Integrin Ligands

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Probing Integrin Selectivity: Rational Design of Highly Active and Selective Ligands for the α5β1 and αvβ3 Integrin Receptor**

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Rational drug design relies on an iterative procedure of initial protein–structure determination, followed by the design, chemical synthesis, and subsequent biological evaluations of specific compounds. However, there is still a large gap between known protein sequences and 3D structures. To date, the most successful theoretical approach to bridge this gap is homology modeling. It is possible to construct an approximate 3D model of the structural unknown protein if the sequence homology to the known 3D structure of the reference protein is higher than 40%. Such a homology-modeled structure is suitable for rational drug design.[1] Herein we describe the successful use of our recently published homology model of the integrin α5β1[2] to design potent (with activities up to the subnanomolar range) and selective ligands for the two highly similar integrin receptors α5β1 and αvβ3. Structural considerations were used to trigger potency and selectivity in both directions. These ligands could allow functional studies in vivo of the role of these two integrin subtypes and might be used as lead structures for antiangiogenic cancer therapy.

Integrins constitute an important class of heterodimeric cell-adhesion receptors that are involved in many severe pathological processes, such as tumor metastasis, thrombosis, inflammation, and osteoporosis.[3] Therefore, they have been attractive therapeutic targets for several years.[4] Since Brooks et al. reported that various low-molecular-weight ligands (for example, our synthesized cyclopentapeptide *cyclo*(-Arg-Gly-Asp-D-Phe-Val-) = *c*(-RGDfV-)[5a]), which are recognized by the αvβ3 and αvβ5 integrins, block angiogenesis in response to growth factors in tumors,[5b] many selective αvβ3-ligands have been developed and some compounds have reached clinical trials.[7] As a result of our research, the cyclic *N*-methylated pentapeptide c(-RGDf[*N*Me]V-),[5b] known as cilengitide, has entered phase II trials for patients with glioblastoma.

Recent knock-out experiments showed, however, that genetically altered mice (lacking the αv integrin) show extensive angiogenesis in some cases, whereas other mice (lacking the β3 or β5 integrins) show no significant effects, and as such, the idea that these two integrins are proangiogenic was seriously questioned.[8,9] On the other hand, the proangiogenic function of the α5β1 receptor has been clearly demonstrated[10,11] so that the α5β1 integrin moved into the focus of research. Although crystal structures of the extracellular domains of the αvβ3 and αIIbβ3 integrins have been solved and provided a deep insight into the ligand binding,[12,13] very little detailed structural information about the α5β1 receptor itself or about ligand–receptor interactions have been obtained until now.[14]

Furthermore, there are only a few small-molecule ligands known to bind α5β1,[15,16] which prompted us to focus our research on this integrin subtype. A first hint for the design of new α5β1 ligands came from our homology model of the α5β1 integrin in complex with a recently reported ligand (SJ749).[2,16] The high sequence similarity between the αvβ3 and α5β1 receptors (αv:α5, 53% identity; β3:β1, 55% identity in the integrin's headgroup) makes it possible to test our hypothetical model by synthesizing a series of rationally designed compounds. Like other ligands targeting the RGD-binding site of integrins, our compounds possess a free a carboxylate function as well as a basic moiety at a distance of about 13 Å. There are two "hot spots" in the binding pockets in which mutations suitable for achieving selectivity between the α5β1 and αvβ3 integrin can be found: in the β subunit, (β3)-Arg214 is replaced by (β1)-Gly217 and additionally (β3)-Arg216 is mutated into (β1)-Leu219 (Figure 1 highlights the important mutations). The substitution of both arginine residues expands this site of the α5β1 binding pocket, which, in comparison with the αvβ3 integrin, allows the introduction of bulky moieties into the ligand's core structure. Secondly, the α5 subunit turned out to be less acidic owing to the mutation of (αv)-Asp150 to (α5)-Ala159. Furthermore, the replacement of (αv)-Thr212 by (α5)-Gln221 results in a different shape of this binding region, which offers the opportunity to gain selectivity by modification of the basic moieties.

Guided by these observations, we synthesized a series of compounds based on a tyrosine scaffold that has already been successfully employed in the integrin field.[17] Putative binding modes were determined by using the AutoDock program (for

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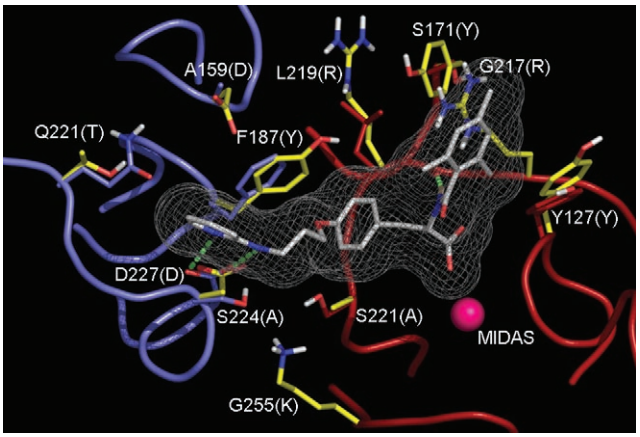


Figure 1. Ribbon representation of the α5β1 integrin binding pocket (α5 blue, β1 red) with the predicted binding pose of **3b** (gray). The side chains of important residues are highlighted and the corresponding residues of the αvβ3 integrin are shown in yellow and labeled in parentheses. The MIDAS metal is represented as a magenta sphere.

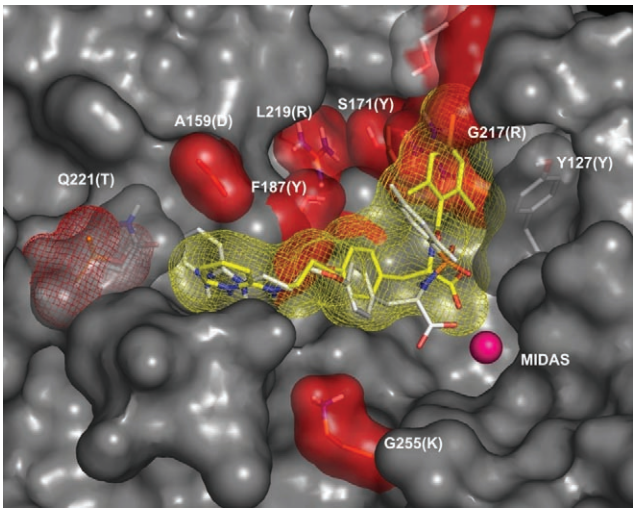


Figure 2. Superposition of the α5β1 (gray) and αvβ3 (transparent red) receptors represented as Connolly surfaces. Compounds **3b** and **3c** were docked in the α5β1 integrin. The mesitylene function of the α5β1-selective compound **3b** would clash with (β3)-Arg 214, which is not present in the α5β1 receptor. Compound **3c** shows no selectivity because of the different position of its bulky mesitylene function.

computational details see the Supporting Information)[18] whose reliability at predicting ligand–receptor complexes has been demonstrated in prior studies on the αvβ3 integrin.[19]

Figure 1 shows compound **3b** in the α5β1 binding pocket together with the mutated residues of αvβ3 (yellow). The carboxylate group of **3b** coordinates the metal (Ca^{2+} or Mn^{2+}) in the MIDAS (metal-ion-dependent adhesion site) region, which resides in the β1 subunit, while the basic amino pyridine inserts into a narrow groove at the top of the (α5)-β-propeller domain, forming hydrogen bonds to the highly conserved (α5)-Asp 227. The tyrosine scaffold enables π–π interaction with (α5)-Phe 187 while the mesitylene function interacts with (β1)-Tyr 127 in the same manner. The α5β1 selectivity of **3b** is determined by the bulky mesitylene group, which cannot be placed at this position in the αvβ3 pocket because of a steric clash with (β3)-Arg 214. The conformational difference between the sulfonamide (substituents are 90° twisted about the SO_2–N bond) in comparison with the planar amide bond causes the mesitylene group in compound **3c** to fold back towards the α subunit (see Figure 2). This position is allowed for both integrins and, hence, the selectivity for α5β1 is lost, although **3c** still shows nanomolar affinity for both integrins.

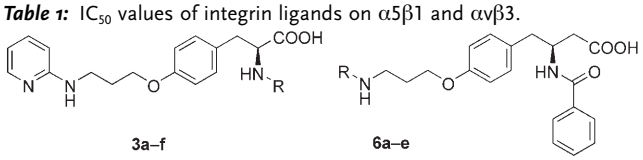
It could be argued that there is no need to put the bulky mesitylene group exactly in this position. Docking calculations show alternative binding modes in which the hydrophobic residue sticks out of the integrin. This, however, would expose the mesitylene to the surrounding water and thus result in a decreased affinity. Attachment of a hydrophobic moiety adjacent to the carboxylate function is of particular importance for integrin binding, which has been demonstrated in prior work.[20] In the case of **3b**, the mesitylene moiety enables a π–π interaction with (β1)-Tyr 127 and forms a hydrogen bond with the backbone carbonyl group of (β1)-Asn 218.

The *ortho* substitution pattern of the attached aromatic residue seems to be important for affinity as well as for selectivity (**3b**, **3d**, and **3f**; Table 1). The former could be explained by an increasing lipophilicity, the latter by a restricted flexibility of the aromatic ring. Among all of the synthesized α-amino acids, **3f** exhibits the best affinity for α5β1 ($IC_{50} = 0.7$ nM) and good selectivity against αvβ3 (≈300-fold). The *para*-isopropyloxy group is placed well for further interaction with the (β1)-SDL (specificity-determining loop), presenting an additional hydrogen-bond acceptor to the serine residue (β1)-Ser 171.

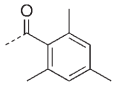
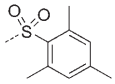
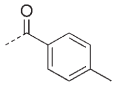
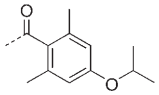
In addition to the α-tyrosine ligands, we synthesized a compound series based on a β-amino acid scaffold. Compound **6e** shows moderate affinity towards α5β1, but high affinity towards the αvβ3 receptor. Considering that various known selective αvβ3 ligands are substituted in some way on the β-position to the carboxylate,[20,21] it is not surprising that this substitution pattern shows high affinity for the αvβ3 integrin and only average affinity for α5β1. In general, all synthesized β-amino acid derivatives exhibit lower α5β1 affinity than the corresponding α-amino acids. As the bulky side chain is more flexible when attached at the β-position, this selectivity might not be caused (at least not only) by steric interaction with (β3)-Arg 214 or adjacent residues. A closer look at the α5 subunit reveals two major differences when compared with the αv subunit (see Figure 1): first, the (α5)-Ala 159 is replaced by the (αv)-Asp 150, which favors an extra hydrogen donor on the basic group opposite to the one interacting with (αv)-Asp 218. Secondly, the mutation of (αv)-Thr 212 to (α5)-Gln 221, which shortens this region of the binding pocket, causes the slightly longer β-amino acids to better fit into the αvβ3 binding site.

To clearly demonstrate that the smaller α5 binding pocket can be used to gain αvβ3 selectivity, we attached a methyl group to all possible positions of the 2-aminopyridine ring (Table 1). As expected, the 4-methylaminopyridine in **6c**

Table 1: IC_{50} values of integrin ligands on α5β1 and αvβ3.



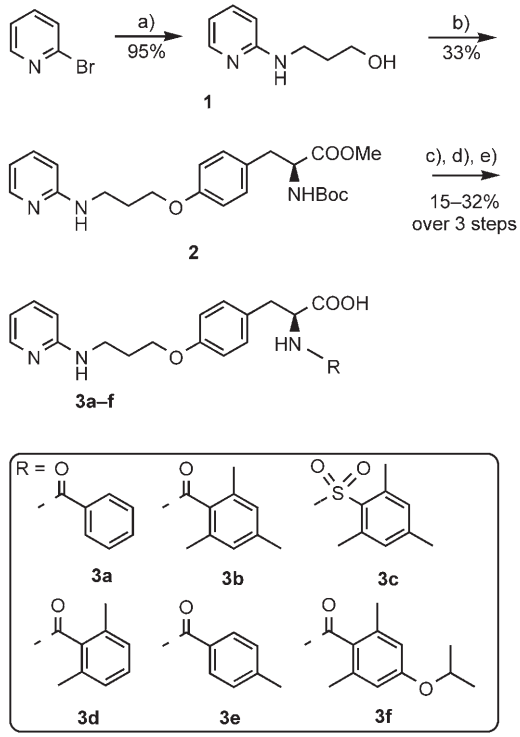
3a–f 6a–e

Entry	Compound	R	IC_{50} [nM][a] α5β1	IC_{50} [nM][a] αvβ3
1	**3a**		243	190
2	**3b**		2.5	703
3	**3c**		46	3.4
4	**3d**		3.1	1624
5	**3e**		416	318
6	**3f**		0.7	279
7	**6a**		3946	13
8	**6b**		215	2.5
9	**6c**		67	0.9
10	**6d**		6969	245
11	**6e**		264	1.2

[a] IC_{50} values are derived from a competitive ELISA test by using the immobilized natural integrin ligands fibronectin and vitronectin and the soluble integrins α5β1 and αvβ3, respectively (for details, see the Supporting Information).

shows no influence on selectivity because the methyl group sticks out of the binding pocket. In contrast, the methyl group at position six of the pyridine ring (**6a**) has a massive impact on α5β1 binding affinity. Compound **6d** shows decreased affinity to both the α5β1 and the αvβ3 integrin. Intramolecular steric hindrance causes the pyridine ring to twist out of the plane, which hampers the formation of a bidentate salt bridge to the conserved (α5)-Asp227 (or Asp218 in αv). Owing to the high sequence identity between αvβ3 and αvβ5 (β3:β5, 65% identity in the integrin's headgroup),[22] we assume that the described modifications might have similar effects on binding the αvβ5 integrin. Compound **6a** is a potent ligand for αvβ3 and has considerably lower activity towards α5β1. Compounds **3f** and **6a** offer the opportunity to investigate the role of both integrins, α5β1 and αvβ3, in biological processes. As expected, both compounds exhibit low binding affinity ($IC_{50} > 10$ μM for **3e** and ≈1 μM for **6a**) towards the platelet integrin αIIbβ3, which is crucial for developing leads for antiangiogenic cancer therapy.

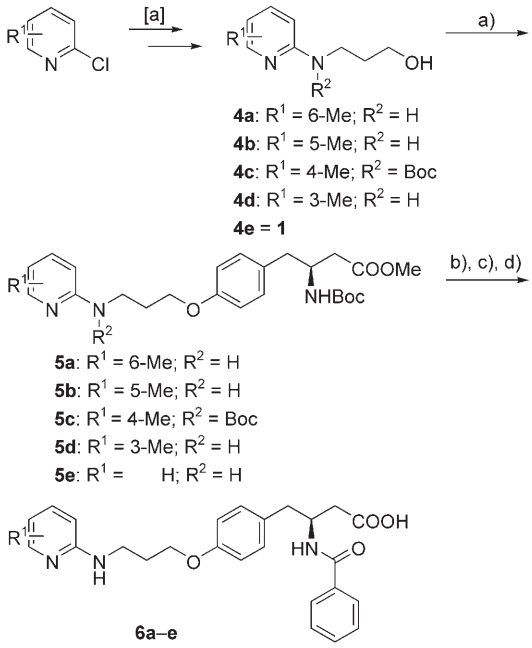
The small compound library (Table 1) was produced by a Mitsunobu reaction of protected of α- or β-tyrosine esters with several basic aminopyridinyl alcohols. The aminopyridinyl alcohol **1** was synthesized by nucleophilic substitution of the commercially available 2-bromopyridine in neat 3-aminopropanol at 150°C in 95% yield (Scheme 1).[23] The



Scheme 1. Synthesis of ligands **3a–f**: a) 3-aminopropanol, 150°C, 12 h; b) *N*-Boc-tyrosine methyl ester, PBu_3, ADDP, THF, 0°C, 8 h; c) aqueous HCl, dioxane, 1 h; d) benzoyl chloride, $NaHCO_3$, dioxane, water, 0.5 h (**a**); aromatic acid, HATU, DIPEA, DMF, 3 h (**b**,**d**,**e**,**f**) or mesitylenesulfonyl chloride, DIPEA, DMF, 8 h (**b**); e) LiOH, methanol, H_2O, HPLC purification. ADDP = azodicarboxylic dipiperidide, Boc = *tert*-butoxycarbonyl, DIPEA = *N*,*N*-diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate.

methyl substituted aminopyridinyl alcohols **4a–d** were synthesized from the corresponding 2-chloromethylpyridines by oxidation to the pyridine-*N*-oxide with *meta*-chloroperbenzoic acid (MCPBA; 78–89%),[24] nucleophilic substitution (>95%), and reduction under hydrogen atmosphere with Pd on carbon (60–82%) (Scheme 2). The aminopyridyl alcohols were coupled to the corresponding *N*-Boc-protected α- or β-tyrosine methyl ester through a Mitsunobu reaction with tributyl phosphine and azodicarboxylic dipiperidid (ADDP) to give the tyrosine ethers **2** and **5a–e** in poor to moderate yields (15–68%).[25]

The yield of the Mitsunobu reaction could be increased by employing an *N*-Boc-protected aminopyridyl alcohol; **5c** could actually only be prepared through this method. The fully protected ligand precursors were Boc-deprotected with



Scheme 2. Synthesis of ligands **6a–e**: a) **4a–e**, N-Boc-β-tyrosine methyl ester, PBu_3, ADDP, THF, 0°C, 8 h; b) aqueous HCl, dioxane, 1 h; c) PhCOCl, dioxane, H_2O, $NaHCO_3$; d) LiOH, methanol, H_2O. [a] Yield: **4a**,**b**,**d**: 66–48% over three steps; **4c**: 30% over five steps.

aqueous HCl in dioxane, acylated with an activated aromatic acid (HATU, DIPEA in DMF) in the case of the ligands **3b**,**d**,**e**,**f** or acylated with benzoyl chloride and $NaHCO_3$ in dioxane/water in the case of **6a–e** and **3a**. The sulfonamide **3c** was produced with mesitylenesulfonyl chloride and DIPEA in DMF. In the last step, the methyl ester was finally cleaved with 5 equivalents of LiOH in methanol/water and the resulting ligands purified by using reverse-phase HPLC techniques (for details regarding synthesis and compound characterization see the Supporting Information).

Taking into account the re-evaluated role of the α5β1 integrin in the development of antiangiogenic drugs for cancer therapy, we herein present the small non-peptidic molecule **3f**, which selectively binds to the α5β1 integrin in the subnanomolar range ($IC_{50} = 0.7$ nM). Minor modifications of the compounds allow the design of highly active ligands with good selectivity for αvβ3 over the α5β1 receptor, whereas very low affinity towards the platelet integrin aIIbb3 has been observed. On the basis of α5β1 homology modeling, analysis of the ligand binding mode, and extensive data on structure–activity relationships, we proposed a model suitable for the rational design of selective α5β1 ligands for the purpose of lead generation and biochemical studies on integrin selectivity.

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