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Ligand Binding Analysis for Human α5β1 Integrin: Strategies for Designing New α5β1 Integrin Antagonists

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Abstract: We report a three-dimensional model of the α5β1 integrin headgroup bound to the most potent and selective ligand (SJ749) known to date. The model was built using the comparative protein modeling method, and it is consistent with experimental data. From this study, we identified two potentially important regions in the α5β1 receptor that are peculiar to this integrin and might be worth considering for drug targeting.

Integrins are ubiquitous cell adhesion receptors that bind ligands on the surface of other cells and in the extracellular matrix and are involved in bidirectional signaling across the plasma membrane, regulating cell adhesion, differentiation, migration, grow, and survival.[1] Evidence exists that such receptors are linked to pathological conditions including tumor progression, thrombosis, immune dysfunction, inflammation, and osteoporosis, so integrins have been attractive therapeutic targets for several diseases.[2,3] Integrin α5β1 and especially αv integrins have attracted attention as targets for antiangiogenic therapy.[4] Brooks et al. have reported that various low molecular weight ligands, which are recognized by αvβ3 and αvβ5 integrins, block angiogenesis in response to growth factors in tumors and suppress the cancer growth and metastasis.[5] On the basis of these findings, the αvβ3 receptor has been the main integrin target in the search for new anticancer drugs in the past 2 decades, and significant progress has been made in the identification of selective and potent αvβ3 integrin antagonists.[6] As a result of our research, the cyclic peptide cyclo(−RGDf[NMe]V−), known as cilengitide, is now in phase II clinical trials for patients with glioblastoma.[7,14]

Nonetheless, recent evidence that mice lacking of β3 or β5 integrins exhibit in some cases enhanced angiogenesis[8] led to a reevaluation of αvβ3 and αvβ5 integrin function in angiogenesis.[9] The different hypothesis made in an effort to explain the discrepancy between the genetic results and those obtained using low-molecular-weight ligands are not fully convincing, and the debate is still open. Reconsidering each integrin as a regulator of angiogenesis, an important issue came out. The α5β1 receptor is the only unambiguously proangiogenic integrin; genetic ablation experiments and pharmacological results are consistent and strongly support its importance in neovascularization processes.[10] Hence, the α5β1 integrin is expected to move into the forefront of research for new effective anticancer drugs.

The research of α5β1 antagonists is not as advanced as that of αvβ3, and only few ligands are known to bind the α5β1 integrin.[11] This constitutes a considerable obstacle to the rational ligand-based drug design. Moreover, the 3D structure of membrane spanning receptors such as α5β1 is hard to obtain through X-ray crystallography or NMR methods. Consequently, few detailed structural information about ligand−receptor interactions have been obtained until now. However, it is well-know that integrins, which are heterodimers of different combinations of noncovalently bound α and β chains, share extensive structural homology. It was also demonstrated that the ligand binding to αv, α5β1, and αIIbβ3 integrins is mediated through the Arg-Gly-Asp (RGD) recognition motif.[12,13]

A first glimpse at the binding between integrins and the RGD tripeptide sequence was provided by the crystal structure[13] of the extracellular domain of αvβ3 integrin in complex with cyclo(−RGDf[NMe]V−).[14] Experiments using X-ray scattering and single-particle electron microscopy pointed out that the overall shape, the domain organization, and the way in which the α and β subunits assemble are very similar between αvβ3 and α5β1.[13,15] Fibronectin, which is the physiological ligand of α5β1, was found to bind its receptor through the RGD-containing module 10 (Fn10) in a manner similar to cyclo(−RGDf[NMe]V−) in the αvβ3 crystal complex.[13] These findings, the high sequence similarity between α5β1 and αvβ3 receptors (αv:α5 53% identity; β3:β1 55% identity in the integrin's headgroup), and the need to get more information about the α5β1 selectivity requirement prompted us to build a 3D model of the α5β1 receptor. A multiple sequence alignment was performed utilizing evolutionary information of all α and β subunits in different organisms. The crystal structure of αvβ3 in the bound conformation was used as a template, obtaining 10 3D models of α5β1 integrin by means of comparative protein modeling methods. The models, which differ mostly in the side chain orientation, were used one at a time for ligand docking studies using the Autodock program (see Supporting Information). Compound **1** (SJ749) was chosen as ligand because of its potency, receptor selectivity, conformational restrictions, and the recently published SARs (Table 1).[16] Docking results obtained for the 10 receptor models were carefully inspected to evaluate the agreement with the experimental data (mutagenesis, cross-linking, SARs) and the convergence and the binding free energy achieved for each simulation. SJ749 was found to fit in one α5β1 model preferentially over the others. Such a complex was energetically minimized using 3000 steps of steepest descent algorithm with the CVFF force field, permitting only the ligand and the side chain atoms of the protein within a radius of 5 Å around the ligand to relax. The stereochemical quality of the resulting model was checked with the program PROCHECK. The majority of the residues occupied the most favored regions (81.7%) of the Ramachandran plot. Other residues

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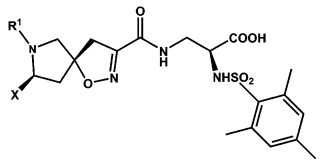
Table 1



compd	X	R^1	$\alpha5\beta1^d$ IC_{50} (nM)	$\alpha v\beta3^e$ IC_{50} (nM)	$\alpha IIb\beta3^f$ IC_{50} (nM)
1 (SJ749)	2-pyridyl $NHCH_2$[a]	Cbz	0.18	49	>100 000
2	2-imidazolyl $NHCH_2$[b]	Cbz	18	41	34 000
3	2-imidazolyl $NHCH_2$[b]	H	230	180	1400
4	2-imidazolyl $NHCH_2$[b]	CO_2Me	21	57	7300
5	2-imidazolyl $NHCH_2$[b]	CO_2nBu	14	130	11 000
6	2-imidazolyl $NHCH_2$[b]	CO_2CH_2(3-pyr)	39	32	5600
7	2-dihydroimidazolyl $NHCH_2$	Cbz	13	57	7300
8	2-dihydroimidazolyl $NHCH_2$[c]	Cbz	1200	>1000	9200
9	2-benzimidazolyl $NHCH_2$	CO_2Me	1.4	34	>100 000
10	2-benzimidazolyl $NHCH_2$	CO_2nBu	0.39	87	>100 000

[a] (*S*,*S*,*S*)-isomer. [b] Mix of diastereomers at C7 (spiro). [c] (*S*,*R*,*S*)-isomer. [d] The IC_{50} values were obtained using an ELISA assay (see ref 16). [e] The IC_{50} values were obtained using a functional $\alpha v\beta3$ antagonism assay involving adhesion of 293 b3-transfected cells to fibrinogen (see ref 16). [f] The functional assay was performed in human platelet-rich plasma (GPIIb/IIIa hPRP) (see ref 16).

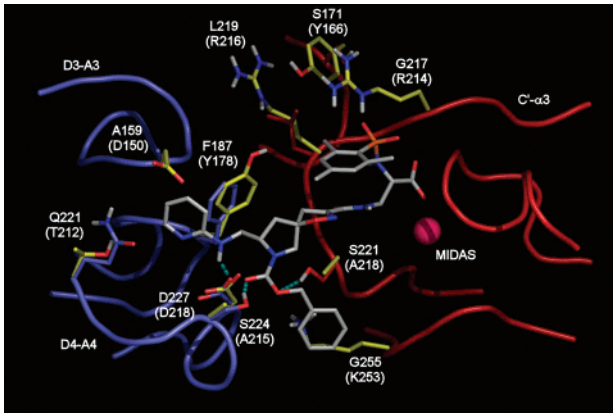


Figure 1. Three-dimensional representation of the complex between the $\alpha5\beta1$ receptor and **1**. The α subunit is represented by blue ribbon diagram, while β subunit is in red. In both subunits, important side chains are shown and are in red and blue. The metal ion in the MIDAS region is represented as a magenta sphere. The $\alpha v\beta3$ was superposed on $\alpha5\beta1$ considering all the backbone atoms, with side chains carbons in yellow. Mutated residues in the binding pocket are labeled with the one-letter amino acid code. The letters and the number in parentheses refer to the $\alpha v\beta3$ receptor.

occupied additional allowed regions (14.9%) or generously allowed regions (2.5%). Only the 0.3% were in disallowed regions. The obtained complex is shown in Figure 1. Compound **1** binds in the RGD binding site at the interface between the β-propeller of the α5 subunit (blue) and the βA domain of the $\beta1$ subunit (red). The 2-aminopyridin moiety of **1** inserts into a groove at the top of the β-propeller formed primarily by the D3−A3 and D4−A4 loops, while the carboxylate group of the ligand coordinates the metal ion at the metal-ion-dependent adhesion site (MIDAS). The contact of the 2-aminopyridin moiety with the D4−A4 loop residue (α5)-Asp227, which corresponds to ($\beta3$)-Asp218 in $\alpha v\beta3$,[17] and the coordination of the metal ion, which is stabilized by a hydrogen bond to the ($\beta1$)-Asn218 backbone, were also observed for $\alpha v\beta3/\alpha v\beta5$ dual ligands using similar techniques.[18] This is not surprising because of the high conservation of residues in the RGD binding site between the $\alpha v\beta3$, $\alpha v\beta5$, and $\alpha5\beta1$ integrins and is in accordance with mutagenesis data,[19] cross-linking studies,[20] and X-ray structure,[13] which indicate

Loop C'- α3

	210									223
β_3	Q	S	V	S	R	N	R	D	A	P	E	G	G	F
β_1	Q	R	I	S	G	N	L	D	S	P	E	G	G	F

Loop D3-A3

	142															159
α_v	C	R	S	Q	D	I	D	A	D	G	Q	G	F	C	Q	G	G	F
α_5	C	R	S	D	F	S	W	A	A	G	Q	G	Y	C	Q	G	G	F

Loop D4-A4

	210															224
α_v	T	R	T	A	Q	A	I	F	D	D	S	Y	L	G	Y	
α_5	T	R	Q	A	S	S	I	Y	D	D	S	Y	L	G	Y	

Figure 2. Sequence alignment of ligand-contacting loops. Mutated residues in the binding pocket are highlighted in gray.

the metal ion at the MIDAS and the ($\beta3$)-Asp218 of loop D4−A4 as key elements in the interaction with RGD-like ligands.

A closer look at the different activities of SJ749 analogues (**2**−**6** in Table 1) toward the $\alpha5\beta1$ and $\alpha v\beta3$ receptors indicates the importance of a carbamate function for the interaction with both integrins. Replacement of the benzyl moiety with a methyl group (compounds **2** and **4**) has no influence on binding of $\alpha5\beta1$ and $\alpha v\beta3$ integrins, thus excluding any relevant contribution of such a group to the binding. Otherwise, the absence of the carbamate function (**3**) leads to a significant decrease of activity, especially regarding the $\alpha5\beta1$ binding. In our model of **1** in complex with $\alpha5\beta1$, both carbamate oxygens are involved in receptor binding through contacts with (α5)-Ser224 and ($\beta1$)-Ser221 residues within the D4−A4 and C′−α3 loops, respectively (Figure 1). Considering the strength of the hydrogen bond acceptor groups, the carbonyl oxygen interaction is probably more significant than that of the sp^3 oxygen.[21] A comparing analysis of the $\alpha5\beta1$ and $\alpha v\beta3$ receptor sequences shows that the above-mentioned serine residues are replaced by two Ala ((αv)-Ala215 and ($\beta3$)-Ala218) in the $\alpha v\beta3$ receptor (Figures 1 and 2). Nonetheless, the carbamate function seems to play a role in $\alpha v\beta3$ ligand binding as well. Docking of **1** in the X-ray structure of $\alpha v\beta3$[13] reveals that the Cbz group could interact with the ($\beta3$)-Lys253 side chain, which is not present in the $\beta1$ subunit.

In our model the spirocyclic scaffold lies at the interface between the α and β subunits, allowing a proper orientation of the pharmacophoric groups. Ac-

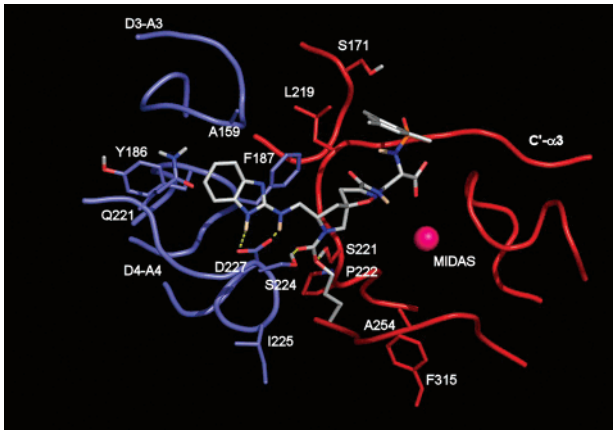


Figure 3. Binding mode of **10** within the α5β1 receptor.

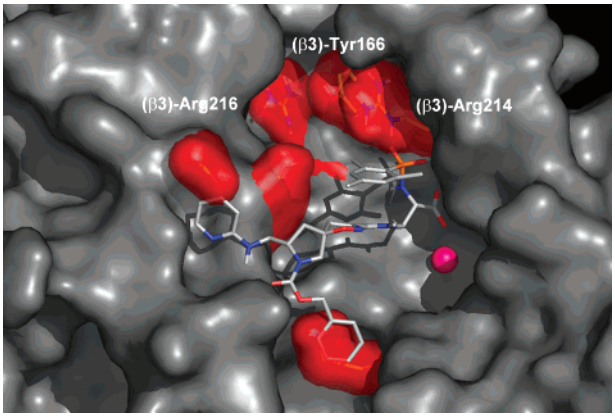


Figure 4. Superposition of α5β1 and αvβ3 receptors represented as Connolly surfaces (gray and transparent red, respectively), with **1** docked into the α5β1 receptor. The three β3 residues, whose mutation in β1 opens a new space adjacent to the RGD binding site, are in white and are visible under the red transparent αvβ3 surface as yellow sticks.

cordingly, its absolute stereochemistry strongly influences the activity toward all three integrins, which is demonstrated by **7** and **8**. Regarding the guanidine mimetic group, Smallheer et al. reported that in contrast to the αvβ3 ligands less basic groups resulted in more potent α5β1 antagonists.[16] This could be explained by a lower acidity in the D3−A3 loop of α5β1 integrin caused by replacement of three (αv)-Asp residues by a Trp, Phe, and Ala residue in the α5 subunit (see Figures 1 and 2). Of special importance might be the mutation of Asp150 to an Ala residue in the α5-subunit: the αvβ3 crystal structure clearly indicates a double-sided coordination of the basic guanidine group by two Asp residues, which is impossible in the α5 binding pocket. Moreover the (α5)-groove, in which the basic ligand group inserts, has a slightly different shape compared to that of αvβ3 because of the above-mentioned substitution of (αv)-Asp150 with an Ala in the α5-subunit and due to the replacement of (αv)-Thr212 by (α5)-Gln221 in the D4−A4 loop (Figures 1 and 2). Replacement of the basic group in **4** and **5** with a benzimidazole ring does not significantly affect the αvβ3 potency (**9** and **10**), while it improves the α5β1 receptor binding (see Table 1). Docking of **10** in the α5β1 integrin revealed the expected electrostatic interactions between the carboxylate group and the metal ion at the MIDAS region and the bidentate salt bridge to the D4−A4 loop residue (α5)-Asp227 (Figure 3). The benzimidazole ring, which was already used as guanidine mimic in other integrin antagonists,[22] was found to form a T-shaped interaction with (α5)-Phe187, which is also present in the αvβ3 integrin ((αv)-Tyr178). The proximity between the (α5)-Gln221 (Thr212 in αv) and the benzimidazole ring allows an amide−aromatic interaction between the carboxamide group of (α5)-Gln221 and the benzimidazole moiety of the ligand. It is worth noting that replacement of (αv)-Asp150 by (α5)-Ala159 increases the lipophilicity of this pocket. Consequently, the higher hydrophobicity of the benzimidazole in comparison to the imidazole moiety could be the reason for the enhanced α5β1 receptor affinity. The *n*-butyloxycarbonyl group inserts between the α5 and β1 subunits; as found for SJ749, carbamate oxygens are involved in receptor binding through contacts with (α5)-Ser224 and (β1)-Ser221 residues. The *n*-butyl chain inserts in a pocket formed mainly by residues (α5)-Ile225, (α5)-Asp228, (β1)-Phe315, (β1)-Pro222, (β1)-Gly255, (β1)-Ala254 and can be involved in weak hydrophobic interactions with the last two residues.

In the αvβ3 receptor, the α-*N*-sulfonyldiaminopropanoic acid subunit may interact with (β3)-Arg214. In the α5β1 integrin, such an interaction would not be conserved because of the substitution of (β3)-Arg214 by (β1)-Gly217 and of (β3)-Arg216 by (β1)-Leu219 (Figures 1 and 2). The superposition of the αvβ3 and α5β1 Connolly surfaces (Figure 4) shows that because of the above cited mutations, a new space opened up adjacent to the RGD binding site, which is absent in αvβ3 receptor. This new pocket is formed mainly by the C′−α3 loop residues (β1)-Gly217, (β1)-Asn218, (β1)-Leu219 and by the SDL residues (β1)-Ile170, (β1)-Ser171, (β1)-Thr189, and (β1)-Pro190. The roof of the pocket comprises the SDL highly conserved sequence NPC ((β3)-Asn175, (β3)-Pro176, (β3)-Cys177)). These findings suggest that a way to achieve αvβ3/α5β1 selectivity is the insertion of properly oriented bulky moieties in the ligand, which should occupy the α5β1 pocket and should not be tolerated by the αvβ3 receptor. The presence of a serine residue within the pocket would offer the opportunity to insert hydrogen bond acceptor groups on the bulky moieties. To our knowledge, no ligand exists so far targeting this pocket.

It is worth noting that most of the α5β1 antagonists possess two branches that mimic the Arg and the Asp residues within the RGD recognition motif and lack of appreciable selectivity.[11] This is not surprising because they are designed to mimic the RGD tripeptide sequence, which is well-known to be recognized by several integrins. On the other hand, we were able to achieve selectivity between αvβ3 and αIIbβ3 by conformational control of the RGD sequence in the ligand.[23a−c] We assumed that selectivity for αvβ3 and αIIbβ3 was caused by changing the distance between the basic and the acid moieties of the peptidomimetics. Our hypothesis has been recently confirmed by the X-ray crystal structures of αIIbβ3 in complex with different ligands.[23d] In the case of αvβ3/α5β1 selectivity, this approach was not successful, so other strategies must be explored. Within the α5β1 binding pocket other regions different from that binding Arg and Asp of the RGD sequence have to be targeted. Compounds **1** and **10** are the most α5β1-selective ligands known to date. Both of them possess an additional chain, represented by a carbamate group, that inserts between strategically located loops, each of

which bears a serine residue (Figures 1 and 2). Thus, (α5)-Ser224 and (β1)-Ser221 offer accessible attachment points for hydrogen bond acceptors. Furthermore, the presence of (α5)-Gln221 and the reduced basicity of the D3−A3 loop open new options for variation of the guanidine mimetic group. The wide pocket along the dimer border, which consists of some α5β1 peculiar amino acids, may be a special feature of such integrin, and it is worth being targeted in the effort to achieve potency and selectivity. As a result of this study, we identified two potentially important regions in the α5β1 receptor that are unique features of this integrin and might be worth considering for drug targeting.

Supporting Information Available: Experimental Section containing details concerning the molecular modeling and docking procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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