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Exploring *N*-Arylsulfonyl-L-proline Scaffold as a Platform for Potent and Selective  $\alpha\beta$ 1 Integrin Inhibitors.

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### Authors

Reed, Nilgun Isik  
Tang, You-Zhi  
McIntosh, Joel  
et al.

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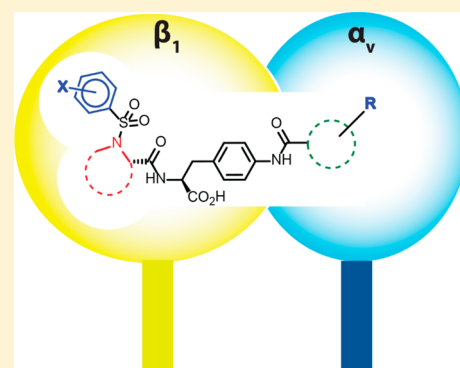
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Exploring *N*-Arylsulfonyl-*L*-proline Scaffold as a Platform for Potent and Selective  $\alpha v\beta 1$  Integrin InhibitorsNilgun Isik Reed,<sup>†</sup> You-Zhi Tang,<sup>‡,||</sup> Joel McIntosh,<sup>‡</sup> Yibing Wu,<sup>‡</sup> Kathleen S. Molnar,<sup>‡</sup> Annafelicia Civitavecchia,<sup>‡</sup> Dean Sheppard,<sup>†</sup> William F. DeGrado,<sup>‡,§</sup> and Hyunil Jo<sup>\*,‡</sup><sup>†</sup>Department of Medicine, University of California—San Francisco, San Francisco, California 94153, United States<sup>‡</sup>Department of Pharmaceutical Chemistry, University of California—San Francisco, San Francisco, California 94158, United States<sup>§</sup>Cardiovascular Research Institute, University of California—San Francisco, San Francisco, California 94158, United States<sup>||</sup>College of Veterinary Medicine, South China Agricultural University, Guangdong 510642, China

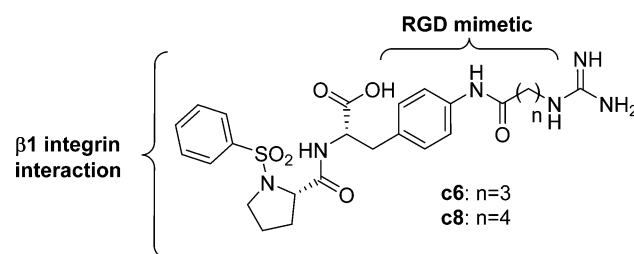
## Supporting Information

**ABSTRACT:** One small molecule inhibitor of  $\alpha v\beta 1$  integrin, **c8**, shows antifibrotic effects in multiple *in vivo* mouse models. Here we synthesized **c8** analogues and systematically investigate their structure–activity relationships (SAR) in  $\alpha v\beta 1$  integrin inhibition. *N*-Phenylsulfonyl-*L*-homoproline analogues of **c8** maintained excellent potency against  $\alpha v\beta 1$  integrin while retaining good selectivity over other RGD integrins. In addition, 2-aminopyridine or cyclic guanidine analogues were shown to be equally potent to **c8**. A rigid phenyl linker increased the potency compared to **c8**, but the selectivity over other RGD integrins diminished. These results can provide further insights on design of  $\alpha v\beta 1$  integrin inhibitors as antifibrotics.



**KEYWORDS:** Fibrosis,  $\alpha v\beta 1$  integrin, phenylsulfonylproline, TGF $\beta$ , integrin antagonist

Integrins are transmembrane proteins that mediate cell–cell and cell–extracellular matrix (ECM) interactions.<sup>1</sup> They exist as heterodimeric proteins consisting of an alpha ( $\alpha$ ) and beta ( $\beta$ ) subunit. Multiple isoforms of each subunit have been identified so far with more than half of the integrins containing either the alpha v ( $\alpha v$ ) or beta 1 ( $\beta 1$ ) subunit.<sup>2</sup> Due to their crucial biological roles in cell adhesion and proliferation, integrins have been extensively studied as therapeutic targets in cardiovascular diseases as well as cancer chemotherapy, and a few integrin inhibitors have reached the clinic.<sup>3</sup> Integrins are also involved in the activation of TGF $\beta$ , a key cytokine in fibrotic diseases. An antibody against the epithelium-specific  $\alpha v\beta 6$  integrin (STX-100) is in phase II clinical trials for idiopathic pulmonary fibrosis (IPF).<sup>4</sup> We recently identified a role for another integrin,  $\alpha v\beta 1$ , in activating TGF $\beta$  to drive organ fibrosis.<sup>5,6</sup> A prototype inhibitor, **c8**, significantly reduced the fibrotic markers in mouse model for liver and lung fibrosis (Figure 1). These findings suggest that inhibitors of  $\alpha v\beta 1$  integrin could be promising new antifibrotic agents. However,  $\alpha v\beta 1$  integrin has not been widely studied, and little structural information on  $\alpha v\beta 1$  integrin is known. To our knowledge, no other selective and potent  $\alpha v\beta 1$  integrin inhibitors have been developed so far. Thus, we turned our attention to developing antifibrotic agents based upon modification of **c8**. Structurally, **c8** (and its congener **c6**) mimics the natural ligand tripeptide sequence Arg-Gly-Asp (RGD) by connecting carboxylic acid and guanidine through a linker. **c8** also contains *N*-



**Figure 1.** Structure and design principle of integrin inhibitors **c8** and **c6**.

arylsulfonyl-*L*-proline scaffold previously shown to confer specificity and potency in inhibition of other  $\beta 1$  integrins ( $\alpha 2\beta 1$  and  $\alpha 4\beta 1$ ).<sup>7–10</sup>

Based upon this analysis, we first investigated how the structural modification in *N*-arylsulfonyl-*L*-prolyl group would influence the potency (Figure 2). It was previously shown that a number of aryl or prolyl analogues in the same *N*-arylsulfonyl-*L*-prolyl scaffold were identified as potent non-RGD  $\beta 1$  integrin inhibitors.<sup>8,9,11</sup> However, it remains unclear if it holds true in the case of RGD integrin inhibitors like **c8**. Second, we were

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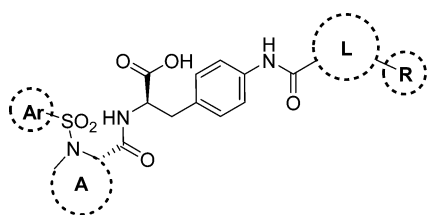
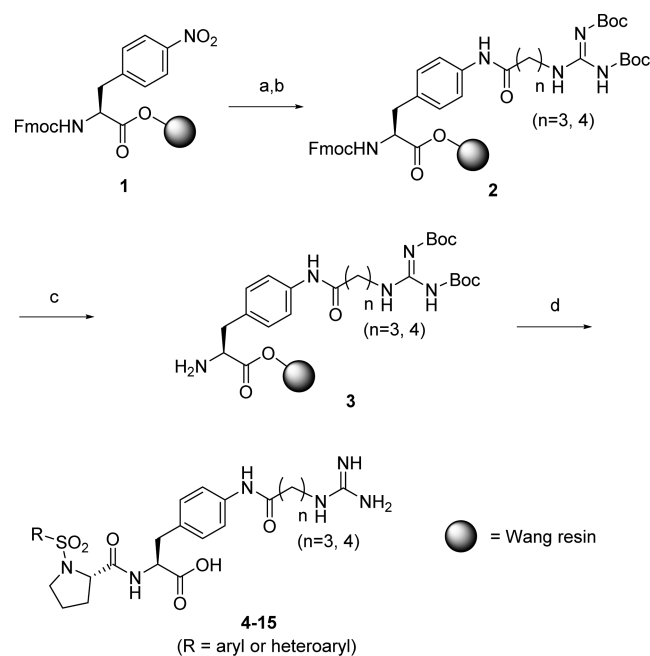


Figure 2. Sites of modification in this study.

interested in SAR of the RGD mimetic moiety since linkers as well as substitution of guanidine basic group could play a significant role in RGD integrin inhibition.<sup>12</sup>

For syntheses of *N*-arylsulfonyl analogues of **c8**, we pursued the solid-phase chemistry (Scheme 1). Conventional Fmoc-

### Scheme 1. Solid Phase Syntheses of *N*-Arylsulfonyl-proline Derivatives<sup>a</sup>



<sup>a</sup>Reagents and conditions: (a) 2 M SnCl<sub>2</sub>, DMF, 60 °C; (b) *N,N'*-di-Boc-guanidino valeric/butanoic acid, HCTU, DIPEA; (c) 20% 4-methylpiperidine, DMF; (d) (i) *N*-arylsulfonyl-L-proline analogues, HCTU, DIPEA, DMF; (ii) TFA/TIPS/H<sub>2</sub>O (95:2.5:2.5).

solid phase peptide synthesis strategy was successfully employed to provide Pro-Phe dipeptide scaffold previously.<sup>9</sup> In addition, *N*-arylsulfonylation of proline on the solid support could facilitate workup and purification. From the commercially available Fmoc-4-(NO<sub>2</sub>)-Phe-Wang resin, SnCl<sub>2</sub>/DMF reduction<sup>12</sup> of the nitro group and HCTU/DIPEA coupling of the resulting amine with *N,N'*-di-Boc-guanidinocarboxylic acid<sup>13</sup> provided the protected guanidine **2**. Fmoc deprotection by 4-methylpiperidine furnished the common intermediate amine **3**. The amine **3** was then coupled to a number of *N*-arylsulfonyl-L-proline analogues using HCTU/DIPEA, and final deprotection and cleavage from the resin with TFA provided the desired analogues **4–15**.

Potency of each compound was then tested by performing cell adhesion assays mediated by  $\alpha$ v $\beta$ 1 integrin using  $\alpha$ 5-deficient/ $\alpha$ v integrin engineered CHO cells as previously described (Table 1).<sup>6,14</sup>

Table 1. Cell Adhesion Assays of *N*-Arylsulfonyl Analogues<sup>a,b,c</sup>

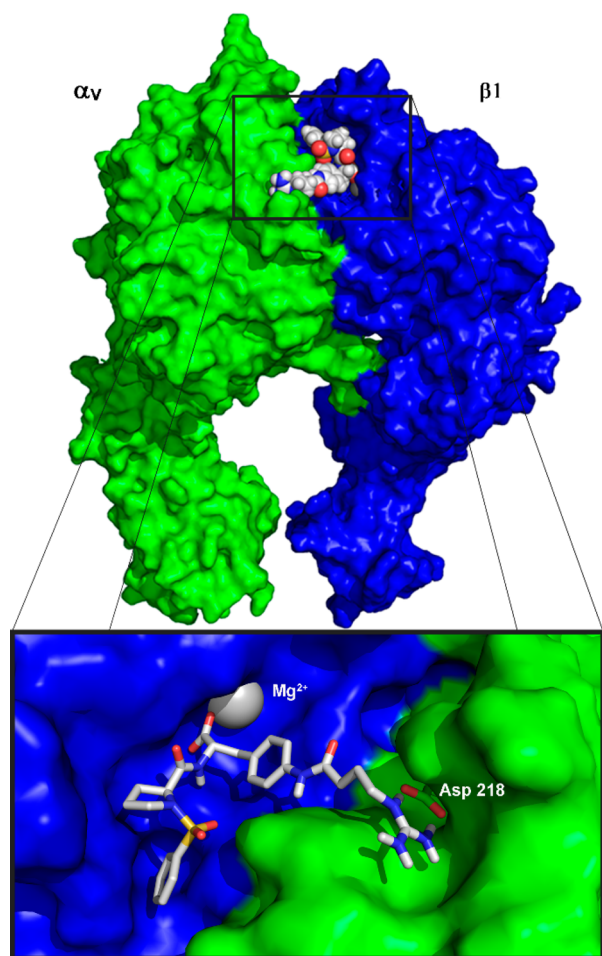
Entry	R	n	$\alpha$ v $\beta$ 1 integrin IC <sub>50</sub> (nM)	$\alpha$ v $\beta$ 1 integrin pIC <sub>50</sub> ± SD
C8		4	0.63	9.2±0.5
C6		3	0.02	10.7±0.1
4		3	630	6.2±0.5
5		3	790	6.1±0.2
6		4	>10000	<5
7		4	40	7.4±0.2
8		4	500	6.3±0.7
9		4	630	6.2±0.5
10		4	13	7.9±0.4
11		4	32	7.5±0.2
12		4	50	7.3±0.3
13		4	2500	5.6±0.2
14		4	79	7.1 ± 0.8
15		4	6300	5.2±0.1

<sup>a</sup> $\alpha$ 5 integrin-deficient/ $\alpha$ v integrin engineered CHO cell and fibronectin were used. Standard deviation ( $n = 3$ ) was calculated for pIC<sub>50</sub>. <sup>b</sup>pIC<sub>50</sub> =  $-\log$ IC<sub>50</sub>(M). <sup>c</sup>Native peptide fragment in fibronectin (GRGDS) showed a moderate IC<sub>50</sub> (ca. 100  $\mu$ M).

It was found that any substitution on the phenyl ring generally decreased potency. In particular, 2- and/or 4-

substitution on the phenyl ring (4, 6, 8, 9, 13) significantly reduced inhibitory activity against  $\alpha v\beta 1$  integrin. This observation differs from our previously reported SAR study on  $\alpha 2\beta 1$  integrin inhibitors where 4-substitution on the phenyl ring was tolerable in the same *N*-arylsulfonyl-L-proline scaffold. By contrast, 3-substitution (7, 10) and phenyl ring replacement by unsubstituted pyridine (11) or thiophene (12) allowed modestly retained potency against  $\alpha v\beta 1$  integrin, similar to results we previously reported for  $\alpha 2\beta 1$ .<sup>8</sup> Interestingly, dansyl group (14) also demonstrated retained potency, but the 4-methyl substituted naphthylsulfonyl (15) markedly reduced potency. Further structural biology studies are needed to explain this difference, but it is likely that *N*-arylsulfonyl-L-proline moiety in  $\alpha v\beta 1$  and  $\alpha 2\beta 1$  integrin may sit in a different orientation. Molecular docking study of **c6** in modeled  $\alpha v\beta 1$  integrin structure also suggested that phenyl sulfonyl ring is situated to allow a good shape complementarity (Figure 3). Thus, substitution on the phenyl ring might cause steric congestion in the pocket in the  $\beta 1$  integrin.

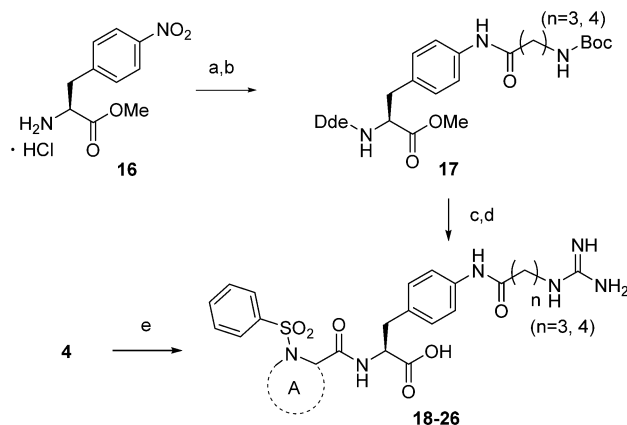
We next turned our attention to the pyrrolidine ring, leaving the phenylsulfonyl group intact. The syntheses were accomplished either in solution or solid phase as described previously



**Figure 3.** Molecular docking structure of **c6** in modeled  $\alpha v\beta 1$  integrin. **c6** binding region in  $\alpha v\beta 1$  integrin was shown in the rectangle.  $\alpha v\beta 1$  integrin structure was constructed by combination of the known  $\alpha v\beta 3$  (PDB code: 1LSG)<sup>15</sup> and  $\alpha 5\beta 1$  (PDB code: 4WK4)<sup>16</sup> integrin structures.  $Mg^{2+}$  and Asp 218 were shown to indicate the key interactions with **c6**.

(Scheme 2). Briefly, dde (*N*-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl) protection<sup>17</sup> of the amine in the

### Scheme 2. Syntheses of *N*-Benzenesulfonyl-L-proline Analogues<sup>a</sup>

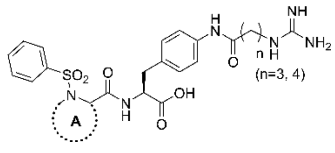













<sup>a</sup>Reagents and conditions: (a) (i) Dde-OH, DIPEA; (ii)  $SnCl_2$ , EtOH, 60 °C (97% over 2 steps); (b) Boc-aminovaleric acid ( $n = 4$ )/Boc-aminobutyric acid ( $n = 3$ ), HCTU, DIPEA, DMF (quant.); (c) (i) TFA, DCM; (ii) *N,N'*-di-Boc-guanidino carboxylic acid, HCTU, DIPEA (56% over 2 steps); (d) (i) 2.5% hydrazine, DMF; (ii) benzenesulfonyl proline analogues, HCTU, DIPEA; (iii) 50% TFA/DCM; (iv) LiOH, THF-H<sub>2</sub>O; (e) (i) *N*-benzenesulfonyl-L-proline analogues, HCTU, DIPEA; (ii) TFA/TIPS/H<sub>2</sub>O (95:2.5:2.5).

commercially available 4-nitrophenylalanine methyl ester **16** yielded the compound **17** after reduction under  $SnCl_2$ /EtOH condition of nitro group followed by coupling with *N,N'*-di-Boc-guanidinocarboxylic acids using HCTU/DIPEA. Dde protecting group was then removed by treatment with 2% hydrazine in DMF and coupled to *N*-benzenesulfonyl-L-proline analogues using HCTU/DIPEA. TFA deprotection of Boc group and hydrolysis of methyl ester provided the desired analogues **18–21** and **26**. Alternatively, the analogues **22–25** were prepared by solid phase synthesis from compound **4** described above.

Potency of *N*-benzenesulfonyl-L-proline analogues (**18–26**) seems to be affected by ring size and substitution (Table 2). For instance, azetidine (**21**) substitution dramatically lowered potency, which is in good agreement with our previous report in  $\alpha 2\beta 1$  integrin inhibitor design. However, 3,4-dehydroprolyl and unsubstituted or 3,3-dimethyl thiaprolyl ring (**21–23**) did not increase the potency,<sup>7</sup> which is opposite to our observation in  $\alpha 2\beta 1$  integrin inhibitor series. This can be partially explained by the different binding mode of *N*-phenylsulfonyl-L-proline moiety between  $\alpha 2\beta 1$  and  $\alpha v\beta 1$  integrins. It is also noteworthy that no strong *exo/endo* conformational effect on the proline ring was observed. Both *cis* and *trans* isomer of 4-fluoro-L-proline (**24**, **25**) exhibited similar potency in cell adhesion assay.<sup>18</sup> On the other hand,  $\alpha$ -methyl substitution (**26**) and  $\beta,\beta$ -dimethyl substitution (**23**) almost completely abolished the inhibitory effect of **c8** (for further comparison, see Supporting Information).<sup>7</sup> While most *N*-arylsulfonyl-L-proline headgroup modifications failed to increase the potency, we did observe that piperidine replacement (**18**, **19**) showed slightly higher potency than **c8**. We speculate that pyrrolidine and piperidine share similar position conformations to locate the hydrophobic phenyl ring into the hydrophobic pocket (Figure 4).

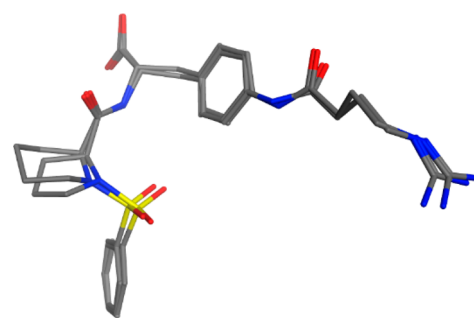
**Table 2. Cell Adhesion Assays of *N*-Benzenesulfonyl-L-prolyl Analogues<sup>a,b,c</sup>**



	A	n	$\alpha v\beta 1$ integrin IC <sub>50</sub> (nM)	$\alpha v\beta 1$ integrin pIC <sub>50</sub> ± SD
C8		4	0.63	9.2±0.5
C6		3	0.02	10.7±0.1
18		4	0.32	9.5±0.7
19		3	0.25	9.6±0.6
20		4	800	6.1±0.4
21		4	160	6.8±0.8
22		4	16	7.8±0.5
23		4	2000	5.7±0.1
24		4	0.5	9.3±0.7
25		4	0.5	9.3±0.7
26		4	>10000	< 5

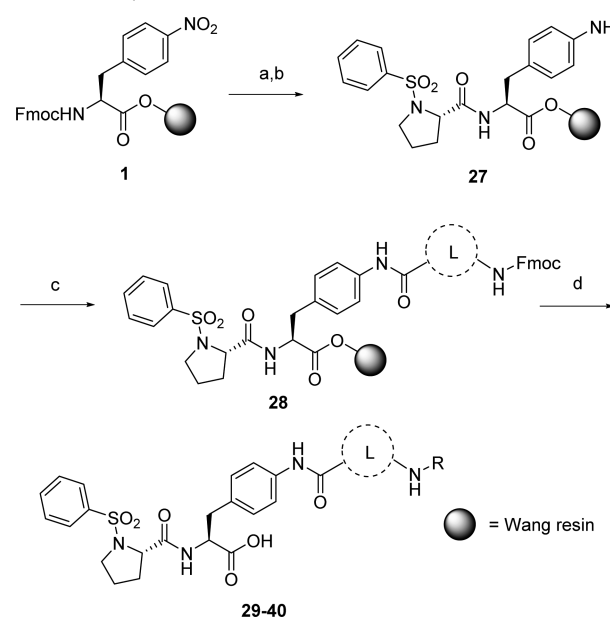
<sup>a</sup> $\alpha 5$  integrin-deficient/ $\alpha v$  integrin-engineered CHO cell and fibronectin were used. <sup>b</sup>Standard deviation ( $n = 3$ ) was calculated for pIC<sub>50</sub>. <sup>c</sup>pIC<sub>50</sub> =  $-\log$ IC<sub>50</sub>(M).

Next, we turned our attention to modification of the linker and guanidine groups in RGD mimetic moiety. We kept the original *N*-benzenesulfonyl-L-proline headgroup intact since it turned out to be one of the most potent compounds. The syntheses of these analogues were performed using conventional solid-phase chemistry (Scheme 3). Starting from commercially available Fmoc-protected *p*-nitrophenylalanine on Wang resin, deprotection by 4-methylpiperidine (20% in DMF) and amide bond formation with *N*-benzenesulfonyl-L-proline produced the common intermediate amine 27.



**Figure 4.** Overlay of docked structures of c6 and piperidine analogue 19 in modeled  $\alpha v\beta 1$  integrin.

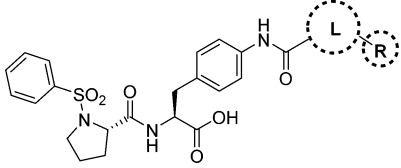
**Scheme 3. Syntheses of Linker and Basic Unit Derivatives<sup>a</sup>**



<sup>a</sup>Reagents and conditions: (a) (i) 4-methylpiperidine/DMF (1/4); (ii) *N*-benzenesulfonyl-L-proline, HCTU, DIPEA; (b) SnCl<sub>2</sub>, DMF, 60 °C; (c) Fmoc-amino acids, HCTU, DIPEA, DMF; (d) (i) 4-methylpiperidine/DMF (1:4); (ii) guanidylating agents, DIPEA or pyridine *N*-oxides, PyBroP, DIPEA; (iii) TFA/TIPS/H<sub>2</sub>O (95:2.5:2.5).

coupling with Fmoc amino acid derivatives and guanidylation of terminal amine after Fmoc deprotection produced the desired analogues 29–40 after final TFA cleavage from resin. For syntheses of 2-aminopyridine analogues (32–35), pyridine *N*-oxide with PyBroP activation was particularly effective.<sup>19</sup>

From analysis of the cell adhesion results (Table 3), it was apparent that cyclic guanidines (29–31) can replace the original guanidine group in c8 with a similar or higher potency. However, high basicity of guanidine and cyclic guanidine could be problematic in further development due to their unfavorable pharmacokinetic properties. We were excited that aminopyridine replacement of guanidine was well tolerated. Indeed one of the aminopyridine analogues (33) showed a higher potency than c8. Furthermore, aminopyridine replacement not only reduces the charge due to its pK<sub>a</sub> (ca. 7), but also renders support on “side-on” binding pattern of basic group in  $\alpha v\beta 1$  integrin rather than “end-on” pattern as seen in  $\alpha IIb\beta 3$  integrin.<sup>20</sup>

**Table 3. Cell Adhesion Assays of RGD Mimetic Analogues of **c8****<sup>a,b,c</sup>


	L	R	$\alpha v\beta 1$ integrin IC <sub>50</sub> (nM)	$\alpha v\beta 1$ integrin pIC <sub>50</sub> ± SD
C8	-(CH <sub>2</sub> ) <sub>4</sub>		0.63	9.2±0.5
C6	-(CH <sub>2</sub> ) <sub>3</sub>		0.02	10.7±0.1
29	-(CH <sub>2</sub> ) <sub>4</sub>		0.32	9.5±0.2
30	-(CH <sub>2</sub> ) <sub>3</sub>		0.13	9.9±0.7
31	-(CH <sub>2</sub> ) <sub>4</sub>		2.0	8.7±0.5
32	-(CH <sub>2</sub> ) <sub>4</sub>		2.0	8.7±0.4
33	-(CH <sub>2</sub> ) <sub>3</sub>		0.2	9.7±0.3
34	-(CH <sub>2</sub> ) <sub>3</sub>		200	6.7±0.2
35	-(CH <sub>2</sub> ) <sub>3</sub>		320	6.5±0.2
36			800	6.1±0.1
37			0.06	10.2±0.3
38			50	7.3±0.1
39			2.0	8.7±0.1
40			2500	5.6±0.1

<sup>a</sup> $\alpha 5$  integrin-deficient/ $\alpha v$  integrin-engineered CHO cell and fibronectin were used. <sup>b</sup>Standard deviation ( $n = 3$ ) was calculated for pIC<sub>50</sub>. <sup>c</sup>pIC<sub>50</sub> =  $-\log IC_{50}$  (M).

Replacement of linear aliphatic linkers to rigid cyclic or aryl linkers also influences the potency. 3-Guanidino benzamide linker (**37**) showed 10-fold higher potency against  $\alpha v\beta 1$  integrin than **c8**, while 3-guanidino phenylacetamide linker (**38**) reduced the potency. Strikingly, almost no inhibition was observed in 4-guanidino benzamide linker (**36**). However, saturation of aromatic ring (**39**) regained the potency that

suggests proper alignment of the basic group is key to maintain the potency. However, the bulky adamantyl linker (**40**) is seemingly ineffective.

Finally, selected potent compounds were tested on a panel of RGD integrins in cell adhesion assay as previously described (Table 4).<sup>6</sup> All of the tested compounds (**18**, **19**, **29**, **33**, **37**)

**Table 4. Selectivity of Selected *N*-Arylsulfonyl-L-prolyl Inhibitors**<sup>a,b,c</sup>

	IC <sub>50</sub> (nM)				
	$\alpha v\beta 1$	$\alpha v\beta 3$	$\alpha v\beta 5$	$\alpha v\beta 8$	$\alpha 5\beta 1$
<b>c8</b>	0.63	>10000	>10000	>10000	>10000
<b>18</b>	0.31	4000	160	2000	3200
<b>19</b>	0.25	2000	1600	2500	3200
<b>29</b>	0.31	>10000	200	>10000	>10000
<b>33</b>	0.2	>10000	>10000	400	>10000
<b>37</b>	0.06	160	>10000	40	63

<sup>a</sup>Cell adhesion mediated by  $\alpha v\beta 1$ ,  $\alpha v\beta 3$ ,  $\alpha v\beta 5$ ,  $\alpha v\beta 8$ , and  $\alpha 5\beta 1$  was measured using pairs of cell lines and ligands selected to isolate the effect of each individual integrin:  $\alpha v\beta 1$ -CHO  $\alpha v$  ( $\alpha 5$ -deficient CHO cells engineered to express  $\alpha v\beta 1$ ) adhering to fibronectin (0.3 mg/mL),  $\alpha v\beta 3$ -SW480 cells transfected with human  $\beta 3$  adhering to fibrinogen (1 mg/mL),  $\alpha v\beta 5$ -wild-type SW480 cells adhering to vitronectin (0.1 mg/mL),  $\alpha v\beta 8$ -glioma cell line (SNB19) adhering to recombinant human TGF $\beta 1$  LAP (1 mg/mL),  $\alpha 5\beta 1$ -colon carcinoma cell line SW480 plated on fibronectin (0.3 mg/mL). <sup>b</sup>See Supporting Information for pIC<sub>50</sub> and standard deviation ( $n = 3$ ). <sup>c</sup>pIC<sub>50</sub> =  $-\log IC_{50}$  (M).

showed a good to excellent selectivity against  $\alpha v\beta 1$  integrin over other RGD integrins. However, it should be noted that the selectivity diminished in 3-guanidino benzamide linker analogue (**37**), while homoproline analogue (**19**) was most selective.

In conclusion, we identified several highly potent  $\alpha v\beta 1$  integrin inhibitors by modification of *N*-arylsulfonyl-L-proline scaffold in **c8**. *N*-Phenylsulfonyl-L-homoproline analogues were shown to be alternative candidates with excellent selectivity toward  $\alpha v\beta 1$  integrin over other RGD integrins. RGD-mimetic modification revealed cyclic guanidine and 2-aminopyridines are excellent basic groups. A 3-substituted benzamide linker showed the increased potency with a little diminished selectivity. Further medicinal chemistry efforts to obtain more potent and selective  $\alpha v\beta 1$  integrin inhibitors by combinations of these features are currently being made and will be reported in due course.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmmedchemlett.6b00196.

Experimental details for syntheses of all new compounds and copies of <sup>1</sup>H and <sup>13</sup>C NMR spectra of key compounds **18**, **19**, **29**, **33**, and **37**. IC<sub>50</sub> curves of key compounds **18**, **19**, **29**, **33**, and **37** against  $\alpha v\beta 1$  integrin and pIC<sub>50</sub> data for RGD integrins and IC<sub>50</sub> comparison table between selected  $\alpha v\beta 1$  and  $\alpha 2\beta 1$  integrin inhibitors (PDF)

## AUTHOR INFORMATION

### Corresponding Author

\*E-mail: [hyunil.jo@ucsf.edu](mailto:hyunil.jo@ucsf.edu)

### Author Contributions

N.I.R. performed the biological assays. H.J., Y-Z.T., J.M., A.C., and W.F.D. designed the molecules and performed the chemical syntheses. H.J., J.M., Y.W., and K.S.M. collected and processed the NMR and high-resolution mass spectrometry data. H.J. and W.F.D. participated in molecular modeling and docking. D.S. and W.F.D. designed the study and supervised it. All the authors reviewed the final draft of the manuscript.

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### Notes

The authors declare the following competing financial interest(s): H.J., N.I.R., Y-Z.T., D.S., and W.F.D. are inventors of a patent application related to this study.

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## ABBREVIATIONS

DMF, dimethylformamide; HCTU, *O*-(6-chlorobenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate; DIPEA, *N,N*-diisopropylethylamine; TIPS, triisopropylsilane; TFA, trifluoroacetic acid; Dde, *N*-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl); DCM, dichloromethane; PyBroP, bromotripyrrolidinophosphonium hexafluorophosphate

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