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SOLID-PHASE SYNTHESIS OF A SELECTIVE $\alpha_v\beta_3$ INTEGRIN ANTAGONIST LIBRARY

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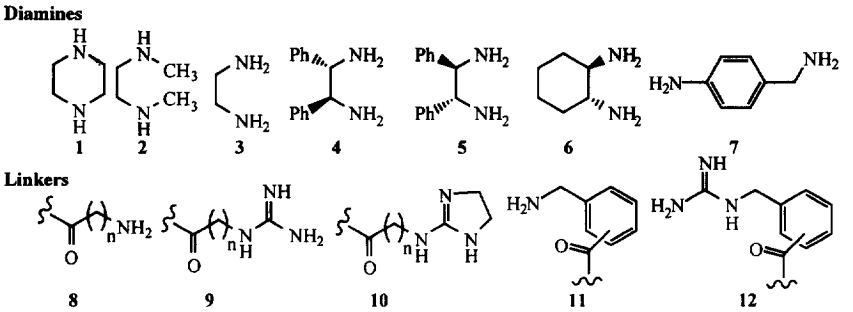
Abstract: Solid-phase synthesis was used to generate a focused RGD peptide mimetic library in an effort to identify potent and selective $\alpha_v\beta_3$ integrin antagonists. Increased activity was observed for compounds possessing a urea linkage to piperazine, with the most active compound (**28**) exhibiting an IC_{50} = 1.1 nM in an $\alpha_v\beta_3$ ELISA assay. © 1997 The DuPont Merck Pharmaceutical Company. Published by Elsevier Science Ltd.

Integrins are heterodimeric transmembrane glycoproteins that have been implicated in the transfer of information from the cellular environment to the cell during a diversity of processes, including embryogenesis, thrombosis, metastasis, and immune responses.[1] Integrins are currently known to be composed of dimers of at least fifteen α subunits and eight β subunits. Two integrins that contain the β_3 subunit are $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$. The $\alpha_{IIb}\beta_3$ is prevalent on platelets, and $\alpha_v\beta_3$ has been implicated in melanoma development and as an important receptor for mediating the attachment of the osteoclast to bone during bone resorption.[2] The ability to selectively inhibit the $\alpha_v\beta_3$ integrin has been postulated to present a therapeutic approach to the treatment of a variety of diseases, including osteoporosis[2b] and diabetic retinopathy.[3]

A variety of integrins have been described that bind the Arg-Gly-Asp (RGD) motif ($\alpha_{IIb}\beta_3$,[4] $\alpha_v\beta_3$,[4] $\alpha_5\beta_1$[5] and $\alpha_v\beta_5$[6]). We set out to develop a library of peptide mimetics that could be used to select ligands with high affinity and *specificity* for any RGD-interactive integrin. Each member of this library consists of (1) a β-alanine group in which the carboxylate mimics the carboxylate of the aspartic acid in RGD; (2) a linking group of varying length, rigidity and physico-chemical properties; and (3) an amine or guanidine to mimic the arginine group in RGD. By systematically varying the linking group and the side chains on β-alanine, it proved possible to identify and optimize the affinity of ligands for a specific integrin. Early in this work (data not shown), we learned that a 2-carbobenzyloxyamino group attached to the β-alanine moiety gave excellent specificity for the β_3 class of RGD-interactive integrins;[7] therefore, α-N-CBZ-diaminopropionic acid was chosen as an aspartic acid mimic. Seven different diamines were chosen as the linking group because of their commercial availability and the ability to couple unprotected diamines to a solid support via a urea linkage (Figure 1). The diversity of the library was increased further by varying the distance between the acidic and basic components by using straight chain amino acids or aminomethylbenzoic acids and by changing the basicity of the amino moiety.[8] A focused

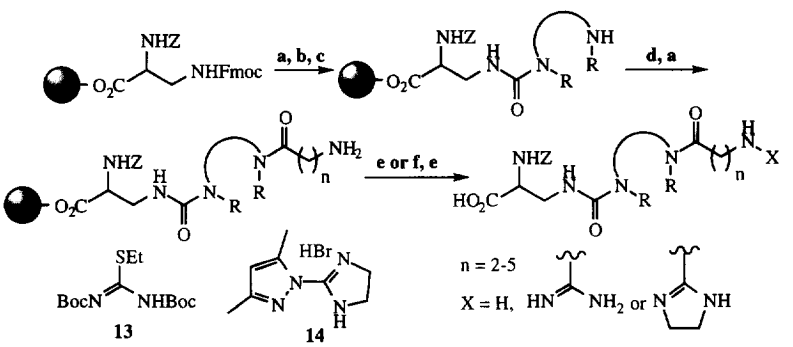
library was created using the constraints described above to discover a potent $\alpha_v\beta_3$ selective antagonist.

Figure 1



α-N-CBZ-β-N-Fmoc-L-diaminopropionic acid (DAP) was attached to bromomethyl Wang resin and the Fmoc group was removed with 20% piperidine/DMF. Activation of the amine with *p*-nitrophenyl chloroformate followed by exposure of the urethane to a diamine delivered the desired urea (Scheme 1).[9,10] Fmoc-protected amino acids were then appended onto the free amine of the diamine through an amide bond under standard peptide coupling conditions. The Fmoc-groups were then removed and the compounds were either cleaved from the resin or treated with N,N-*bis*-Boc-S-ethylthiourea[11] (**13**) or with 2-(3,5-dimethylpyrazolyl)-4,5-dihydroimidazole hydrobromide salt[12] (**14**) followed by acid cleavage from the resin.[13]

Scheme 1



a. 20% Piperidine/DMF; **b.** *p*-Nitrophenyl chloroformate, DIEA, CH_2Cl_2/THF; **c.** Diamine, DIEA, DMF; **d.** $HO_2C(CH_2)_nNHFmoc$ (n = 2-5), HBTU, DIEA, DMF; **e.** 50% TFA/CH_2Cl_2; **f.** **13** or **14**, DMF, DIEA.

The ability of these compounds to inhibit $\alpha_v\beta_3$ was assessed with an ELISA assay,[14] and the specificity of the active compounds was determined by measuring their activities against the related integrin GPIIb/IIIa ($\alpha_{IIb}\beta_3$) in a platelet aggregation assay [15] and against the $\alpha_5\beta_1$ and $\alpha_v\beta_5$ integrins[16] (Table 1). Typical $\alpha_{IIb}\beta_3$ antagonists are about 10 to 50-fold less potent in the platelet aggregation assay as compared to an ELISA assay, so that the $\alpha_{IIb}\beta_3$ values should be divided by this factor when comparing the $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ results (unpublished results). All of the compounds synthesized using diamines **3-7** had less than 50% inhibition at 10 μM in the $\alpha_v\beta_3$ ELISA assay and were deemed inactive. Compounds exhibiting less than 50% inhibition at 100 μM in the GPIIb/IIIa

PRP assay were deemed inactive. The activity of the compounds listed below can be compared to the known $\alpha_v\beta_3$ integrin antagonist cycloRGDfV which exhibited an IC_{50} of 50 nM in the $\alpha_v\beta_3$ ELISA.[17]

Table 1

Compound	Diamine	Linker / n =	$\alpha_v\beta_3$ ELISA IC_{50} (nM)	$\alpha_5\beta_1$ IC_{50} (nM)	$\alpha_v\beta_5$ IC_{50} (nM)	GPIIb/IIIa PRP Data (nM)
15		8 / 2	660 ± 360	>10,000		70,000 ± 17,000
16		8 / 3	1,400 ± 730	>10,000		47,000 ± 9,800
17		8 / 4	2,200 ± 1600	>10,000		29,000 ± 5,400
18		8 / 5	Inactive (n = 1)	>10,000		7,500 (n = 1)
19		*meta*-11	Inactive (n = 1)	>10,000		Inactive (n = 1)
20		*para*-11	Inactive (n = 1)	>10,000		15,000 (n = 1)
21		*meta*-12	Inactive (n = 1)	>10,000		25,000 (n = 1)
22	**1**	*para*-12	Inactive (n = 1)	>10,000		9,000 (n = 1)
23		9 / 2	31 ± 13	>50,000	20,000 ± 9,100	41,000 ± 4,300
24		9 / 3	4.0 ± 2.4	3,200 ± 1,800	4,700 ± 360	9,700 ± 700
25		9 / 4	120 ± 67	>10,000		1,400 ± 200
26		9 / 5	110 ± 67	>10,000		1,900 ± 400
27		10 / 2	5.0 ± 2.1	>10,000	9,800 ± 3,300	73,000 ± 300
28		10 / 3	1.1 ± 0.4	660 ± 100	420 ± 170	20,000 ± 3,300
29		10 / 4	16 ± 7.1	8,200 ± 1,300	17,000 ± 5,100	15,000 ± 1,900
30		10 / 5	130 ± 71	>10,000	>100,000	37,000 ± 8,700
31		8 / 2	1900 (n = 1)	>10,000		82,000 ± 24,000
32		8 / 3	440 (n = 1)	>10,000		89,000 ± 12,000
33		8 / 4	990 (n = 1)	>10,000		89,000 ± 15,000
34	**2**	8 / 5	600 (n = 1)	>10,000		16,000 ± 2,700
35		9 / 2	420 (n = 1)	>10,000		Inactive (n = 1)
36		9 / 3	410 (n = 1)	>10,000		96,000 ± 16,000
37		9 / 4	370 (n = 1)	>10,000		35,000 ± 5,400
38		9 / 5	110 (n = 1)	>10,000		12,000 ± 900

The most active compounds in the $\alpha_v\beta_3$ ELISA contain the piperazine diamine and either the guanidine (**24**) or cyclic guanidine (**28**) functionalities. Furthermore, compounds with n = 3 are more active than the longer chain compounds in the $\alpha_v\beta_3$ ELISA assay. There is also an increase in $\alpha_v\beta_3$ ELISA activity upon decreasing the distance between the carboxylic acid and the basic amino groups along with a corresponding decrease in $\alpha_{IIb}\beta_3$ activity (**23-30**).

In summary, a library of RGD peptide mimetics was synthesized using solid-phase chemistry and a potent $\alpha_v\beta_3$ integrin antagonist (**28**) was identified which showed excellent specificity for $\alpha_v\beta_3$ integrin relative to $\alpha_{IIb}\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$. Furthermore, an increase in selectivity for the $\alpha_v\beta_3$ integrin over the closely related $\alpha_{IIb}\beta_3$ integrin was observed upon decreasing the distance between the carboxylic acid group of DAP and the basic guanidine group in either the acyclic guanidines or the cyclic guanidines.[18] These results show the utility of this approach for the design of conformationally constrained compounds that can serve as first-generation leads in the design of novel pharmaceuticals. Our continuing efforts to identify other integrin antagonists will be reported at a later date.

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9. **Preparation of Bromomethyl Wang Resin:** Triphenylphosphine (5 equiv) was dissolved in DMF (10 mL/g resin). To this solution was added Wang resin (7.3 mmol), obtained from Advanced ChemTech (0.73 mmol/g), followed by carbon tetrabromide (5 equiv) and stirred at rt for 2.5 h. The resin was filtered, washed repeatedly with DMF and MeOH and dried overnight at 35 °C/30 mmHg. **Coupling to Bromomethyl Wang Resin:** The resin was swelled in DMF (10 mL/g resin), α-N-CBZ-β-N-Fmoc-L-α,β-diaminopropionic acid (1.5 equiv), cesium iodide (1 equiv) and Hunig's base (1.5 equiv) were added and the reaction was stirred at rt for 15 h. The resin was filtered, washed repeatedly with DMF and MeOH, and dried overnight at 35 °C/30 mmHg. **Coupling of Diamines:** Following removal of the Fmoc group under standard conditions, the resin was swelled in CH_2Cl_2/THF (1:1, 10 mL/g resin), treated with *p*-nitrophenyl chloroformate (5 equiv) and Hunig's base (2 equiv) and stirred at rt for 45 min or until a negative Kaiser test was observed. The resin was washed with CH_2Cl_2 (4x), swelled in DMF (10 mL/g resin) and Hunig's base (2 equiv) and the diamine (5 equiv) were added. The reaction was mixed at rt for 40 min, or until a positive Kaiser test was obtained and the resin was washed repeatedly with DMF and isopropanol. The resin was dried overnight at 35 °C/30 mmHg. **Formation of guanidines:** The resin (0.042 mmol) was swelled in DMF (10 mL/g resin) and Hunig's base (5 equiv) and N,N-*bis*-Boc-S-ethylthiourea (**13**) (2 equiv) were added. The resin was mixed at rt for 15 h, filtered, washed repeatedly with DMF followed by isopropanol and air dried. The same procedure was followed when 2-(3.5-dimethylpyrazolyl)-4,5-dihydroimidazole · HBr (**14**) was used, except the reaction was heated at 70 °C for 15 h.
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13. All compounds were >80% pure by reverse phase HPLC and molecular composition was determined by low resolution MS.
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16. The $\alpha_v\beta_5$ and $\alpha_5\beta_1$ assays were performed as described in: Mousa, S.A.; Forsythe, M.; Lorelli, W.; Bozarth, J.; Xue, C.-B.; Wityak, J; Sielecki, T. M.; Olson, R. E.; DeGrado, W.; Kapil, R.; Hussain, M.; Wexler, R.; Thoolen, M. J.; Reilly, T. M. *Coronary Artery Disease* **1996**, *7*, 767. In brief, the $\alpha_v\beta_5$ assay was performed by plating a Costar 3590 plate with vitronectin (100 µL, 0.25 µg/well) overnight at 4 °C. After coating overnight, each well was washed twice with PBS (200 µL) and nonspecific binding was blocked by adding 200 µL of PBS + 50% BSA per well for 1 h at rt. SKBR3 cells were detached with 0.005% trypsin/0.1% EDTA, washed, and resuspended inserum free McCoy's 5A standard media (Gibco BRL) at 1 x 10^6 cells/mL. The cells were labeled with 2 µM Calcein-AM (Molecular Probes #3100, 50 µg/vial) for 30 min at 37 °C humidified incubator. The cells were washed and centrifuged. The cells (1 x 10^6 cells/mL) were preincubated with either 150 µL of test compounds or media, gently mixed, then incubated for 15 min at rt. Drug treated SKBR3 cells were added to the assay plate an incubated for 60 min at rt. The media was removed from the wells and washed twice with McCoy's 5A media (200 µL). Following washing, McCoy's 5A media (100 µL) was added to each well and the fluorescence was read on

a Cytofluor 2300 at sensitivity 2, Ex = 485 nm and EM = 530 nm. The $\alpha_5\beta_1$ receptor, obtained from human placenta, was diluted (1:2000) with coating buffer and coated (100 μL/well) onto Costar (3590) plates overnight at 4 °C. The plates were washed once with buffer (B/B buffer) and the wells were then blocked with B/B buffer containing 1% BSA (200 μL). After washing with B/B buffer, 100 μL of biotinylated fibronectin and 11 μL of either inhibitor or B/B buffer containing 1.0% BSA was added to each well. After 1 h at rt, the plate was washed with B/B buffer (2x) and incubated for 1 h at rt with 100 mL anti-biotin alkaline phosphatase (1:12000) dilution in B/B buffer. The plates were washed with B/B buffer and incubated for 1 h at rt with 100 μL alkaline phosphatase substrate. Color was developed at rt for approximately 45 min. The reaction was stopped by adding 2 N NaOH (25 μL/well) and the absorbance was read at 450 nM.

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