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Small Molecule Inhibitors of Integrin α2*β*1

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Interactions between the integrin, α2*β*1, and extracellular matrix (ECM), particularly collagen, play a pivotal role in platelet adhesion and thrombus formation. Platelets interact with collagen in the subendothelial matrix that is exposed by vascular damage. To evaluate the potential of α2*β*1 inhibitors for anticancer and antithrombotic applications, we have developed a series of small molecule inhibitors of this integrin based on a prolyl-2,3-diaminopropionic acid (DAP) scaffold using solid-phase parallel synthesis. A benzenesulfonamide substituent at the N-terminus of the dipepetide and a benzyl urea at the DAP side chain resulted in tight and highly selective inhibition of α2*β*1-mediated adhesion of human platelets and other cells to collagen.

Introduction

The blood vessel wall is lined by a continuous layer of endothelial cells that not only interfaces directly with the blood but also serves as a barrier that separates cells and proteins in the blood from proteins in the subendothelial extracellular connective tissue matrix (ECM). Thus, when vascular trauma disrupts the endothelium, platelets are exposed to a variety of ECM proteins, initiating the formation of a hemostatic plug under physiologic circumstances[1] or thrombosis when endothelial disruption perturbs the integrity of an atherosclerotic plaque.[2] Collagen, among the ECM proteins, plays a pivotal role in platelet-mediated hemostasis and thrombosis, because it is not only a ligand for platelet adhesion and an agonist for aggregation but is also the binding site for von Willebrand factor (vWF) in the ECM.[3] Following vascular trauma, circulating platelets slow, tether, and roll on collagen-bound vWF, allowing the platelet collagen receptor GPVI to interact with collagen. Collagen binding to GPVI, a member of the immunoglobulin receptor superfamily, then initiates a signaling pathway that triggers the activation of a second platelet collagen receptor α2*β*1, as well as the platelet adhesion receptor αIIb*β*3.[4,5]

α2*β*1 is a widely expressed integrin that binds to type I collagen, as well as type II−V and to laminins 1 and 5, depending on the cellular context.[6] It has been implicated in hemostasis and thrombosis, as well as cancer metastasis, wound healing, and angiogenesis.[7,8] α2*β*1 is one of nine integrins whose α-subunit has an inserted (I)-domain that is responsible for binding of the integrin to its natural ligand. Importantly, α2*β*1 has multiple conformational states that can be regulated by intracellular signaling pathways.[4,9−11]

Although α2*β*1 was the first collagen receptor to be identified on platelets,[12,13] the relative roles of α2*β*1 and GPVI in mediating platelet response to collagen has been extensively debated in recent years. Using mouse models, Nieswandt et al. reported that α2*β*1 is essential for platelet adhesion on type I collagen solubilized by pepsin but may be dispensable when platelets adhere to physiologically relevant fibrillar collagen under low and high shear conditions. For example, they observed only a slight delay in *ex vivo* aggregation of *β*1-null mouse platelets stimulated by fibrillar collagen.[4,5,14,15] On the other hand, overexpression of α2*β*1 in humans due to polymorphisms in the α2 gene has been associated with stroke and nonfatal myocardial infarction.[16] Moreover, additional experiments suggest that both α2*β*1 and GPVI play a significant role in hemostasis and thrombosis *in vivo*.[17] For example, Kahn et al. observed that pharmacologic inhibition of human platelets and of α2-deficient mouse platelets resulted in reduced adhesion on fibrillar collagen under flow conditions. Also *in vivo* α2-deficient mice displayed delayed thrombotic responses in the tail-bleeding model.[18,19]

Given that α2*β*1 functions in platelets after they first adhere to collagen and that patients with α2*β*1 deficiency only exhibit a mild bleeding diathesis,[12,20−22] α2*β*1 appears to be a good target for the development of safer and more effective small-molecule antithrombotic agents, especially in light of the increase in the overall incidence of cardiovascular disease.[23] Previously, benzenesulfonyl-Pro-Phe dipeptide derivatives were developed as inhibitors of the closely related integrin α4*β*1 (Figure 1a).[24,25] By varying the Phe derivative, it was possible to obtain high potency and selectivity for α4*β*1. In related studies, inhibitors of the integrin αIIb*β*3 have been developed with 2,3-diaminopropionic acid (DAP) occupying a roughly equivalent position as the Phe derivative in the α4*β*1 inhibitors (Figure 1b).[26,27]

Here, we combine the prolyl-sulfonylamide fragment with derivatives of DAP to develop selective inhibitors of α2*β*1. These compounds are potent inhibitors of the adhesion of platelets and transfected cells to immobilized soluble collagen type I. However, they do not inhibit the binding of the isolated α2 I-domain to immobilized Type I collagen. This behavior suggests that they inhibit adhesion via binding to the *β*1 I-like domain.

Results and Discussion

Chemistry. The prolyl-diaminopropionic acid derivatives were synthesized by the solid-phase route described in Scheme 1. Fmoc-DAP(Alloc)-OH was attached to bromomethyl Wang

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(a) $\alpha4\beta1$ antagonist

(b) $\alpha IIb\beta3$ antagonists

Figure 1. Chemical structure of antagonists of $\alpha4\beta1$ and $\alpha IIb\beta3$ integrins.

Scheme 1[a]

[a] Reagents and conditions: (a) Fmoc-DAP(Alloc)-OH, CsI, DIEA, DMF; (b) 20% piperidine in DMF; (c) Fmoc-Pro-OH, HATU, HOAT, DIEA, DMF; (d) $PhSO_2Cl$ for **3** or R_2Cl for **18**−**28**, DIEA, CH_2Cl_2; (e) $Pd(PPh_3)_4$, $PhSiH_3$, CH_2Cl_2; (f) R_1Cl or isocyanate derivatives, DIEA, DMF; (g) TFA; (h) $SnCl_2{\cdot}2H_2O$, DMF; (i) R_3Cl or isocyanate, DIEA, CH_2Cl_2.

resin using CsI as the catalyst.[28] Following deprotection of the Fmoc group with 20% PIP in DMF, Fmoc-Pro was appended under standard peptide coupling conditions. Deprotection of the Fmoc group followed by the sulfonamide formation gave intermediate **3** for the modification of the 3-position of the DAP fragment. The allyloxycarbonyl (Alloc) group of DAP was removed using $Pd(PPh_3)_4$ and $PhSiH_3$ in degassed CH_2Cl_2 under argon gas. Acylation or urea formation of the released amine with concomitant cleavage from the resin generated the desired $\alpha2\beta1$ inhibitors. The side chain of the DAP fragment was varied in a similar manner. The nitro-substituted compound **28** was treated with $SnCl_2{\cdot}2H_2O$ to generate the amine which was modified for compounds **30**−**32**.

Inhibition of Human Platelet Adhesion. A series of compounds were tested to determine their ability to inhibit the adhesion of washed human platelets to immobilized soluble collagen type I (Tables 1 and 2). Because the expression level of $\alpha2\beta1$ on platelets can vary among normal individuals by a factor of 4 and affect $\alpha2\beta1$-mediated platelet function,[16,29] the inhibitory activities for each compound in the platelet adhesion assay reported in Table 1 and 2 were evaluated using platelets from a single individual. Parallel experiments with platelets from other individuals showed a very similar trend in affinities relative to a standard compound (**9**), but the midpoints of the curves

Table 1. In Vitro Human Platelet Adhesion Inhibitory Activity

Compound	R_1	IC_{50}(μM)	Compound	R_1	IC_{50}(μM)
5		0.660	**12**		0.296
6		0.367	**13**		0.023
7		>10	**14**		0.107
8		0.185	**15**		0.467
9		0.015	**16**		0.160
10		0.357	**17**		0.108
11		0.185			

Table 2. In Vitro Human Platelet Adhesion Inhibitory Activity

Compound	R_2	IC_{50} (μM)	Compound	R_2	IC_{50} (μM)
9		0.015	**25**		0.287
18		>10	**26**		0.036
19		0.029	**27**		0.070
20		0.039	**28**		0.105
21		0.035	**29**		0.144
22		0.017	**30**		0.131
23		>10	**31**		0.151
24		0.050	**32**		0.236

Table 3. Potency and Selectivity of Selected Inhibitors against Related Integrins

integrin	cell line	ligand	IC_{50} (μM)				
			9	**13**	**19**	**22**	**24**
α1β1[a]	K562[b]	collagen IV	29%	22%	26%	32%	30%
α2β1	platelet	collagen I	0.015	0.023	0.029	0.017	0.050
α2β1	K562[b]	collagen I	0.138	0.102	0.089	0.083	0.177
α4β1	Jurkat	VCAM	2.128	1.966	0.770	0.637	4.334
α5β1	K562	fibronectin	> 10	> 10	> 10	> 10	> 10

[a] % inhibition at 3 μM. [b] Transfected K562 cell.

were systematically shifted toward higher concentrations by as much as a factor of 3 to 4.

The first modification was conducted in the side chain of the DAP fragment to determine the preferred interaction for good α2β1 binding (Table 1). Benzamide compound **5** showed moderate activity, which was enhanced by an ethyl linker between the amide and the hydrophobic aromatic ring. It is clearly seen that the carbonyl group is essential for α2β1 binding. Changing the carbonyl group (**6**) to a sulfonyl group (**7**) resulted in more than a 25-fold decrease in potency.

Replacing one of the CH_2 groups with oxygen or NH to provide urethane **8** and urea **9** showed an improvement in potency. The 10-fold increase in potency of urea **9** over the urethane **8** suggests that the hydrogen bonding donor NH group of the urea might play a critical role in enhancing binding potency, although the rigidity and electronic properties are also affected. Aliphatic substitution within the urea (**10** and **11**) or conversion of the benzyl urea to a phenyl urea (**12**) resulted in loss of potency. We also investigated the effect of substituents on the phenyl ring of the benzyl urea group. Substitution at the para position (**13**) with an amino group had little effect on potency. However, data from chloro- and methoxy-subsitituted compounds **14**−**17** resulted in more than a 10-fold decrease in potency over unsubstituted compound **9**.

Encouraged by these results, we next determined the effect of varying the N-terminal benzenesulfonyl group, while holding the urea side chain constant (Table 2). It is clearly seen that the sulfonyl group is essential for α2β1 binding. Replacing the sulfonyl group (**9**) with a carbonyl group (**18**) caused a greater than 600-fold decrease in potency. Therefore, further analogues focused on the sulfonylated Pro-DAP. Generally, the activity of the compounds was very sensitive to the position (ortho, meta, and para) of the substituents. The 3,5-disubstituted compounds (**19**−**22**) were well tolerated, regardless of the properties of substituents (electron-donating and electron-withdrawing groups). This series displayed similar levels of potency to the unsubstituted lead compound **9**. However, 2,6-dichlorobenzenesulfonyl compound **23** was more than 600-fold less active than 3,5-dichlorobenzenesulfonyl compound **22**. It is likely that the two substituents at the 2 and 6 positions cause an unfavorable orientation of the phenyl ring relative to the pyrrolidine ring of the Pro residue. In contrast to the planar 2-naphthalene sulfonyl compound **24**, staggered biphenyl sulfonyl compound **25** showed a 5-fold decrease in potency, suggesting the importance of the spatial hydrophobic functionality for potency. Although the substitutions at meta and para positions were well tolerated (**19**−**22**, **24**), an attempt to improve the activity of **9** by introducing various substituents at the para position (**26**−**32**) did not significantly improve potency.

Selectivity of the Inhibitors. Integrins are heterodimers composed of α and β subunits. There are eighteen different integrin α subunits and eight different β subunits that combine to form twenty-four structurally and functionally diverse receptors. To confirm that our compounds are specific α2β1 inhibitors, we examined the specificity of five selected potent α2β1 inhibitors against three closely related integrins, α1β1, α4β1, and α5β1 (Table 3).[30] Because our goal was to determine the specificity of the compounds, we selected from the compounds with the highest potency (Tables 1 and 2), selecting only one compound, **24**, with moderate potency as a control. To evaluate α1β1-mediated adhesion to collagen IV, we used K562 cells transfected with the α1 integrin subunit. None of the tested compounds inhibited K562 cell adhesion to collagen VI at 1 μM concentration, and only parial effects were observed at 3 μM concetnrations of the compounds. Similarly, the compounds had no effect on α5β1-mediated K562 adhesion to fibronectin.

Table 4. Selectivity of Selected Inhibitors for α2β1 versus α4β1

	9	**13**	**19**	**22**	**24**
IC_{50} (platelets, μM)	0.01 5	0.02 3	0.02 9	0.01 7	0.05 0
IC_{50} (α4β1)/IC_{50} (α2β1) Jurkat versus platelets	142	85	27	38	87
IC_{50} (α4β1)/IC_{50} (α2β1) Jurkat versus tranfected K562 cells	15	19	8.6	7.7	24

A number of groups have recently reported a series of structurally similar α4β1 antagonists.[24,25] Therefore, we examined the specificity of our compounds for α2β1 versus α4β1, employing Jurkat cells, which use α4β1 to specifically adhere to VCAM-1. We were encouraged to find that all five compounds exhibited a high degree of specificity for α2β1 versus α4β1 (Table 4). The absolute degree of specificity depends on whether one compares the results of α4β1-mediated Jurkat cell adhesion to α2β1-mediated platelet adhesion or K562 cell adhesion data; however, in both cases, the compounds showed considerable specificity for the desired target. Unsubstituted benzenesulfonyl compounds **9** and **13** showed high selectivity for α2β1 over α4β1, as did 2-naphthalenesulfonyl compound **24**. It is noteworthy that 3,6-disubstituted benzenesulfonyl compounds **19** and **22** exhibited reduced selectivity over α4β1. This modification had previously been shown to increase potency for α4β1.[25] Interestingly, we determined that a previously reported α4β1 antagonist (3,5-dichlorobenzenesulfonyl-Pro-Tyr)[25] showed no selectivity under this condition (the IC50's of α4β1 antagonist to α2β1 and α4β1 were 0.336 and 0.356 μM, respectively).

We also examined α2β1-mediated adhesion using K562 cells transfected with α2. Untransfected cells do not adhere to type I collagen under our experimental conditions; thus, the transfected cells adhere to immobilized type I collagen in an α2β1-dependent manner. Comparison of adhesion with these cells to adhesion with platelets provides a measure of the sensitivity of the assay to the type of cell employed. The first four compounds listed in Table 4 are all approximately equipotent within the errors of the assays (up to a factor of 2), but the fifth compound (naphthyl-substituted sulfonamide **24**) is 2- to 3-fold less potent using both cell types. Thus, there is reasonable agreement concerning the ability of the assay to discriminate between

compounds with different affinities (although more low-affinity compounds would need to be tested to further support this statement). However, all compounds in the human platelet adhesion assay were approximately 1 order of magnitude more potent than in the assay with transfected K562 cells. Presumably, the different potency of antagonists against $\alpha 2\beta 1$ in platelets versus K562 cells reflects differences in levels of expression as well as the degree of integrin activation.[31]

We also examined the specificity of the most potent compound **9** using ADP stimulated platelet aggregation, an effect that is ultimately mediated by $\alpha IIb\beta 3$. These studies showed that the inhibitor did not affect platelet aggregation stimulated by addition of ADP agonist in the presence of the compound (Supplementary Figure 1). These result shows that the compounds do not affect signaling through this pathway, and that the compounds do not inhibit the function of activate $\alpha IIb\beta 3$.

Possible Location of the Binding Site of the Inhibitor. The affinity state of $\alpha 2\beta 1$ can be regulated by inside-out signaling pathways that affect the position of $\beta 1$ C7 helix.[32] Unlike the metal ion-dependent adhesion site (MIDAS) on the $\alpha 2$ I-domain which is involved in direct binding of collagen, the MIDAS on the $\beta 1$ I-like domain provides an intrinsic ligand binding site to activate the I domain.[10] To define the binding site and mode of action, all compounds were evaluated in a recombinant human $\alpha 2$ I-domain/soluble collagen I enzyme-linked immunosorbant assay (ELISA).[33] These studies showed that the present inhibitors did not inhibit I-domain binding to immobilized type I collagen, although a number of unrelated compounds have been shown to inhibit binding to this site. This behavior is consistent with binding to the $\beta 1$ I-like domain MIDAS located near a key regulatory interface with the $\alpha 2$ I-domain. Given the specificity of these compounds for $\alpha 2\beta 1$ over other $\beta 1$ integrins, it would appear that this site requires the presence of both integrin subunits.

Conclusions

In conclusion, we have identified a series of prolyl-2,3-diaminopropionic acid (DAP) derivatives that bind tightly to integrin $\alpha 2\beta 1$. Solid-phase parallel synthesis was used to optimize the different portions of the compound and obtain potent antagonists. The SAR on this series of diamino-propionic acid derivatives shows that the following features were sufficient to provide high potency and selectivity: (a) a urea group with a distal benzylic group linked by a proper spacer was critical for $\alpha 2\beta 1$ binding; (b) the sulfonyl group at the N-terminal residue was necessary for tight binding; (c) the potency was extremely sensitive to the position of substituents on the aryl sulfonyl moiety (ortho *vs* meta and para position).

The goal of this work is to develop selective small molecule inhibitors of $\alpha 2\beta 1$ to allow pharmacological investigations of the role of this integrin in various disease processes. Our compounds are of sufficient potency for this purpose and are selective for $\alpha 2\beta 1$ over $\alpha 1\beta 1$, $\alpha 4\beta 1$, $\alpha 5\beta 1$, and $\alpha IIb\beta 3$. In contrast, a known $\alpha 4\beta 1$ antagonist showed no selectivity under our assay conditions. Currently, we are using these $\alpha 2\beta 1$ inhibitors to study the mechanism of $\alpha 2\beta 1$ mediated-thrombus formation *in vitro* and *in vivo*. Further development of these compounds might ultimately lead to clinically useful agents for treatment of thrombosis and/or cancer.

Experimental Section

General. Unless otherwise indicated, all reactions were run under argon gas. Anhydrous solvents were obtained via passage through an activated alumina column[34] and from commercial suppliers. ^1H and ^{13}C NMR spectra were recorded on an AM-500 or DRX-500 spectrometer. Chemical shifts are reported relative to internal DMSO-d_6 (δ 2.50 for ^1H and δ 39.52 for ^{13}C). High-resolution mass spectra were accomplished using an Autospec high resolution double focusing electrospray ionization/chemical ionization spectrometer with either DEC 11/73 or OPUS software data system. Preparative HPLC was performed on a Varian HPLC system, using a GRACEVYDAC C-18 column, 250 × 22 mm, 100 Å, and a flow rate of 10 mL/min; $\lambda = 254$ nm; mobile phase A, 0.1% TFA in H_2O, and mobile phase B, 0.1% TFA in CH_3CN. The purified fractions were lyophilized. Compound purities were determined by analytical RP-HPLC using a GRACEVYDAC C-18 column eluted at a rate of 1 mL/min with a gradient of solvent B varying at no faster than 1%/min. All compounds had a purity of 95% or greater based on the integrated peak area (detection at 210 nm).

General Procedure for the Preparation of Inhibitors 5−32. The 4-(bromomethyl)phenoxymethyl polystyrene resin was swelled in DMF (15 mL/g resin). Fmoc-DAP(Alloc)-OH (1.5 equiv), CsI (1.0 equiv), and DIEA (2 equiv) were added, and the reaction was stirred at 25 °C for 18 h. The resin was filtered and washed repeatedly with DMF and MeOH. After deprotecting the Fmoc group by treatment of 20% PIP in DMF, the resin was washed with DMF. This resin was then suspended with DMF and stirred with Fmoc-Pro-OH or proline analogue (3 equiv), HATU (3 equiv), HOAT (3 equiv), and DIEA (6 equiv) for 3 h. The resin was filtered and washed with DMF. After deprotecting the Fmoc group by treatment of 20% PIP in DMF, the resin was washed with DMF. This resin was then suspended with CH_2Cl_2 and stirred with benzenesulfonyl chloride derivatives (3 equiv) and DIEA (6 equiv) for 18 h. The resin was filtered, washed with CH_2Cl_2 and DMF, and dried overnight. To a peptide resin washed with oxygen-free CH_2Cl_2 in the presence of argon was added a solution of $PhSiH_3$ (25 equiv), and the resin was stirred for 2 min. Subsequently, Pd-$(PPh_3)_4$ (0.5 equiv) was added under argon. The reaction was stirred for 2 h under argon. Then, the resin was washed repeatedly with CH_2Cl_2 and DMF. This resin was then suspended with DMF and stirred with isocyanate derivatives (3 equiv) for 18 h. The resin was filtered, washed with DMF and CH_2Cl_2, and dried.

Compounds **18−32** were prepared through a similar manner. The nitro-substituted compound **28** in DMF was treated with $SnCl_2 \cdot 2H_2O$ (20 equiv, 2 M) and stirred at 25 °C for 20 h to generate the amine. After filtration and washing, the resin in CH_2Cl_2 was treated with R_3Cl (2 equiv) or isocyanate (2 equiv) and DIEA (3 equiv) to obtain compounds **30−32**. The final compounds were cleaved from the resin by treatment of 100% TFA.

Human Platelet Adhesion Assay. Flat bottom microtiter plates (96-well) (Immulon 2, Dynatech Laboratories, Chantilly, VA) were coated with soluble type I collagen dissolved in 50 mM $NaHCO_3$ buffer, pH 8.0, containing 150 mM NaCl as previously described.[35] Unoccupied protein binding sites on the wells were blocked with 5 mg/mL bovine serum albumin dissolved in the same buffer. Human platelets were isolated from blood anticoagulated with 0.1 volume 3.8% sodium citrate by gel-filtration using GFP buffer (4 mM HEPES buffer, pH 7.4, containing 135 mM NaCl, 2.7 mM KCl, 5.6 mM glucose, 3.3 mM NaH_2PO_4, 0.35 mg/mL bovine serum albumin, and 2 mM $MgCl_2$). Aliquots (100 μL) of the gel-filtered platelet suspension containing 1.25×10^8 platelets/mL were added to the protein-coated wells in the absence or presence of an inhibitor. Following incubation for 30 min at 37 °C without agitation, the plates were washed with the Tris-buffered NaCl, containing 2 mM $MgCl_2$, pH 7.4, and the number of adherent platelets measured using the colorimetric assay reported by Bellavite et al.[36] Briefly, 150 μL of a 0.1 M citrate buffer, pH 5.4, containing 5 mM *p*-nitrophenyl phosphate and 0.1% Triton X-100 was added to the wells after washing. After incubation for 60 min at 25 °C in the absence of ambient light, color was developed by the addition of 100 μL of 2 N NaOH and the absorbance at 405 nm was read using an EL800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, Vermont).

Cell Adhesion Assay for Specificity of Inhibitors.[30] The ligands (3 μg/mL of collagen IV for α1β1 or 3 μg/mL of collagen I for α2β1) were immobilized on 96-well flat microtiter plates (100 μL for each well) in PBS buffer solution overnight at 4 °C. In the case of VCAM (3 μg/mL, for α4β1) and fibronectin (10 μg/mL, for α5β1), 20 mM acetic acid was used instead of PBS buffer solution. In the case of α1β1 and α2β1, wells were blocked with 1% BSA in HBSS buffer solution without Ca^{2+} containing Mg^{2+} for 1 h. In the case of α4β1 and α5β1, 1% BSA in HyQ HBSS buffer solution containing Ca^{2+} and Mg^{2+} was used. Cells in the same buffer solution without BSA were labeled with incubation of 12.5 μM CMFDA at 37 °C for 30 min. After centrifugation and washing with buffer solution containing 1% BSA, cells were resuspended in the same buffer solution (1×10^6 cells/mL) and incubated in the presence of different concentrations of inhibitors at 25 °C for 15 min. Cells were added to the wells (100 μL/well) and incubated at 37 °C for 30 min. Unbound cells were washed out, and bound cells were lysed by the addition of 0.5% Triton X-100. The plates were read using a Cytofluor 2350 fluorescence plate reader (Millipore, Bedford, MA) with 485 nm (excitation) and 530 nm (emission).

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Supporting Information Available: Analytical data for new compounds and platelet aggregation. This material is available free of charge via the Internet at http://pubs.acs.org.

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