Discovery of *N*-{*N*-[(3-Cyanophenyl)sulfonyl]-4(*R*)-cyclobutylamino-(L)-prolyl}-4-[(3′,5′-dichloroisonicotinoyl)amino]-(L)-phenylalanine (MK-0668), an Extremely Potent and Orally Active Antagonist of Very Late Antigen-4

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Abstract: Extremely potent very late antigen-4 (VLA-4) antagonists with picomolar, whole blood activity and slow dissociation rates were discovered by incorporating an amino substituent on the proline fragment of the initial lead structure. This level of potency against the unactivated form of VLA-4 was shown to be sufficient to overcome the poor pharmacokinetic profiles typical of this class of VLA-4 antagonists, and sustained activity as measured by receptor occupancy was achieved in preclinical species after oral dosing.

VLA-4[a] (α4β1, CD49d/CD29) is a member of the integrin family that is expressed on all lymphocytes.[1] VLA-4 normally exists at a resting state or unactivated state. Although the precise mechanism of activation in vivo is not clear, VLA-4 can be activated in vitro by divalent cation Mn^{2+} or activating antibodies.[2] Once activated, VLA-4 binds strongly to ligands including (1) vascular cell adhesion molecule-1 (VCAM-1), which is expressed on activated endothelial cells at sites of inflammation and is produced in response to inflammatory cytokines, and (2) the CS-1 domain of fibronectin (FN) which arises by alternative splicing within a region of FN. The binding interactions between VLA-4 and VCAM-1/FN are proposed to be key mediators of cell−cell and cell−matrix adhesion. As such, these cell adhesion interactions may be essential for the activation, migration, proliferation, and differentiation of lymphocytes during normal and/or pathophysiological processes. Thus, inhibition of VLA-4 may result in a reduction in the migration and/or activation of cell types important to sustaining a prolonged inflammatory response.[3] Anti-α4 antibodies and blocking peptides have been reported to be efficacious in a number of animal models of inflammatory diseases, including antigen-induced bronchial hyperresponsiveness,[4] experimental allergic encephalomyelitis,[5] adjuvant arthritis,[6] and spontaneous chronic colitis.[7]

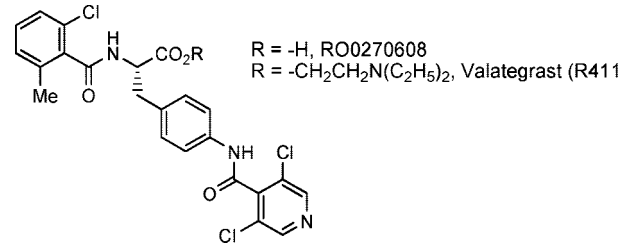

Figure 1. Structure of a clinical VLA-4 antagonist.

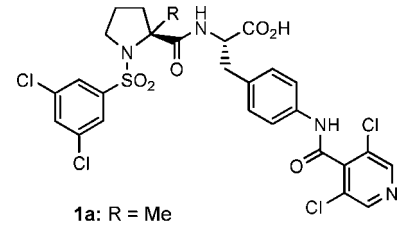

Figure 2. Lead structures of tight binding VLA-4 antagonists.

Clinical proof of concept was first achieved with an anti-α4 neutralizing antibody, natalizumab, which has been shown to be effective in human clinical trials of multiple sclerosis (MS) and Crohn's disease (CD) and has been approved by FDA for both indications.[8,9] At a monthly iv dose of 3 and 6 mg, approximately 80% and 90% saturation, respectively, of α4β1 receptors (receptor occupancy or RO) on peripheral blood lymphocytes was reported.[8] Subsequently, a small molecule VLA-4 antagonist, valategrast (R411) (Figure 1), was shown to be beneficial for the treatment of asthma in a 12-week phase II clinical trial.[10−12] The active metabolite of valategrast (RO0270608, Figure 1) was a dual α4β1−α4β7 antagonist with high affinity for the activated states of both α4β1 and α4β7 integrins, but the affinity was lower and the dissociation was rapid when the receptors were not activated.[11]

Previously, we identified a tight binding VLA-4 antagonist **1a** (Figure 2) with picomolar affinity on the unactivated form of VLA-4 along with a slow dissociation rate or off-rate.[13] However, it suffered from poor bioavailability and a short half-life in preclinical species. After iv dosing (0.5 mpk) in rats, only 15% receptor occupancy was observed at 8 h postdosing. On the basis of the reported experience with natalizumab, we hypothesized that a small molecule antagonist would need to achieve ≥80% RO at 12−24 h to be effective in the treatment of MS and CD. Therefore, much improvement in whole blood activity and/or pharmacokinetics over **1a** would be necessary to achieve this level of RO. Because of the peptidic nature and molecular weight of this lead, improving pharmacokinetic properties was a significant challenge. However, if a compound possessed very potent whole blood activity with a fast on-rate and also had a very slow off-rate once bound, sustained receptor occupancy might be achievable even after the drug was rapidly eliminated from circulation.

To test this hypothesis, we sought to improve the whole blood activity and the off-rate of lead structure **1a**. Compound **1a** is a carboxylic acid and is highly bound to human plasma proteins

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[a] Abbreviations: VLA-4, very late antigen-4; VCAM-1, vascular cell adhesion molecule-1; FN, fibronectin; MS, multiple sclerosis; CD, Crohn's disease; RO, receptor occupancy; LOQ, limit of quantitation; TDI, time dependent inhibition.

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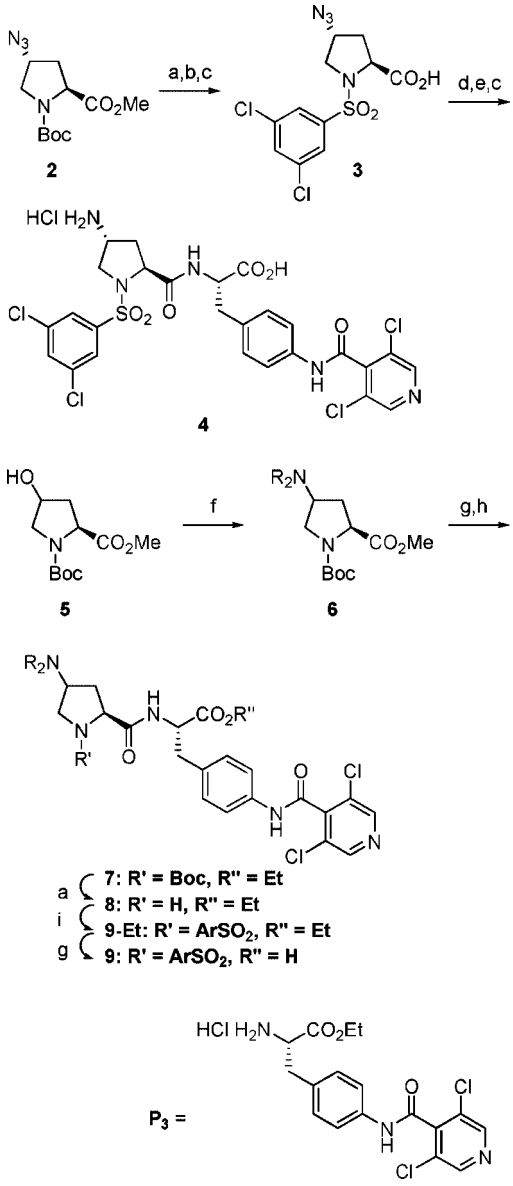
Scheme 1[a]

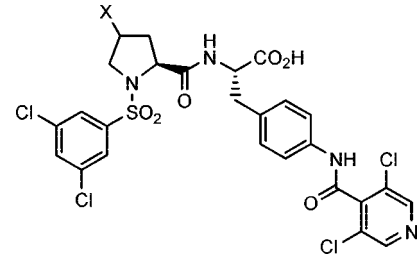


[a] (a) HCl, dioxane; (b) 3,5-Cl_2-$PhSO_2Cl$, Et_3N, DMAP (cat.), CH_2Cl_2/THF; (c) LiOH, MeOH/THF/H_2O; (d) P_3, PyBOP, *i*-Pr_2NEt, CH_2Cl_2; (e) Me_3P, THF; (f) Tf_2O, *i*-Pr_2NEt, CH_2Cl_2, −40 °C, 1 h; R_2NH, −20 °C to room temp, 16 h; (g) LiOH, CH_3CN, H_2O; (h) P_3, EDC, HOBt, CH_2Cl_2, H_2O; (i) $ArSO_2Cl$, Et_3N, DMAP (cat.), CH_2Cl_2/THF.

(i.e., albumin), so the introduction of an amino group was pursued. The syntheses of these amino substituted compounds were well precedented, and the synthetic sequence is outlined in Scheme 1. The key transformation was from **5** to **6** via a stereospecific S_N2 displacement of the triflate of readily available (4*S*)- or (4*R*)-hydroxyproline by an amine nucleophile.

The affinity of these new compounds was assessed by competitive binding to human VLA-4 in buffer and in whole blood without addition of activating reagents. The dissociation kinetics of these tight binding antagonists was also evaluated at 37 °C and was expressed as % bound at a specified time. The data are summarized in Table 1.[13,14] As reported previously,[13] removing the α-methyl group of the proline fragment reduced protein binding and improved whole blood activity, but it had little effect on the off-rate (**1b** vs **1a**). Incorporation of an unsubstituted amino group at the C-4 position of proline further improved the whole blood activity as expected, presumably because of reduced plasma protein binding, but it led to a

Table 1. In Vitro Potency of VLA-4 Antagonists[14]



Compd	X	IC_{50} (nM)[a]		Off-rate (37°C) (%bound@ Time[a])
		buffer	whole blood	
1b	—H	0.08 ± 0.01	1.5	62%@1 h
4	—NH_2	0.09	0.4	47%@1 h
9a	—N□	0.08 ± 0.02	0.2	77 ± 4%@3 h
9b	—N□	0.2		15%@1 h
9c	—N⬠	0.1	0.2	82%@3 h
9d	—N⬡	0.09 ± 0.02	0.3	89%@3 h
9e	—NH□	0.10 ± 0.03	0.1 ± 0.1	82%@3 h

[a] Data expressed as mean ± SD ($n \geq 3$ independent experiments) or single value ($n = 1$).

slightly diminished off-rate (**4** vs **1b**). Alkyl substitutions on the amino group dramatically enhanced the off-rate while maintaining potent whole blood activity (**9a** vs **4**). This effect appeared to be sensitive to the stereochemistry at C-4 as the other diastereomer **9b** had poor affinity and a faster off-rate, suggesting that the 4-alkylamino substituent was involved in specific interactions with VLA-4. Slight increases in off-rate were observed by increasing the ring size of the cyclic amino group with minimal impact on the whole blood activity (**9c**,**d** vs **9a**). Monosubstituted amino group was also effective in enhancing VLA-4 affinity and off-rate, as reflected by **9e**.

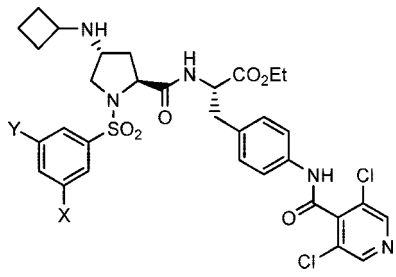
Compound **9a** was selected to be profiled in vivo. Thus, **9a** was dosed iv (0.5 mg/kg) in rats and blood samples were collected at different time points postdosing. The RO of VLA-4 on the circulating lymphocytes was assessed by competitive binding with a radiolabeled VLA-4 ligand.[16] In the same study, the plasma concentration of **9a** was also determined by LC−MS. As summarized in Table 2, administration of **9a** resulted in 64 ± 2% RO at 8 h postdosing.[15,16] As expected on the basis of experience with this class of compounds,[13] **9a** exhibited rapid plasma clearance and a short plasma half-life ($t_{1/2} < 1$ h). No **9a** was detectable beyond 2 h (LOQ < 0.1 nM). Compound **9a** was further evaluated in dogs and rhesus monkeys using a similar protocol. In dogs, RO of 72 ± 6% was observed at 8 h postdosing (0.75 mg/kg iv) while no compound was detectable beyond 1 h. Similarly, RO of 87 ± 6% was observed at 8 h postdosing in rhesus monkeys (0.75 mg/kg iv) while the concentration of **9a** dropped below the limit of detection at 6 h. The peak RO of **9a** reached 100% in all three species. These results suggested that sustained RO can be achieved despite the high clearance and low plasma concentration when the compound possesses exceptional affinity to the receptor.[15]

Table 2. Receptor Occupancy and Pharmacokinetic Parameters of VLA-4 Antagonists[16]

compd	species	dose (mg/kg)	route	RO at *X* h (%)[a]	*X* (h)[b]	Cl_p ((mL/min)/kg)[c]	$t_{1/2}$ (h)[c]	t_{min} (h)[d]
9a	rat	0.5	iv	64 ± 2	8	65	1.0	2
9a	dog	0.75	iv	72 ± 6	8	29	0.5	1
9a	monkey	0.75	iv	87 ± 6	8	33	1.1	6
9a	rat	1	po	46 ± 12	8	ND	ND	ND
9a-Et	rat	1	po	62 ± 16	8	ND	ND	ND
9e-Et	rat	1	po	51 ± 5	8	ND	ND	ND
9f	rat	5	po	67 ± 11	12	65	1.0	0
9f	dog	5	po	83 ± 8	12	29	0.5	6
9f	monkey	5	po	88 ± 1.8	8	33	1.1	0
9f-Et	rat	5	po	77 ± 13	12	ND	ND	0
9f-Et	dog	5	po	97 ± 7	12	ND	ND	12
9f-Et	monkey	5	po	95 ± 4	8	ND	ND	0

[a] % receptor occupancy at *X* h. [b] Time when receptor occupancy was measured. [c] Cl_p and plasma half-life were determined after iv dose. [d] Latest time point when **9a** or **9f** could be detected (LOQ ≈ 0.1 nM).

Table 3. Cytochrome P450 Inhibition and clogP of VLA-4 Antagonists[17]



compd	X, Y	CYP3A4 IC_{50} (μM)	TDI rate (min^{-1})	clogP[a]
9e-Et	Cl, Cl	<0.4	0.05	5.5
9f-Et	CN, H	1.7, 2.4	0.04	3.5

[a] Calculated by ACD8.1 software.

Compound **9a** and its ethyl ester **9a**-Et were then studied via oral dosing in rats. Receptor occupancies of 46 ± 12% and 62 ± 16%, respectively, were observed at 8 h postdosing (1 mg/kg). The ethyl ester of **9e** (**9e**-Et) was also studied by oral dosing (1 mg/kg) in rats and afforded comparable RO (51 ± 5% at 8 h postdosing). Although still moderate in comparison with clinical observations of natalizumab, the level of oral activity of **9a**/**9a**-Et and **9e**-Et as assessed by receptor occupancy represented a breakthrough in the search for an orally active small molecule antagonist of the unactivated form of VLA-4.

Because the azetidine ring of **9a**/**9a**-Et was found to be acid labile, **9e**/**9e**-Et was selected for additional characterization toward the identification of a development candidate. Metabolism studies revealed that **9e**-Et is a potent and time-dependent inhibitor (TDI) of CYP3A4 with a TDI rate of 0.05 min^{-1} (Table 3).[17] The parent acid **9e** also displayed a propensity for bioactivation upon incubation with liver microsomes, and covalent adducts were formed with microsomal proteins after incubation for 1 h (rat, 108 pmol/mg; human, 150 pmol/mg).[18] Because CYP inhibition and covalent adduct formation pose significant risks of drug−drug interactions and idiosyncratic toxicities, respectively[19,20] alternative structures were sought. It is well-known that CYP inhibition is often associated with high lipophilicity.[21] Replacing the 3,5-dichlorophenyl group of **9e**-Et with a 3-cyanophenyl group (**9f**-Et) resulted in significant reduction in CYP3A4 inhibition, while the clogP value decreased from 5.5 to 3.5. Furthermore, **9f** also demonstrated decreased levels of covalent adduct formation when incubated with microsomal preparations (rat, 39 ± 2 pmol/mg; human, 56 ± 2 pmol/mg).

The amino acid **9f** (MK-0668) was a potent antagonist of the unactivated state of VLA-4 (IC_{90} in whole blood: human 0.13 ± 0.03 nM, dog 0.19 ± 0.01 nM, rhesus 0.21 nM, rat 0.4 ± 0.1 nM; off-rate at 37 °C on human VLA-4, 64 ± 10% bound at 3 h). When evaluated in vivo (5 mg/kg po), **9f**-Et demonstrated sustained RO of 77−97% at 8−12 h postdosing across three species (Table 2). As a comparison, the acid **9f** was also studied and afforded somewhat lower levels of RO (67−88% at 8−12 h postdosing). Analogous to other compounds in the series, **9f**/**9f**-Et displayed poor pharmacokinetic properties in the preclinical species (F < 1%, $t_{1/2} \leq$ 1 h). Nevertheless, RO was maintained even after no compound was detectable in the plasma, consistent with slow dissociation rate of **9f** from VLA-4 receptors. If similar PK/PD relationship holds in humans, with a twice-daily oral dosing schedule, **9f** or its prodrug **9f**-Et would be expected to achieve RO coverage comparable to that of intravenously dosed natalizumab.

In summary, extremely potent VLA-4 antagonists with picomolar, whole blood activity and slow off-rates were discovered by incorporating an amino substituent on the proline fragment of the initial lead structure. This level of potency was shown to be sufficient to overcome the poor pharmacokinetic profiles typical of this class of VLA-4 antagonists, and sustained activity as measured by receptor occupancy was achieved after oral dosing.[22]

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Supporting Information Available: Synthetic procedures and analytical data of VLA-4 antagonists and conditions for all the biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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