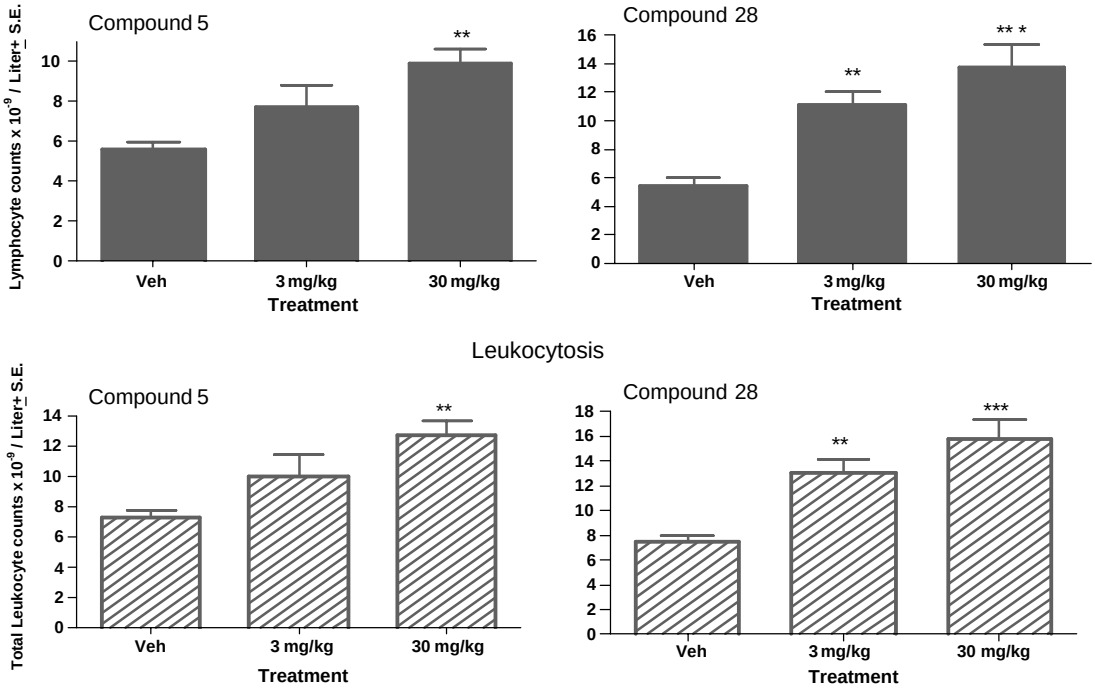


Figure 3. In vivo leukocytosis studies. Dose–responsive lymphocytosis occurs following subcutaneous administration of **5** and **28**. Female, Balb/c mice, $n = 8$, were given vehicle, 3 mg/kg or 30 mg/kg of Compound **5** or Compound **28** in 0.5% methyl cellulose. One hour later, blood samples were drawn and were analyzed for lymphocyte numbers. Numbers represent means and standard error of counts $\times 10^{-9}$/L: ** = $p < 0.01$, *** = $p < 0.001$ versus vehicle-treated group; ANOVA.

6. Conclusion

In summary, we have identified a novel series of potent non-sulfonamide α_4 integrin antagonists. While amine and urea substitutions provided moderately active compounds, amides were equipotent or even more potent than the corresponding sulfonamides. Several compounds had low-nanomolar or even sub-nanomolar potency; the ratio of $\alpha_4\beta_1/\alpha_4\beta_7$ activity varied from 0.1 to 6.7. In vivo administration of amide **28** as well as sulfonamide **5** produced a significant elevation in circulating lymphocytes and total white cells. Further investigation of such compounds may lead to new treatments of diseases mediated by α_4 integrins.

7. Experimental

All commercially available chemicals were used as purchased. Melting points were obtained with a Mel-Temp capillary melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded with a Bruker AC-400 spectrometer with TMS as an internal standard. Electrospray ionization mass spectra (ESI) were obtained using a Fisons spectrometer (Hewlett-Packard HPLC driven electrospray MS instrument). Compounds were purified using ISCO CombiFlash® Sq16x system with silica RediSep columns or Gilson HPLC system with YMC ODS-H80 column. The purities of each compound were determined using a Hewlett-Packard LC 1100 system (YMC J'Sphere H80 S4 column, 4.0 × 50 mm, 4 mm C_{18}; mobile phase of 90% H_2O (0.1% TFA) to 10% H_2O (0.1% TFA) with a flow rate of 1 mL/min; detection at 220 and 254 nM). Elemental analyses were conducted by Robertson Microlit Laboratories.

7.1. *N*-Benzyloxycarbonyl-[2.2.2]azabicyclooctane-1 ethyl carboxylate (10)

Amino ester **9** (5.90 g, 0.0322 mol) was dissolved in 100 ml of dry DCM containing 9.43 mL (0.067 mol) of Et_3N and solution was cooled in the ice bath. Benzyl chloroformate (5.77 g, 4.83 mL, 0.0338 mol) was added dropwise by syringe. The reaction was stirred 2 h at 0 °C, then warmed up to room temperature and stirred overnight. The reaction mixture was washed with 0.1 N HCl, 5% $NaHCO_3$, and water, dried over $MgSO_4$ and concentrate, resulting **10** as viscous oil. Product was analyzed by TLC (hexane/EtOAc 1:1, R_f 0.75). The crude material was purified by column chromatography (silica, heptane/EtOAc 2:1) resulting 7.73 g (76%) of yellow solid.

^{1}H NMR ($CDCl_3$): δ 7.37–7.26 (5H, m), 5.20–5.10 (2H, m), 4.71–4.69 (1H, m), 4.26–4.00 (3H, m), 2.24–2.22 (1H, m), 2.16–2.15 and 2.13–2.04 (1H, m), 2.00–1.40 (m, 9H), 1.25 and 1.15 (3H, J = 7.3 Hz).

7.2. *N*-Benzyloxycarbonyl-[2.2.2]azabicyclooctane-1 carboxylic acid (11)

Ester **10** (7.73 g, 24.36 mmol) was dissolved in 100 ml MeOH and 5 equiv of 1.0 N NaOH aq were added as one portion. Reaction was warmed to 70 °C for 5 h and evaporated. The residue was dissolved in 100 mL H_2O, acidified by 1 N HCl to pH 2 and extracted by EtOAc (3× 25 mL). Organic fractions were combined, dried over $MgSO_4$, filtered, and evaporated, providing white solid material (6.34 g, 90%).

^{1}H NMR ($CDCl_3$): δ 7.34–7.26 (5H, m), 5.29-5-.10 (2H, m), 4.70–4.13 (2H, m), 2.29–2.23 (1H, m), 2.09–2.00 (1H, m), 2.00–1.40 (8H, m); MS (ES^-) 288.

7.3. 4-Amino-*N*-Boc phenylalanine methyl ester (13)

N-Boc-4-nitro-L-phenylalanine, **12**, (5 g) was dissolved in 100 ml MeOH/chloroform 1:1 mixture, the solution was cooled in the ice bath. Trimethylsilyl diazomethane (1 M solution in hexane) was added dropwise until the solution remained yellow. The reaction mixture was evaporated in vacuum, the residue was dissolved in 50 ml MeOH/ethyl acetate 1:1 mixture and was hydrogenated at 30 psi overnight over Pd/C 10% (100 mg). After filtration the solvent was evaporated, providing 5.1 g of **13** as white solid.

^{1}H NMR ($CDCl_3$): δ 6.89 (d, J = 8.2 Hz, 2H), 6.60 (d, J = 8.3 Hz, 2H), 5.05–4.95 (m, 1H), 4.59–4.50 (m, 1H), 3.01–2.95 (m, 2H), 1.41 (s, 9H); MS (ESI^+) m/z 295 $(M+H)^+$. Anal. Calcd for $C_{15}H_{22}N_2O_4$: C, 61.21; H, 7.53; N, 9.52. Found: C, 61.24; H, 7.80; N, 9.46.

7.4. 4-Dichloroisonicotinamido phenylalanine methyl ester (14)

Compound **13** (5.0 g, 0.017 mol) was dissolved in 50 ml CH_2Cl_2 containing 3 ml of Et_3N followed by 5.31 g (0.025 mol) of 3,5-dichloroisonocotinoyl chloride,[11] The reaction mixture was kept overnight at room temperature, washed with 0.1 N HCl, 10% $NaHCO_3$, dried over $MgSO_4$, filtered, and evaporated. The product was purified by crystallization from hexane/ethyl acetate, providing 6.22 g (78% yield) of *N*-BOC-4-dichloroisonicotinamido phenylalanine methyl ester as white solid, mp 124–126 °C.

^{1}H NMR (DMSO-d_6): δ 8.79 (s, 2H), 7.56 (d, J = 8.4 Hz, 2H), 7.30 (d, J = 8.1 Hz, 1H), 7.24 (d, J = 8.4 Hz, 2H), 4.17–4.13 (m, 1H), 2.97 (dd, J = 13.7 and 5.0, 1H), 2.83 (dd, J = 13.6 and 9.9, 1H); 1.33 (s, 9H); MS (ESI^+) m/z 469 $(M+H)^+$. Anal. Calcd for $C_{21}H_{23}Cl_2N_3O_5 \cdot 0.8\ Et_2O$: C, 55.09; H, 5.92; N, 7.96. Found: C, 54.94; H, 5.86; N, 8.00.

N-BOC-4-dichloroisonicotinamido phenylalanine methyl ester (4.68 g, 0.01 mol) was dissolved in 30 ml of CH_2Cl_2 followed by 1 ml of TFA. Reaction was kept overnight at room temperature, evaporated in vacuum, and the viscous residue was recrystallized from CH_2Cl_2/ether, providing **14** as white solid (5.1 g, 80% yield); mp 257–259 °C.

^{1}H NMR (DMSO-d_6): δ 8.80 (s, 2H), 8.44 (br s, 3H), 7.63 (d, J = 8.4 Hz, 2H), 7.63 (d, J = 8.5 Hz, 2H), 7.24 (d, J = 8.5 Hz, 2H), 4.33 (t, J = 6.4 Hz, 1H), 3.09 (d, J = 6.4, 2H), MS (ESI^+) m/z 369 $(M+H)^+$. Anal. Calcd

for $C_{16}H_{15}Cl_2N_3O_3 \cdot CF_3CO_2H$: C, 44.83; H, 3.34; F, 11.82; N, 8.71. Found: C, 44.49; H, 3.23; F, 11.78; N, 8.61.

7.5. 2-[(2-Aza-bicyclo[2.2.2]octane-3-carbonyl)-amino]-3-(4-[(3,5-dichloro-pyridine-4-carbonyl)-amino]-phenyl)-propionic acid methyl ester (15)

The acid **11** (2.50 g, 8.6 μmol) and di-TFA salt of amine **14** (5.14 g, 8.6 μmol) were dissolved in 15 mL CH_2Cl_2, containing 500 μL of Et_3N, followed by bis(2-oxo-3-oxazolidinyl)phosphinic chloride (4.40 g, 2 equiv). The reaction was stirred overnight at room temperature under nitrogen and then washed H_2O. The residue after evaporation of solvent was subjected to column chromatography on silica gel eluted with ethyl acetate to give 3-(2-(4-[(3,5-dichloro-pyridine-4-carbonyl)-amino]-phenyl)-1-methoxycarbonyl ethylcarbamoyl)-2-aza-bicyclo[2.2.2]octane-2-carboxylic acid benzyl ester (4.66 g, 85%) as a white solid.

^{1}H NMR (300 MHz, CD_3CN): δ 8.90 (1H, s), 8.65 (2H, s), 7.65–7.50 (2H, m), 7.45–7.15 (9H, m), 6.97 (1H, d, J = 8.0 Hz), 5.12–4.99 (2H, m), 4.75–4.60 (1H, m), 4.09–4.01 (2H, m), 3.69–3.62 (3H, m), 3.20–2.90 (1H, m); MS (ESI$^+$) m/z 639 $(M+H)^+$.

This benzyloxycarbonyl derivative (3.65 g, 5.72 mmol) was added to 33% HBr in AcOH (45 mL) under vigorous stirring. The reaction was kept at room temperature for 3 h (reaction became homogeneous after 45 min). The viscous liquid was evaporated under vacuum, and the resulting residue was dissolved in water (250 mL), then extracted with Et_2O. The organic layer was discarded. The aqueous layer was basified to pH 7 with Na_2CO_3 and extracted with EtOAc (5× 20 mL). The organic layers were combined, dried (Na_2SO_4), and evaporated to provide Compound **15** (2.85 g) as a pale yellow solid. Compound **15** was purified by column chromatography on silica gel eluted with 9:1 $CHCl_3$/MeOH to give 2.5 g (87%) of pure compound **15** a white solid. The analytically pure material was purified by HPLC, providing after lyophilization a salt with 1.3 equivalents of TFA.

^{1}H NMR (CD_3OD): δ 8.66 (s, 2H), 7.59 (d, J = 8.5 Hz, 2H), 7.24 (d, J = 8.5 Hz, 2H), 4.83 (m, 1H), 3.92 (s, 1H), 3.74 (s, 3H), 3. 42 (s, 1H), 3.25 (m, 1H), 2.97 (m, 1H), 2.28 (s, 1H), 2.01–1.54 (m, 8H), MS (ESI$^+$) m/z 506 $(M+H)^+$. Anal. Calcd for $C_{24}H_{26}Cl_2N_4O_4 \cdot 1.3\ CF_3COOH$: C, 48.88; H, 4.21; Cl, 10.85; F, 11.34; N, 8.57. Found: C, 48.53; H, 3.82; Cl, 10.90; F, 11.12; N, 8.35.

7.6. Typical procedure for preparation of amides (6)

Compound **15** (73 mg, 0.10 mmol), the acid (0.105 mmol), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (21 mg, 0.11 mmol), and 1-hydroxybenzotriazole hydrate (14 mg, 0.105 mmol) were suspended in DCM (1 mL) at room temperature and *N*-methyl-morpholine (14 μL, 0.120 mmol) was added in one portion. The reaction was kept at room temperature for 4 h and loaded into a silica column. Flash chromatography (silica gel, EtOAc) provided target amide with 65–90% yield.

This amide was dissolved in MeOH/water (2 mL, 1:1) and LiOH (4 mg, 0.1 mmol) was added in one portion. The reaction was homogenized in an ultrasonic bath and kept overnight at room temperature. The reaction mixture was diluted with water (20 mL), extracted with Et_2O (10 mL), and the organic layer was discarded. The aqueous layer was acidified with 1 N HCl to pH 2 and extracted with EtOAc (2× 10 mL). The organic layers were combined, dried ($MgSO_4$), filtered, and evaporated to give a white residue which was purified by HPLC. The desired fractions were pooled and lyophilized to yield target product **6**, which was characterized by NMR, MS, and elemental analysis.

7.7. Typical procedure for preparation of ureas (7)

Compound **15** (73 mg, 0.10 mmol), the isocyanate (0.105 mmol) and 150 μL of Et_3N were suspended in DCM (1 mL). The reaction was kept at room temperature for 12 h and loaded into a silica column. Flash chromatography (silica gel, EtOAc) provided target amide with 50–85% yield.

This urea was dissolved in MeOH/water (2 mL, 1:1) and LiOH (4 mg, 0.1 mmol) was added in one portion. The reaction was homogenized in an ultrasonic bath and kept overnight at room temperature. The reaction mixture was diluted with water (20 mL), extracted with Et_2O (10 mL), and the organic layer was discarded. The aqueous layer was acidified with 1 N HCl to pH 2 and extracted with EtOAc (2× 10 mL). The organic layers were combined, dried ($MgSO_4$), filtered, and evaporated to give a white residue which was purified by HPLC. The desired fractions were pooled and lyophilized to yield target product **7**, which was characterized by NMR, MS, and elemental analysis.

7.8. Typical procedure for preparation of amines (8)

A 10-mL vial (SmithProcess) containing a magnetic stir bar was charged with **15** (73 mg, 0.10 mmol), aldehyde/ketone (0.15 mmol), acetic acid (10 μL), sodium triacetoxyborohydride (32 mg 0.15 mmol) in ethylene dichloride (0.5 mL). The vial was sealed and the mixture was heated under microwave (SmithSynthesizer) at 120 °C for 5 min. The reaction mixture was concentrated and treated with 1 N LiOH (0.5 mL) in MeOH (0.5 mL) at room temperature for 4 h. Acidification and purification gave desired compound, which was purified by HPLC. The desired fractions were pooled and lyophilized to yield target product **8**, which was characterized by NMR, MS, and elemental analysis.

After lyophilization compounds usually contained 0.2–1.5 equivalent of TFA. Most compounds existed as rotamers, which complicated NMR spectra. Below are presented examples of the target compounds characterization (*S*,*S*-isomers).

16: ^{1}H NMR (DMSO-d_6): δ 0.80 (s, 1H); 8.73 (s, 2H), 7.71 (d, J = 6.97 d, 1H), 7.49 (d, J = 8.5 Hz, 2H), 7.21 (d, J = 8.5 Hz, 2H), 4.23–4.28 (m, 1H), 4.09–4.05 (m, 2H), 3.00–2.80 (m, 2H), 2.03 (s, 1H), 1.90–1.73 (m, 1H),

1.65–1.40 (m, 6H), 1.35–1.15 (m, 1H), 1.08 (s, 9H). MS (ESI^+) *m/z* 575 $(M+H)^+$. Anal. Calcd ($C_{28}H_{32}Cl_2N_4O_5 \cdot$ 0.25 TFA): C, 56.67; H, 5.38; F, 2.36; Cl, 11.74; N, 9.28. Found: C, 56.22; H, 5.01; F, 2.30; Cl, 12.02; N, 9.02.

17: ^1H NMR (CD_3OD, rotamers): δ 8.65 (s, 2H), 7.60–7.55 (m, 2H), 7.34–7.25 (m, 2H), 4.71 (m, 0.5H), 4.46 (m, 0.5H), 4.32 (m, 0.5H), 4.26 (m, 0.5H), 4.03 (m, 1H), 3.20–2.90 (m, 2H), 2.20–1.20 (m, 12H), 1.05 and 0.94 (s, 9H). MS (ESI^+) *m/z* 589 $(M+H)^+$. Anal. Calcd ($C_{29}H_{34}Cl_2N_4O_5 \cdot$ 1.2 TFA): C, 51.92; H, 4.88; F, 9.42; Cl, 9.76; N, 7.71. Found: C, 52.55; H, 4.80; F, 10.08; Cl, 10.14; N, 7.78.

18: ^1H NMR (DMSO-d_6, rotamers): δ 10.74 and 10.72 (s, 1H); 8.71 (s, 2H), 7.42–7.35 (m, 2.5H), 7.14–7.06 (m, 2.5H), 4.24 (m, 0.5H), 4.08–4.00 (m, 1H), 3.98–3.95 (m, 0.5H), 3.87–3.85 (m, 0.5H), 3.79 (m, 0.5H), 3.00–2.90 (m, 2H), 2.32–2.22 (m, 0.5H), 2.16–2.05 (m, 0.5H), 2.05 and 2.00 (m, 1H), 1.96–1.89 (m, 0.5H), 1.85–1.75 (m, 0.5H), 1.65–1.15 (m, 11H), 0.80 (d, J = 6.6 Hz, 3H), 0.72 (dd, J = 6.4 and 3.7 Hz, 3H). MS (ESI^+) *m/z* 589 $(M+H)^+$. Anal. Calcd ($C_{29}H_{34}Cl_2N_4O_5 \cdot$ 2.5 TFA$\cdot$ 0.5 H_2O): C, 46.22; H, 4.28; Cl, 8.02; F, 16.13; N, 6.34. Found: C, 46.21; H, 4.34; Cl, 7.73; F, 15.69; N, 6.46; KF 1.04.

21: ^1H NMR (CD_3OD, rotamers): δ 8.65 (m, 2H), 7.60–7.50 (m, 2H), 7.35–7.20 (m, 2H), 4.75–3.70 (m, 3H), 3.20–2.80 (m, 2H), 2.65–2.35 (m, 2H), 2.15 (m, 2H), 2.00–1.20 (m, 19H). MS (ESI^+) *m/z* 662 $(M+H)^+$. Anal. Calcd ($C_{31}H_{37}Cl_2N_5O_7 \cdot$ 1.1 TFA): C, 50.60; H, 4.87; Cl, 9.00; F, 7.96; N, 8.89. Found: C, 50.72; H, 4.80; Cl, 9.43; F, 8.00; N, 9.27.

28: ^1H NMR (DMSO-d_6, rotamers): δ 10.75 (s, 1H), 8.72 (s, 1H), 7.66 (m, 1H), 7.45–7.39 (m, 2H), 7.20–7.15 (m, 3H), 7.10–7.03 (m, 2H), 4.25 (m, 1H), 4.04 (m, 1H), 3.85–3.75 (m, 1H), 3.10–2.50 (m, 4H), 2.00–1.10 (m, 10H). MS (ESI^+) *m/z* 623 $(M+H)^+$. Anal. Calcd ($C_{32}H_{32}Cl_2N_4O_5 \cdot$ 1 TFA): C, 55.37; H, 4.51; Cl, 9.61; F, 7.73; N, 7.60. Found: C, 55.29; H, 4.46; Cl, 9.45; F, 7.55; N, 7.42.

30: ^1H NMR (DMSO-d_6, rotamers): δ 0.79 (s, 1H), 8.78 (m, 2H), 7.52–7.38 (m, 4H), 7.22–6.94 (m, 4H), 4.30–3.60 (m, 4H), 3.15–2.95 (m, 2H), 2.07 (m, 1H), 1.75–1.20 (m, 9H). MS (ESI^+) *m/z* 615 $(M+H)^+$. Anal. Calcd ($C_{29}H_{28}Cl_2N_4O_5S \cdot$ 2 H_2O, 1.5 TFA): C, 46.72; H, 4.10; Cl 8.62; F, 10.39; N, 6.81; S 3.90. Found: C, 46.24; H,3.96; Cl, 9.02; F, 10.07; N, 6.61; S, 3.84; KF 4.06.

33: ^1H NMR (CD_3OD, rotamers): δ 8.64–8.63 (m, 2H), 7.59–7.52 (m, 2H), 7.32–7.28 (m, 2H), 6.92–6.75 (m, 3H), 4.70–4.73 (m, 1H), 4.43–4.21 (m, 1H), 3.91 (s, 1H), 3.81–3.72 (m, 6H), 3.61–3.48 (m, 1H), 3.39–3.31 (m, 1H), 3.16–2.91 (m, 2H), 2.17 (s, 1H), 1.93–1.45 (m, 8H). MS (ESI^+) *m/z* 669 $(M+H)^+$. Anal. Calcd ($C_{33}H_{34}Cl_2N_4O_7 \cdot$ 1 H_2O, 1 TFA): C, 52.44; H, 4.65; Cl, 8.85; F, 7.11; N, 6.99. Found: C, 52.20; H, 4.35; Cl, 9.00; F, 6.76; N, 6.59; KF 2.20.

35: ^1H NMR (CD_3OD, rotamers): δ 8.79–8.71 (m, 1H), 8.62 (m, 2H), 8.47–8.34 (m, 1H), 7.92–7.77 (m, 1.5H), 7.67 (m, 0.5H), 7.52–7.42 (m, 2H), 7.33–7.21 (m, 2H), 4.68–4.62 (m, 1H), 4.42–4.30 (m, 1H), 4.00–3.95 (m, 1H), 3.56–3.29 (m, 1H), 3.17–2.92 (m, 3H), 2.30–2.17 (m, 1H), 2.08–1.15 (m, 8H). MS (ESI^+) *m/z* 610 $(M+H)^+$. Anal. Calcd ($C_{30}H_{29}Cl_2$ $N_5O_5 \cdot$ 1.5 H_2O, 1.3 TFA): C, 49.83; H, 4.27; Cl, 9.02; F, 9.43; N, 8.91. Found: C, 49.92; H, 3.78; N, 8.87; Cl, 8.95; F, 9.68; KF 3.30.

37: ^1H NMR (CD_3OD, rotamers): δ 8.78–8.26 (m, 5H), 8.00–7.93 (m, 1H), 7.56–7.48 (m, 2H), 7.35–7.29 (m, 2H), 4.70–4.67 (m, 1H), 4.41–4.35 (m, 1H), 4.21–4.17 (m, 1H), 4.05–3.95 (m, 1H), 3.70–3.34 (m, 1H), 3.20–2.97 (m, 2H), 2.28–2.20 (m, 1H), 2.03–1.13 (m, 8H). MS (ESI^+) *m/z* 610 $(M+H)^+$. Anal. Calcd ($C_{30}H_{29}Cl_2N_5O_5 \cdot$ 1 H_2O, 1 TFA): C, 51.76; H, 4.34; F, 7.68; N, 9.43. Found: C, 51.46; H, 4.69; F, 7.53; N, 9.69; KF 2.35.

39: ^1H NMR (DMSO-d_6, rotamers): δ 10.85 and 10.83 (s, 1H), 8.76 and 8.75 (s, 2H), 7.50–7.43 (m, 2H), 7.30–7.18 (m, 7H), 4.25 (m, 0.5H), 4.20 (dd, J = 12.7 and 7.2 Hz, 0.5H), 4.05 (m, 0.5H), 4.20 (dd, J = 7.2 and 5.2 Hz, 0.5H), 3.90 (m, 0.5H), 3.84 (m, 0.5H), 3.06–3.00 (m, 1H), 2.95–2.55 (m, 5H), 2.25–2.10 (m, 1H), 1.65–1.15 (m, 9H). MS (ESI^+) *m/z* 671 $(M+H)^+$. Anal. Calcd ($C_{32}H_{31}Cl_3N_5O_5 \cdot$ 3.5 H_2O, 3.5 TFA): C, 41.30; H, 3.69; Cl, 9.38; F, 17.58; N, 6.18. Found: C, 41.26; H, 3.43; Cl, 9.07; F, 17.92; N, 6.36; KF 5.35.

41:^1H NMR (CD_3OD, rotamers): δ 8.63 (s, 2H), 7.60–7.50 (m, 2H), 7.40–7.15 (m, 7H), 5.11 and 5.07 (m, 1H), 4.80–4.65 (m, 1H), 4.17 (m, 1H), 4.04 (m, 1H), 3.25–3.15 (m, 1H), 3.03–2.80 (m, 1H), 2.20–1.20 (m, 10H). MS (ESI^+) *m/z* 625 $(M+H)^+$. Anal. Calcd ($C_{31}H_{30}Cl_2N_4O_6$): C, 59.53; H, 4.83; Cl, 11.34; N, 8.96. Found: C, 59.26; H, 4.56; Cl, 11.59; N, 8.70.

48:^1H NMR (CD_3OD, rotamers): δ 8.65–8.61 (m, 2H), 7.65–7.55 (m, 2H), 7.31–7.08 (m, 7H), 3.81–3.72 (m, 1H), 3.58–3.31 (m, 3H), 3.16–2.78 (m, 2H), 2.75–2.48 (m, 2H), 2.23 (br s, 1H), 2.05–1.11 (m, 11H). MS (ESI^+) *m/z* 609 $(M+H)^+$. Anal. Calcd ($C_{32}H_{34}Cl_2N_4O_4 \cdot$ 0.5 H_2O, 1.6 TFA): C, 52.78; H, 4.61; Cl, 8.85; F, 11.38; N, 6.99. Found: C, 52.72; H, 4.04; Cl, 9.00; F, 11.05; N, 6.97; KF 1.20.

49: ^1H NMR (CD_3OD, rotamers): δ 8.63 (m, 2H), 7.57 (M, 2H), 7.34–7.24 (m, 7H), 3.97 (m, 1H), 3.16–3.04 (m, 3H), 2.52–2.29 (m, 4H), 2.29 (br s, 1H), 2.20–1.50 (m, 9H). MS (ESI^+) *m/z* 609 $(M+H)^+$. Anal. Calcd ($C_{32}H_{34}Cl_2N_4O_4 \cdot$ 0.4 H_2O, 1.4 TFA): C, 53.84; H, 4.70; Cl, 9.13; F, 10.28; N, 7.25. Found: C, 53.92; H, 4.91; Cl, 9.29; F, 10.35; KF 1.01.

50: ^1H NMR (CD_3OD, rotamers): δ 8.67 (s, 2H), 7.57 (m, 2H), 7.42 (m, 2H), 7.39–7.23 (m, 3H), 7.02 (m, 2H), 4.43–4.32 (m, 2H), 4.30–4.20 (m, 1H), 3.80 (s, 1H), 3.57 (m, 1H), 3.03–2.92 (m, 1H), 2.77–2.67 (m, 1H), 2.42–2.21 (m, H), 2.18–2.02 (m, 1H), 1.98–1.72 (m, 4H), 1.63–1.52 (m, 2H). (ESI^+) *m/z* 581 $(M+H)^+$. Anal. Calcd ($C_{30}H_{30}Cl_2N_4O_4 \cdot$ 1.0 H_2O, 1.6 TFA): C, 51.00; H, 4.33; Cl, 9.07; F, 11.66; N, 7.17. Found: C, 50.93; H, 3.75; Cl, 9.25; F, 11.45; N, 7.12; KF 2.15.

51: ^{1}H NMR(DMSO-d_6): δ 10.91 (s, 1H); 9.06–9.00 (m, 1H); 8.83 (d, J = 7.8 Hz, 1H); 8.80 (s, 2H), 8.00–7.92 (m, 1H), 7.57 (d, J = 8.5 Hz, 2H), 7.27 (d, J = 8.5 Hz, 2H), 4.57–4.52 (m, 1H), 3.92 (s, 1H), 3.37 (s, 1H), 3.13 (dd, J = 14.0 and 4.6 Hz, 1H), 2.91 (dd, J = 14.0 and 9.6 Hz, 1H, 2.12 s, 1H), 1.85–1.48 (m, 8H). MS (ESI$^+$) m/z 492 (M+H)$^+$. Anal. Calcd ($C_{23}H_{24}Cl_2N_4O_4$· 1.1 TFA): C, 49.07; H, 4.10; Cl, 11.50; F, 10.16; N, 9.08. Found: C, 48.85; H, 3.72; Cl, 11.49; F, 10.25; N, 8.85.

7.9. Ramos cell adhesion assay ($\alpha_4\beta_1$ mediated adhesion/VCAM-1)

Immulon 96-well plates (Dynex) were coated with 100 μL recombinant hVCAM-1 at 4.0 μg/mL in 0.05 M $NaCO_3$ buffer, pH 9.0, overnight at 4 °C (R&D Systems). Plates were washed two times in PBS with 1% BSA and blocked for 1 h at room temperature in this buffer. PBS was removed and compounds to be tested (50 μL) were added at 2 times concentration. Ramos cells (50 μL at 2×10^6/mL), labeled with 5 μM Calcein AM (Molecular Probes) for 1 h at 37 °C, were added to each well and allowed to adhere for 1 h at room temperature. Plates were washed 4 times in PBS + 1% BSA and cells were lysed for 15 min in 100 μL of 1 M Tris, pH 8.0, with 1% SDS. The plate was read at 485 nm excitation and 530 nm emission.

7.10. $\alpha_4\beta_7$-K562 cell adhesion assay ($\alpha_4\beta_7$ mediated adhesion/MAdCAM-1)

M2 anti-FLAG antibody coated 96-well plates (Sigma) were coated for 1 h at 4 °C with 2–8 μL/well recombinant FLAG-hMAdCAM-1 contained in 100 μL of Dulbecco's PBS, pH 7.4, with 1% BSA and 1 mM Mn^{2+} (PBS–BSA–Mn). Plates were washed once with PBS–BSA–Mn. Buffer was removed and compounds to be tested (50 μL) were added at 2 times concentration. Stably transfected K562 cells expressing human $\alpha_4\beta_7$ integrin, (50 μL at 2×10^6/mL) that had been labeled with 100 μg/mL carboxymethyl fluorescein diacetate succinimidyl ester (CFDA-SE; Molecular Probes) for 15 min at 37 °C were added to each well and allowed to adhere for 1 h at room temperature. Plates were washed 4 times in PBS–BSA–Mn and then cells were lysed for 2 min by addition of 100 μL of PBS without Ca, Mg supplemented with 0.1 M NaOH. The plate was read on a 96-well fluorescent plate reader at 485 nm excitation and 530 nm emission.

7.11. Pharmacokinetic assay

Rats were dosed intravenously (IV) at 3 mg/kg and by oral gavage at 30 mg/kg with tested compound. Blood samples (0.5–1.0 ml) were collected post dose into heparinized tubes and centrifuged for cell removal. Precisely 200 μL of plasma supernatant was then transferred to a clean vial, placed on dry ice, and subsequently stored in a −70 °C freezer prior to analysis. Plasma samples were prepared by adding 400 μL of acetonitrile containing internal standard to 200 μL of plasma to precipitate proteins. Samples were centrifuged and supernatant was removed for analysis by LC–MS–MS. Calibration standards were prepared by adding appropriate volumes of stock solution directly into plasma and treated identically to collected plasma samples. Calibration standards were prepared in the range of 0.01–10 μM for quantitation. LC–MS–MS analysis was performed utilizing multiple reaction monitoring for detection of characteristic ions for each tested compound, additional related analytes, and internal standard. The low detection limit was 0.01 μM.

7.12. Leukocytosis studies

Selected compounds were administered subcutaneously in 0.5% methyl cellulose, at doses of 3 and 30 mg/kg, to 8-week-old female Balb/C mice (Charles River Laboratories, Kingston, NC). One hour later, 250 μL of blood were removed and immediately added to microtainer tubes containing lithium heparin (Becton–Dickinson, Franklin Lakes, NJ). Samples were analyzed for total white blood cell counts and for lymphocyte counts using an Advia 120 Hematology System (Bayer Diagnostics, Tarrytown, NY). ANOVA was used to evaluate statistical significance.

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