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Discovery of small molecule inhibitors of integrin αvβ3 through structure-based virtual screening

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Abstract—Inhibitors of integrin αvβ3 have been implicated in the treatment of a variety of diseases, including tumor metastasis, neovascularization, osteoporosis, and rheumatoid arthritis. It is therefore desirable to develop new types of small molecule inhibitors of integrin αvβ3. Here we describe the discovery of novel classes of small molecule inhibitors, via structure-based virtual screening, that target the ligand binding site of integrin αvβ3. Application of the docking procedure for screening of a commercially available compound database resulted in a 1774-fold reduction in the size of the screening set (88695 to 50 compounds) and gave a hit-rate of 14% upon biological evaluation (IC_{50} value ranging from 30 to 200 μM). The best hit, compound **37**, 3,4-dichloro-phenylbiguanide, showed inhibitory activity, in a time- and dose-dependent manner, in both cell motility and angiogenesis assays. Based on the best hit, compound **37**, a more effective derivative compound **62** has been identified. Furthermore, molecular graphics analyses of a series of substituted phenylbiguanides were carried out to predict the binding mode between the active compounds and integrin αvβ3. Our results indicate that the substituted phenylbiguanides might be involved in the inhibition of bivalent cation-mediated ligand binding of integrin αvβ3.

Integrins are non-covalent heterodimeric receptors that mediate divalent cation-dependent cell-cell and cell-matrix adhesion through tightly regulated interactions with ligands.[1,2] Integrin αvβ3, also known as the vitronectin receptor, is expressed on the surface of a variety of cell types, including osteoclasts, vascular smooth muscle cells, endothelial cells, and tumor cells. Integrin αvβ3 is associated with angiogenesis,[3,4] tumor metastasis,[5–7] inflammation,[8,9] vascular smooth muscle cell migration,[10] and bone resorption,[11,12] representing a promising molecular target to develop inhibitors which could be used in the treatment of diseases including cancer,[4,13] macular degeneration and diabetic retinopathy,[14] osteoporosis,[15] and restenosis following percutaneous transluminal coronary angioplasty (PTCA).[10,16]

Integrin αvβ3 is known to recognize the Arg-Gly-Asp (RGD) tripeptide sequence,[17] and a number of groups have reported the discovery of potent non-peptide αvβ3 antagonists based on mimetics of the RGD sequence.[18–20] The crystal structure of integrin αvβ3 in complex with an RGD ligand was reported by Xiong et al.[21] This information provided us the structural basis to the identification of novel small molecule integrin αvβ3 inhibitors based on the receptor–ligand complex, not only the ligand sequence alone.

Here, we describe the identification of novel small molecule inhibitors of integrin αvβ3 through the application of virtual screening protocol based on the crystal structure of the extracellular segment of integrin αvβ3 in complex with a RGD ligand (PDB entry, 1L5G, was retrieved from the Protein Data Bank). A preliminary DOCK screening against ligand binding site of integrin αvβ3 was performed on a database containing 88,695 commercially available organic compounds (www.specs.net), which were converted into 3D MOL2 format via in-house procedures described previously.[22] The same binding site as pentapeptide cyclo(RGDF = N{Me}V) was chosen for dock screening. Water, metal ions, and complexed ligand were deleted; hydrogens and charges were added by SYBYL6.6. All docking calculations were carried out with DOCK4.0.1 using flexible ligands based on the anchored search method. The standard docking protocol

Abbreviations: PTCA, percutaneous transluminal coronary angioplasty; RGD, Arg-Gly-Asp; bFGF, basic fibroblast growth factor; HUVECs, human umbilical vein endothelial cells; ECM, extracellular matrix; MIDAS, metal ion-dependent adhesion site; ADMIDAS, adjacent to MIDAS.

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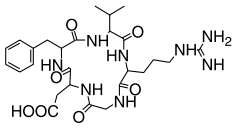
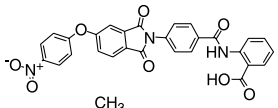
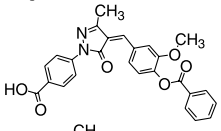
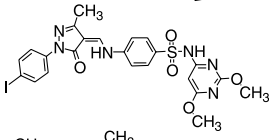
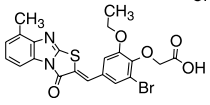
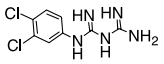
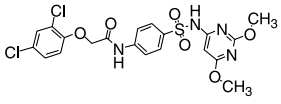
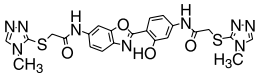
was as follows: (1) target preparation, (2) sphere set generation, (3) force-field grid calculation, and (4) docking and scoring. Top 20,000 compounds with steric complementarity to the ligand binding site were selected for rigorous secondary DOCK screening. Docking results were clustered and ranked in terms of energy score. Top 1000 compounds with the best energy score were selected for further structural diversity and drug-like analysis, and 50 compounds were finally selected for biological testing.

Integrin αvβ3-mediated cell adhesion assay was performed to assess the adhesion ability of human melanoma cells M21 to vitronectin-coated plates in the presence of these compounds.[23] Table 1 presents the chemical structures and the IC_{50} values for inhibitory activity of active compounds in this cell adhesion assay. Seven of the 50 compounds demonstrated notable inhibitory activity against adhesion of M21 cells on vitronectin (IC_{50} < 200μM), representing a hit-rate of 14%. The best hit, compound **37**, 3,4-dichloro-phenylbiguanide, was selected for further studies including scrape motility assay[24] and in vitro angiogenesis assay.[25]

As shown in Figure 1, compound **37** significantly inhibited cell migration on vitronectin-coated plates. After treatment of M21 cells with 7.5, 15.0, and 30.0 μM of compound **37** for 8 h, the migration distances were 27.8 ± 6.5, 8.3 ± 7.2, and 1.1 ± 4.3 μm, compared to that of 30.6 ± 8.1 μm in the absence of compound **37**, representing a ~9.1, 73.1, and 96.4% of inhibition, respectively (Fig. 1A). After 16 h, the migration distances were 72.2 ± 10.6, 38.9 ± 9.4, and 16.7 ± 7.2 μm in the presence of 7.5, 15.0, and 30.0 μM compound **37**, compared to that of 119.4 ± 11.6 μm in the absence of compound **37**, representing a ~39.5, 67.4, and 86.0% of inhibition, respectively (Fig. 1B). These results clearly indicate that compound **37** inhibited the migration of M21 cells in a time- and dose-dependent manner.

Angiogenesis depends on both cytokine stimulation and vascular cell adhesion events. Integrin αvβ3 has been identified to be involved in angiogenesis initiated by basic fibroblast growth factor (bFGF).[26] In this study, in vitro angiogenesis was stimulated by incubation of human umbilical vein endothelial cells (HUVECs) on

Table 1. Chemical structures and inhibitory effect of active compounds

Compound	Structure	αvβ3-mediated cell adhesion IC_{50}[a] (μM)
c(RGDfV)[b]		0.6 ± 0.07
11		180.4 ± 9.5
27		143.1 ± 8.2
30		53.5 ± 1.3
32		132.0 ± 3.4
37		38.5 ± 1.7
39		137.0 ± 11.1
40		162.1 ± 26.9

Non-tissue culture plastic 96-well plates were coated with 2 μg/ml vitronectin overnight. Then, M21 cells were seeded onto coated plates in the absence or presence of different concentrations of compounds and allowed to adhere at 37 °C for 1 h. Adherent cells were fixed, stained, and the plates were read with a microplate reader at 570 nm. Percentage of cell adhesion inhibition was calculated using wells without compounds as 100% of adhesion.

[a] The results represent the means ± SD of triplicate determinations.

[b] c(RGDfV) as a positive control.

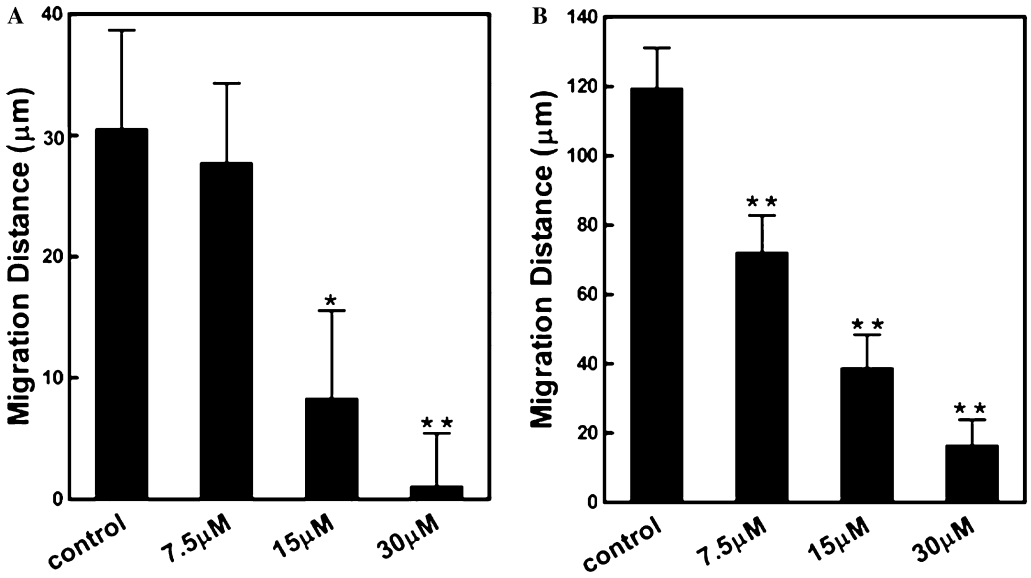


Figure 1. Cell migration in the presence or absence of compound **37** in scrape motility assay. The migration distance within 8 h (A) or 16 h (B) was measured under phase-contrast microscopy. The results represent means ± SD of nine determinations from three individual experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to untreated cells.

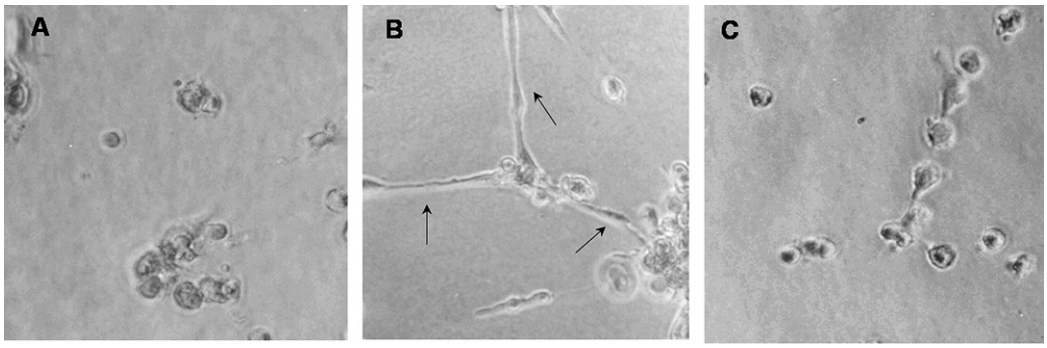


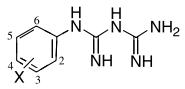
Figure 2. Effects of compound **37** on bFGF-induced capillary-like structure formation of HUVECs on ECM gel. HUVECs in ECM gel were treated with PBS (A), 100 ng/ml bFGF (B) or 100 ng/ml bFGF in combination with 30 μM compound **37** (C) for 6 h. The resulting capillary-like structures (arrowheads) were viewed by phase-contrast microscopy.

extracellular matrix (ECM) gel in the presence of bFGF. When HUVECs were plated on ECM gel without the addition of bFGF, most of them appeared in a cobblestone shape (Fig. 2A). In the presence of bFGF (100 ng/ml), HUVECs formed an anastomosing capillary-like network within 6 h (Fig. 2B). Compound **37**, at a concentration of 30 μM, significantly inhibited the capillary-like structure formation of HUVECs (Fig. 2C).

To elucidate the structural features required for the activity of compound **37** against integrin αvβ3, we investigated the effects of a series of substituted phenylbiguanides based on the chemical structure of compound **37**. Table 2 presents the chemical structures and IC_{50} values of several substituted phenylbiguanides. Compound **62**, 3,5-dichloro-phenylbiguanide, showed the best inhibitory activity, while the derivative **68** had similar activity with compound **37**. Taken together, the results indicate that dichlorosubstituted derivatives (compounds **37**, **62**, **67**, **64**, and **60**) had better activity than monosubstituted series (compounds **61**, **59**, and **65**), and unsubstituted phenylbiguanide **66** was the least active compound ($IC_{50} > 200$ μM).

Molecular graphics analysis was carried out to deduce a probable binding model for substituted phenylbiguanides with integrin αvβ3. Integrins mediate divalent cation-dependent interactions with their extracellular ligands, and the presence of bivalent cations in the metal ion-dependent adhesion site (MIDAS) and ADMIDAS (adjacent to MIDAS) of integrin αvβ3 has been shown to be very important for the binding of ligands (Fig. 3A).[21] We visually checked the 25 top scoring poses for compound **62**, the most effective compound of substituted phenylbiguanides, and two major binding modes were identified. The binding pose that the positive charged biguanide group mimics the arginine side chain in RGD ligand (energy score, −44.66 kcal/mol) is not as favorable as that the biguanide group inserts deeply into MIDAS (energy score, −48.04 kcal/mol) (Fig. 3B). As shown in Figure 3C, in the most energetic favorable binding pose of compound **62**, the biguanide group is forming three H-bonds with the key residues Ser121, Ser123, and Glu220 in MIDAS, and two more H-bonds with Ala218 and Asn215 around MIDAS and ADMIDAS. This binding pose suggests that compound **62** might block the bivalent cation interaction

Table 2. Structures and activities of substituted phenylbiguanides



Compound	X	αvβ3-mediated cell adhesion IC_{50}[a] (μM)
62	3,5-Cl_2	33.5 ± 3.1
68	3,5-$(CF_3)_2$	39.5 ± 2.5
67	2,4-Cl_2	66.9 ± 5.3
64	2,3-Cl_2	87.3 ± 3.6
63	3-Cl, 4-F	97.3 ± 3.6
60	2,5-Cl_2	100.2 ± 8.4
53	4-SCF_3	101.4 ± 7.8
58	2-Cl, 5-CF_3	127.2 ± 7.5
61	4-Cl	148.6 ± 13.0
54	4-$SCHF_2$	178.1 ± 10.8
59	3-Cl	190.6 ± 16.0
51	2-CH3, 5-Cl	>200
55	3-SCH_3	>200
56	3-$COOCH_2CH_3$	>200
52	2-OCH_2CH_3	>200
65	2-Cl	>200
57	2-SCH_3	>200
66	H	>200

[a] The results represent the means ± SD of triplicate determinations.

with integrin αvβ3, in such a way that it inhibited the cation-mediated binding of integrin αvβ3 with its ligands, and the eventual stabilization of the agonist-bound conformation. Similarly, the alternate docking poses for other phenylbiguanide compounds were also explored, and the best scoring poses were always docked into MIDAS instead of arginine binding site (data not shown).

However, considering the limitations of docking method, the predicted binding mode between the substituted phenylbiguanides and integrin αvβ3 needs to be validated by further studies, for example, to solve the crystal structure of compound **62** with integrin receptor. Better understanding of the molecular basis of such interactions would lead to better lead optimization process.

In conclusion, here we describe the discovery of novel small molecule inhibitors against integrin αvβ3 via structure-based virtual screening. Based on the best hit, compound **37**, we identified a more effective derivative, compound **62**, with a strong inhibitory activity in integrin αvβ3-mediated cell adhesion assay (IC_{50}, 33.5 ± 3.1 μM). Furthermore, the strong interactions calculated between biguanide group and MIDAS pocket might be the structural basis for the inhibitory activity of the substituted phenylbiguanides. The results of the molecular graphics analysis indicate that the substituted phenylbiguanides might bind the receptor in a significantly different binding mode from other known inhibitors of integrin αvβ3, thus providing an alternate approach for the discovery of novel potent inhibitors. Taken together, our results suggest that the structure-based virtual screening strategy employed in this study may represent an effective approach for identifying

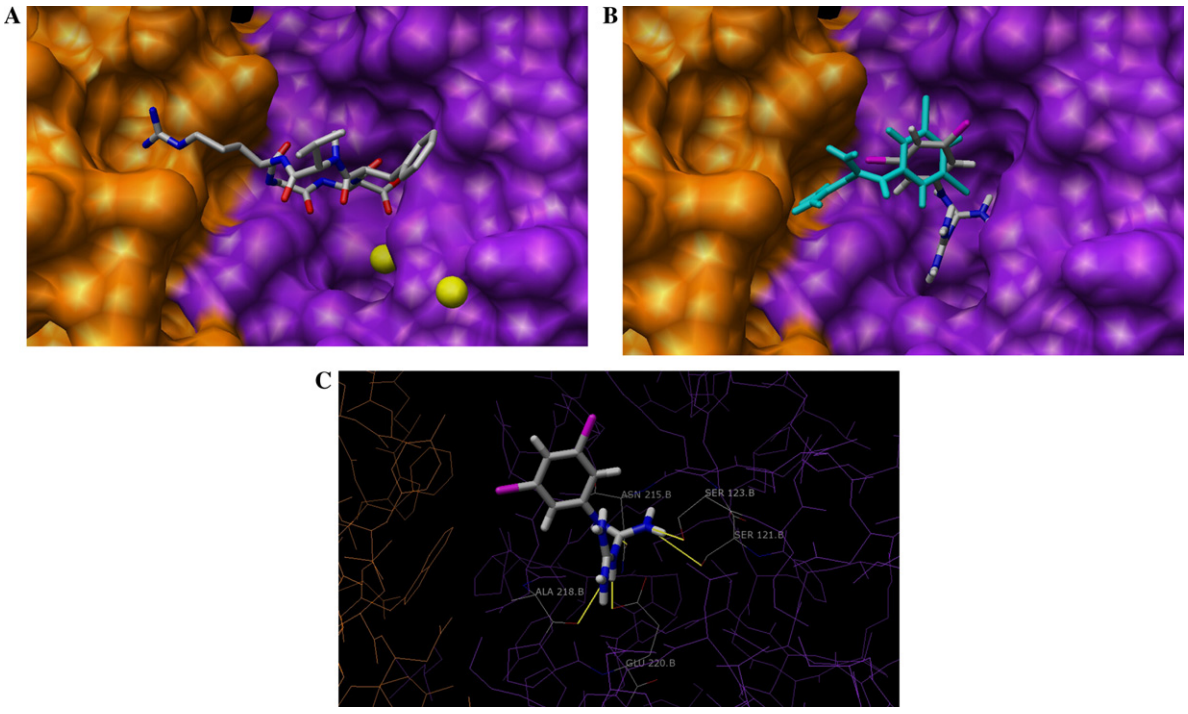


Figure 3. Binding mode analysis of compound **62**. (A) Ligand binding site of integrin αvβ3. Ligand (pentapeptide cyclo(RGDF = N{Me}V)) is colored by atom type; metal ions at ADMIDAS and MIDAS are colored in yellow. In this and subsequent figures, αv and β3 are colored in orange and purple, respectively. (B) Comparative view of the best docking pose of compound **62** (colored by atom type) where biguanide group binds with MIDAS pocket and an alternative docking pose (in green) where biguanide group is docked into arginine binding site, the energy scores of these two binding poses are −48.04 and −44.66 kcal/mol, respectively. (C) The calculated atomic interactions between the docked compound **62** and integrin αvβ3. Compound **62** is colored by atom type, and hydrogen bonds are shown in yellow lines.

novel, structurally diverse, non-peptide small molecule inhibitors targeting integrin αvβ3.

Acknowledgments

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