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N-Isonicotinoyl-(L)-4-aminophenylalanine Derivatives as Tight Binding VLA-4 Antagonists

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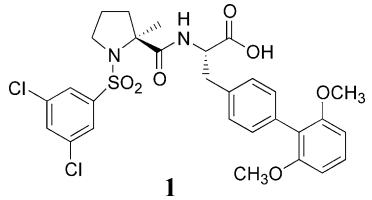
Abstract—A series of isonicotinoyl-(L)-aminophenylalanine derivatives was prepared and evaluated as VLA-4 antagonists. These compounds exhibit subnanomolar binding affinity to VLA-4 and significant off-rates. The interplay between off-rate, protein binding and pharmacokinetics is discussed.

VLA-4 ($\alpha_4\beta_1$; CD49d/CD29; 'very late antigen-4') is a key cell surface integrin present on leukocytes which binds vascular cell adhesion molecule-1 (VCAM-1) on endothelial cell surfaces and leads to leukocyte infiltration to extravascular tissue. Antibodies against VLA-4 have been shown to block leukocyte infiltration and prevent tissue damage in inflammatory disease models of asthma,[1] multiple sclerosis,[2] rheumatoid arthritis (RA),[3] and inflammatory bowel disease (IBD).[4] Potent small molecule inhibitors of VLA-4 might therefore serve as useful agents in the treatment of these diseases.

The mechanism by which lymphocytes are recruited to sites of inflammation has been emerging from many different studies.[5,6] It appears to involve tethering, rolling, arresting and migration of lymphocytes along and through the vascular endothelium. During the tethering and rolling phase, it is thought that VLA-4 may be in a low activation or resting state. Upon encountering endothelial surfaces adjacent to an inflammatory site, a higher density of ligand and/or chemokine signaling prompts a rapid conformational change in VLA-4 producing a higher affinity or 'activated' state. This in turn leads to tight binding or arresting of the lymphocytes on the vascular surface followed by migration into the extravascular tissue. In the laboratory, an activated state of VLA-4 is produced by the addition of Mn^{2+} or other stimuli including antigens, anti-T-cell antibodies, and phorbol esters.

In a previous communication, we disclosed the identification of **1** as a potent VLA-4 antagonist.[7] This compound exhibited picomolar binding activity in an assay using Mn^{2+} activated human Jurkat cells ($IC_{50} = 80$ pM). Given the robust response to antibodies in various animal models of VLA-4 activity, it was felt that a small molecular weight compound that inhibited both the resting and activated states of VLA-4 might provide the exceptional potency obtained with VLA-4 antibodies. Assays to assess the potency of **1** against the resting state of VLA-4 as well as measuring off-rates were developed and are described herein.



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The Mn^{2+} activation state of VLA-4 was determined in a competitive binding assay between inhibitor and

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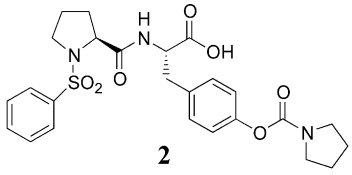
^{125}I-VCAM-Ig with Jurkat cells.[7] Unfortunately, when the incubation buffer lacks Mn^{2+}, ^{125}I-VCAM-Ig does not bind to Jurkat cells. Previously described studies identified **2** as a potent inhibitor of VLA-4.[8,9] ^{35}S-Labeled-**2** was also a potent inhibitor of unactivated VLA-4 ($K_D = 1$ nM) and a competitive binding assay between ^{35}S-**2** and inhibitor with Jurkat cells in a Ca^{2+}, Mg^{2+} buffer was established.[10]



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Compound **1** exhibited good binding activity against both activated and unactivated states of VLA-4, but unfortunately exhibited very poor pharmacokinetics in the rat after intravenous and oral dosing.[7] Nearly all sulfonylated dipeptide derivatives described as VLA-4 antagonists share this liability. On the other hand, if a compound has a very slow off-rate, prolonged physiological effects may result. Thus, a compound could exhibit exceptional pharmacological effects with less than ideal pharmacokinetics. With this in mind and given the generally poor pharmacokinetic profiles of this class of compounds, VLA-4 antagonists with exceptionally slow off-rates were sought that might be administered by aerosol inhalation.

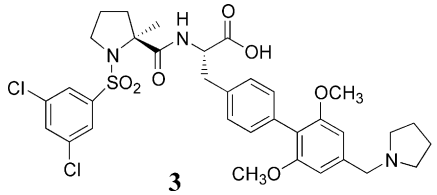
To measure the off-rate of compound **1**, Jurkat cells were saturated with the compound, washed, and the percentage of compound still bound to VLA-4 after 1 h at 37 °C was determined.[11] The higher the percentage of compound bound at 1 h, the slower the off-rate. Although **1** showed subnanomolar activity in the binding assays, it was found to have a relatively fast off-rate (only 1% bound @ 1 h, 37 °C) (Table 1). Compounds **3**, **4**, and **5**, which contain a sulfonylated α-methylproline coupled with phenylalanine derivatives demonstrated to be potent binders of VLA-4,[12–15] were screened in the off-rate and binding assays. The compounds showed similar binding affinities against both the activated (Mn^{2+}) and resting (Ca^2, Mg^{2+}) forms of VLA-4, but very different off-rates (Table 1). Thus, the binding assays were no longer able to differentiate between these compounds.

The off-rate data provided two important features about the SAR of binding to VLA-4. First, the acylated aminophenylalaine derivative **4** was a much tighter binder to VLA-4 than the dimethoxybiphenylalanine **1**. Second, incorporation of a polar group onto either the dimethoxybiphenyl alanine **3** or the aminophenylalanine **5** led to a significant decrease in off-rate. This second observation suggested that an important binding site can be accessed from the phenylalanine which may contribute to slower off-rates.[13]

To rapidly generate a series of acylated 4-aminophenylalanine derivatives, aniline **6** was prepared as outlined in Scheme 1. PyBOP coupling of *N*-(3,5-dichlorobenzenesulfonyl)-2(*S*)-methyl-proline with *p*-nitrophenylalanine methyl ester followed by tin(II)chloride reduction provided ready access to the key aniline intermediate **6**. This aniline was readily acylated with acyl chlorides or with carboxylic acids and PyBOP to give the final compounds after ester hydrolysis.

Since the pyridine nitrogen had apparently provided a key binding interaction which led to slower off-rates, modifying its basicity by changing the electronics of the pyridine ring might effect the off-rate. Removal of either one or both the chlorine atoms would result in a more basic pyridine nitrogen and might be expected to decrease the off-rate, but as noted with **5**→**7**→**8**, less tight binding occurred (Table 2 and Fig. 1). However, this effect may also be a function of a change in rotational tautomers associated with the lack of ortho substituents. Replacement of one of the electron withdrawing chlorines with an electron donating methoxy group **9** would have a similar effect on pyridine basicity, but this also led to a modest decrease in off-rate relative to **5**. A survey of Table 2 again confirmed that the binding assays were unable to differentiate among the more potent compounds. Although significant differences were seen in off-rates, the compounds showed similar potencies in the binding assays. Converting the pyridine to the pyridine *N*-oxide

Table 1. Inhibition of VLA-4[a] by sulfonylated 2-(*S*)-methyl-proline phenylalanine derivatives



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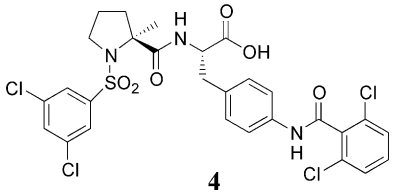

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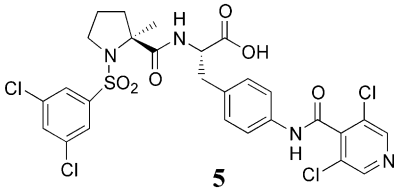

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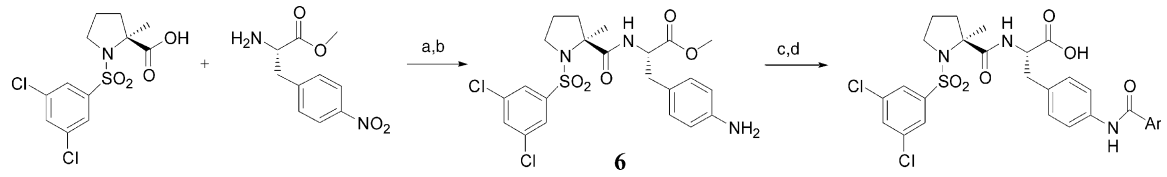
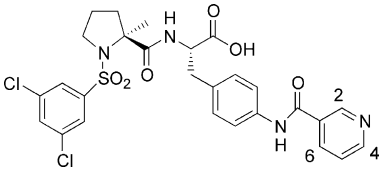
Compd	VLA-4 (IC_{50}, nM)		Off-rate (37 °C)% bound @ 1 h
	Mn^{2+}[a]	Ca^{2+},[a] Mg^{2+},[b]	
1	0.08	0.09	1
3	0.10	0.09	43
4	0.21	0.15	15
5	0.11	0.11	57

[a] ^{125}I-VCAM-Ig, $n = 3$.
[b] ^{35}S-**2**, $n = 3$.



Scheme 1. (a) PyBOP, DIPEA, CH_2Cl_2; (b) $SnCl_2$, MeOH; (c) acid chloride, NEt_3, CH_2Cl_2, or carboxylic acid, PyBOP, DIPEA, CH_2Cl_2; (d) NaOH, MeOH.

Table 2. Inhibition of VLA-4 by sulfonylated 2(*S*)-methyl-proline-4-isonicotinoyl-aminophenylalanine derivatives



Compd	2	4	6	VLA-4 (IC_{50}, nM)		Off-rate (37 °C)% bound @ 1 h
				Mn^{2+} [a]	Ca^{2+},Mg^{2+} [b]	
5	Cl	—	Cl	0.11	0.11	57
7	H	—	Cl	0.14	0.21	26
8	H	—	H	0.27	0.25	12
9	Cl	—	OMe	0.13	0.08	40
10	Cl	O	Cl	0.10	0.08	61

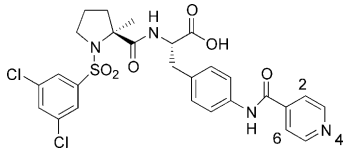
[a] ^{125}I-VCAM-Ig, $n=3$.
[b] ^{35}S-**2**, $n=3$.

10 provided similar potency to the parent pyridine, while greatly increasing the polarity of the compound.

Positional isomers of **5** were also explored. Utilizing intermediate **6** as a precursor, the 3-isonicotinoyl derivatives (**11**–**13**) were synthesized. Again, the importance of the 2,6-dichlorine substitution was reaffirmed. While compound **11** showed an off-rate equivalent to that of **5**, removal of one of the chlorines resulted in a complete loss of bound compound at 1 h. Although **11** demonstrated nearly equal potency to **5**, the potential for reaction of the 2-chloropyridine with nucleophiles diminished our interest in this series and offered no advantages (Table 3).

Having established that the 2,6-dichloroisonicotinic acid and its *N*-oxide were the optimal substitution for the right half of the molecule, we briefly explored modifi-

Table 3. Inhibition of VLA-4 by sulfonylated 2(*S*)-methyl-proline-3-isonicotinoyl-aminophenylalanine derivatives



Compd	2	4	6	VLA-4 (IC_{50}, nM)		Off-rate (37 °C)% bound @ 1 h
				Mn^{2+} [a]	Ca^{2+},Mg^{2+} [b]	
11	Cl	Me	Cl	0.19	0.10	61
12	H	Me	Cl	0.16	0.31	0
13	H	H	SMe	0.36	1.56	0

[a] ^{125}I-VCAM-Ig, $n=3$.
[b] ^{35}S-**2**, $n=3$.

cations to the proline half of the molecule. The α-methyl group on the proline was initially introduced to stabilize the amide bond to improve the pharmacokinetics. Since oral pharmacokinetics was less of a concern, the des-α-methyl proline analogue **14** was made. As demonstrated previously, this methyl group slightly decreased potency and increased protein binding. Removal of one or both of the chlorines from the benzenesulfonyl group would reduce the hydrophobicity and the molecular weight of the compounds, but unfortunately, both the monochloro **15** and des-chloro derivative **16** in the des-α-methyl series demonstrated a significant increase in off-rate (Table 4).

The pharmacokinetic properties of **5** was measured in rats, dog and rhesus monkey (Table 5). The compound exhibited low oral bioavailability, moderate to high plasma clearance and a short half-life in all three species tested. The binding of **5** with plasma proteins was high and showed some species dependency varying from 97.8% in the dog to 99.85% in the rhesus monkey. The

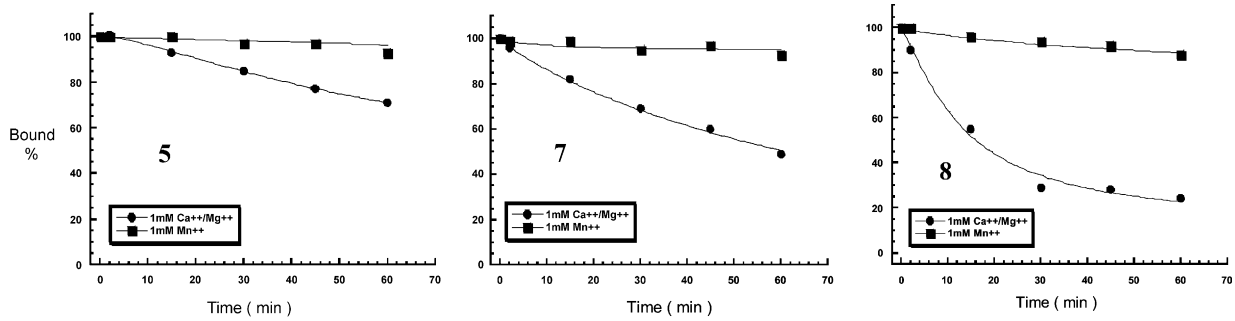
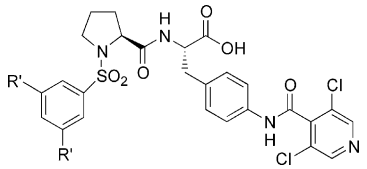


Figure 1. Relative off-rates for compounds **5**, **7**, and **8** under activating (Mn^{2+}) and non-activating (Ca^{2+}, Mg^{2+}) conditions. See ref 11 for assay.

Table 4. Inhibition of VLA-4 by sulfonylated 2(*S*)-methyl-proline-3-isonicotinoyl-aminophenylalanine derivatives



Compd	R′	R″	VLA-4 (IC_{50}, nM)		Off-rate (37 °C)% bound @ 1 h
			Mn^{2+} [a]	Ca^{2+},Mg^{2+} [b]	
14	Cl	Cl	0.10	0.07	62
15	Cl	H	0.09	0.10	37
16	H	H	0.15	0.13	19

[a] ^{125}I-VCAM-Ig, $n = 3$.
[b] ^{35}S-**2**, $n = 3$.

Table 5. Pharmacokinetic parameters of **5**

Species	F (%)[a]	Cl_p[b]	$t_{1/2}$ (h)[c]	% Unbound plasma
Sprague–Dawley				
Rats	1.6	19	1.7	0.2
Beagle dog	0.4	32	0.73	2.2
Rhesus monkey	4.0	7.5	2.3	0.15

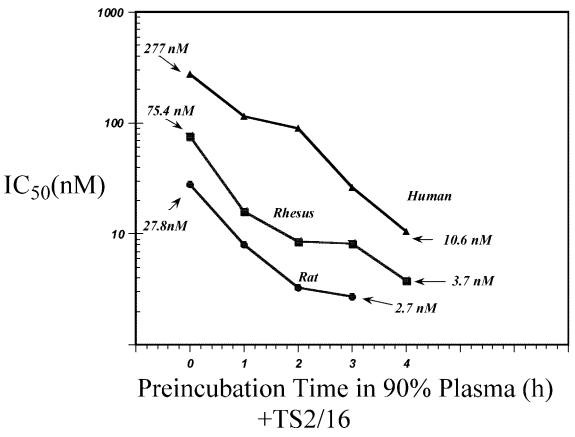
[a] Dose 1 mg/kg iv; 2 mg/kg po.
[b] mL/kg/min.
[c] $t_{1/2}$ = plasma half-life$_{(0–8\ h)}$.

clearance rates correlated to the percent protein bound which indicated the lower clearance numbers may be a result of high plasma protein binding.

When **5** was tested in a plasma shift assay where plasma was added to the binding assay, the IC_{50} showed a marked time dependence.[16] When the cells had not been preincubated with **5**, large IC_{50}'s were obtained, however, as the preincubation times increased, the IC_{50}'s decreased (Table 6). Thus, the presence of plasma proteins effected the initial binding of **5** to VLA-4. However, pharmacological effects would still be determined by the long off-rates since compounds in this class generally have high plasma clearance rates.

The effectiveness of **5** to inhibit eosinophil trafficking to the lung in an ovalbumin (OVA) sensitization model was determined. Mice sensitized with OVA were dosed with **5** intranasally twice daily for 3 days, then challenged with ovalbumin.[17] A sample of the bronchial aveolar lavage fluid was recovered from the lungs and the level of eosinophils present was determined to be lower compared with animals treated with vehicle alone ($ED_{50} = 1$ mg/kg intranasal).

Table 6. Potency of **5** in 90% rat, rhesus and human plasma + TS2/16



In summary, we have demonstrated that isonicotinoyl-(L)-aminophenylalanine derivatives are potent VLA-4 antagonists which possess slow off-rates. Compound **5** shows activity in reducing eosinophil trafficking in an OVA asthma model even though it is highly protein bound and rapidly cleared from the plasma.

Acknowledgements

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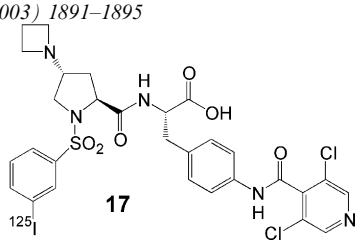
10. ^{35}S-**2** (1200 Ci/mmol) was synthesized by the Merck Labeled Compound Synthesis Department. The assay was performed in Millipore Multiscreen 96-well plates (MHVBN4550). Jurkat cells (from American Type Culture

Collection, Rockville, MD, USA) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C in a tissue culture incubator. Cells were pelleted by centrifugation, washed one time with Hepes buffer (25 mM Hepes pH 7.4, 150 mM NaCl, 3 mM KCl, 2 mM glucose), and resuspended in binding buffer (1 mM $CaCl_2$, 1mM $MgCl_2$ and 0.1% Bovine serum albumin in Hepes buffer). Dilutions of test compounds were prepared in DMSO at 100 times the desired concentration. Jurkat cells at a final concentration of 0.5×10^6 cells/well were added to wells containing 150 pM ^{35}S-**2**, binding buffer and test compound. The plates were incubated at room temperature for 30 min. The assay suspension was filtered using a vacuum apparatus, and the plates were washed once with 100 μL of binding buffer. The plates were then transferred to Packard Multiscreen adapter plates (Cat# 6005178) and 100 μL of MicroScint-20 was added to the wells. Radioactive counts were quantified in a Packard Topcount. Data Analysis was performed using the MRLCalc algorithm.

11. ^{125}I-**17** (2000 Ci/mmol) was purchased from Amersham Biosciences. The assay was performed in Millipore Multiscreen 96-well plates (MAFB NOB50). Jurkat cells (from American Type Culture Collection, Rockville, MD, USA) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C in a tissue culture incubator. Cells were pelleted by centrifugation, washed one time with Hepes buffer (25 mM Hepes pH 7.4, 150 mM NaCl, 3 mM KCl, 2 mM glucose), and resuspended in binding buffer (containing either 1mM $CaCl_2$ and $MgCl_2$ or 1 mM $MnCl_2$ and 0.1% Bovine serum albumin in Hepes buffer). Jurkat cells at a final concentration of 1×10^6 cells/well were incubated with 50nM test compounds at 37 °C for 1 h. The plates then were filtered and washed one time with 200 μL of cold binding buffer containing the appropriate cations. The cells were resuspended in 200 μL of appropriate binding buffer including 5 nM ^{125}I-**17** and were further incubated at 37 °C for 2, 15, 30, 45, 60 and up to 180 min. Cells were collected by filtering at each time point and washed two times with 200 μL of cold binding buffer containing the appropriate cations. The plates were then transferred to Packard Multiscreen adapter plates (Cat# 6005178) and 100 μL of MicroScint-20 was added to the wells. The radioactivity associated with the cells was measured in a Packard Topcount. Cells incubated with 1% dimethyl sulfoxide were used as positive control and cells incubated with 10 μM unlabeled **17** were served as the negative control (non-specific background). The amount of non-specific radioactivity associated with the negative control cells was subtracted from all observations. The amount of radioactivity associated with the positive control cell was equated to 0% bound receptor sites. Receptor occupancy (% bound) of test compound was determined from the positive cell control. The data is the mean of duplicates.



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16. ^{125}I-**17** (2000 Ci/mmol) was used to determine the potency of compounds in the presence of 90% human plasma and was purchased from Amersham Biosciences. The assay was performed in the presence or absence of the activating antibody, TS2/16 (mouse anti-human β1 integrin monoclonal antibody) and was carried out in Millipore Multiscreen 96-well plates (MAFCNOB50). Jurkat cells (from American Type Culture Collection, Rockville, MD, USA) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C in a tissue culture incubator. Cells were pelleted by centrifugation and resuspended in binding buffer (including 90% human plasma, 25 mM Hepes pH 7.4, 150 mM NaCl, 3 mM KCl and 2 mM Glucose) in the presence or absence of TS2/16. Jurkat cells at a final concentration of 1.0×10^6 cells/well were incubated with test compound for various time intervals prior to the addition of ^{125}I-**17** (400 pM). The plates were further incubated at room temperature for 60 min. At the end of the incubation period, the assay suspension was filtered using a vacuum apparatus, and the plates were washed twice with 250 μL of cold Hepes buffer containing 1 mM $CaCl_2$ and $MgCl_2$ in the presence or absence of TS2/16. The plates were then transferred to Packard Multiscreen adapter plates (Cat# 6005178) and 100 μL of MicroScint-20 was added to the wells. Radioactive counts were quantified in a Packard Topcount. Data analysis was performed using the MRLCalc algorithm.

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