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Probing Integrin Selectivity: Rational Design of Highly Active and Selective Ligands for the $\alpha 5\beta 1$ and $\alpha v\beta 3$ Integrin Receptor**

Dominik Heckmann, Axel Meyer, Luciana Marinelli, Grit Zahn, Roland Stragies, and Horst Kessler*

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 $\alpha 5\beta 1$ ^[2] to design potent (with activities up to the subnanomolar range) and selective ligands for the two highly similar integrin receptors $\alpha 5\beta 1$ and $\alpha v\beta 3$. 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 $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins, block angiogenesis in response to growth factors in tumors,^[5b] many selective $\alpha v\beta 3$ -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[NMe]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 $\beta 3$ or $\beta 5$ 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 $\alpha 5\beta 1$ receptor has been clearly demonstrated^[10,11] so that the $\alpha 5\beta 1$ integrin moved into the focus of research. Although crystal structures of the extracellular domains of the $\alpha v\beta 3$ and $\alpha IIb\beta 3$ integrins have been solved and provided a deep insight into the ligand binding,^[12,13] very little detailed structural information about the $\alpha 5\beta 1$ 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 $\alpha 5\beta 1$,^[15,16] which prompted us to focus our research on this integrin subtype. A first hint for the design of new $\alpha 5\beta 1$ ligands came from our homology model of the $\alpha 5\beta 1$ integrin in complex with a recently reported ligand (SJ749).^[2,16] The high sequence similarity between the $\alpha v\beta 3$ and $\alpha 5\beta 1$ receptors (αv : $\alpha 5$, 53% identity; $\beta 3$: $\beta 1$, 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 $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrin can be found: in the β subunit, ($\beta 3$)-Arg 214 is replaced by ($\beta 1$)-Gly 217 and additionally ($\beta 3$)-Arg 216 is mutated into ($\beta 1$)-Leu 219 (Figure 1 highlights the important mutations). The substitution of both arginine residues expands this site of the $\alpha 5\beta 1$ binding pocket, which, in comparison with the $\alpha v\beta 3$ integrin, allows the introduction of bulky moieties into the ligand's core structure. Secondly, the $\alpha 5$ subunit turned out to be less acidic owing to the mutation of (αv)-Asp 150 to ($\alpha 5$)-Ala 159. Furthermore, the replacement of (αv)-Thr 212 by ($\alpha 5$)-Gln 221 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

[*] Dipl.-Chem. D. Heckmann, Dr. A. Meyer, Prof. Dr. H. Kessler
Department Chemie
TU München
Lichtenbergstrasse 4, 85747 Garching (Germany)
Fax: (+49) 89-2891-3210
E-mail: kessler@ch.tum.de

Dr. L. Marinelli
Dipartimento di Chimica Farmaceutica e Tossicologica
Università di Napoli “Federico II”
Via D. Montesano, 49-80131 Napoli (Italy)

Dr. G. Zahn, Dr. R. Stragies
Jerini AG
Invalidenstrasse 130, 10115 Berlin (Germany)

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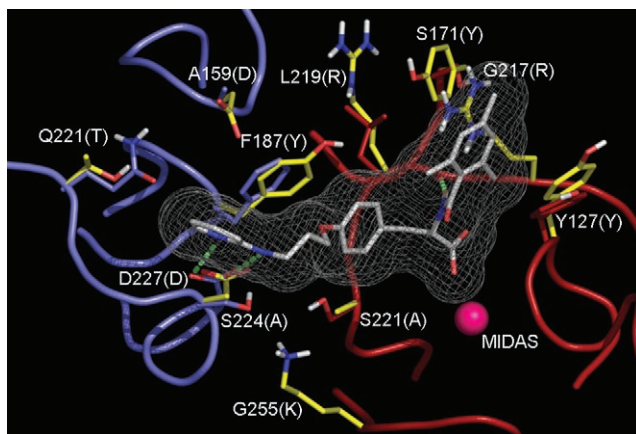


Figure 1. Ribbon representation of the $\alpha 5\beta 1$ integrin binding pocket ($\alpha 5$ blue, $\beta 1$ red) with the predicted binding pose of **3b** (gray). The side chains of important residues are highlighted and the corresponding residues of the $\alpha v\beta 3$ integrin are shown in yellow and labeled in parentheses. The MIDAS metal is represented as a magenta sphere.

computational details see the Supporting Information)^[18] whose reliability at predicting ligand–receptor complexes has been demonstrated in prior studies on the $\alpha v\beta 3$ integrin.^[19]

Figure 1 shows compound **3b** in the $\alpha 5\beta 1$ binding pocket together with the mutated residues of $\alpha v\beta 3$ (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 $\beta 1$ subunit, while the basic amino pyridine inserts into a narrow groove at the top of the ($\alpha 5$)- β -propeller domain, forming hydrogen bonds to the highly conserved ($\alpha 5$)-Asp227. The tyrosine scaffold enables π – π interaction with ($\alpha 5$)-Phe187 while the mesitylene function interacts with ($\beta 1$)-Tyr127 in the same manner. The $\alpha 5\beta 1$ selectivity of **3b** is determined by the bulky mesitylene group, which cannot be placed at this position in the $\alpha v\beta 3$ pocket because of a steric clash with ($\beta 3$)-Arg214. 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 $\alpha 5\beta 1$ 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 ($\beta 1$)-Tyr127 and forms a hydrogen bond with the backbone carbonyl group of ($\beta 1$)-Asn218.

The *ortho* substitution pattern of the attached aromatic residue seems to be important for affinity as well as for

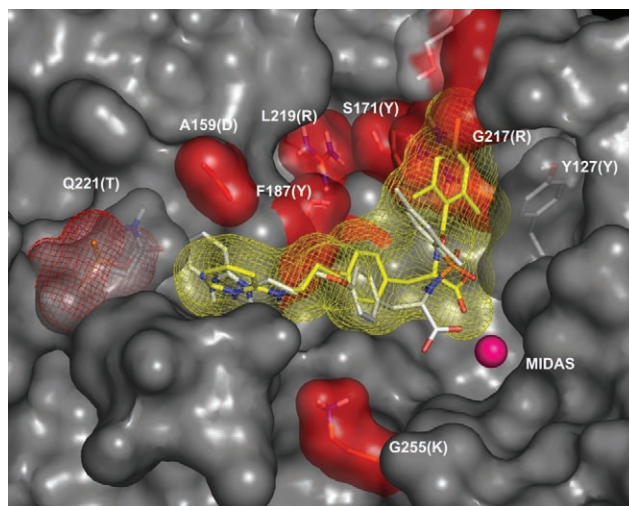


Figure 2. Superposition of the $\alpha 5\beta 1$ (gray) and $\alpha v\beta 3$ (transparent red) receptors represented as Connolly surfaces. Compounds **3b** and **3c** were docked in the $\alpha 5\beta 1$ integrin. The mesitylene function of the $\alpha 5\beta 1$ -selective compound **3b** would clash with ($\beta 3$)-Arg214, which is not present in the $\alpha 5\beta 1$ receptor. Compound **3c** shows no selectivity because of the different position of its bulky mesitylene function.

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 $\alpha 5\beta 1$ ($\text{IC}_{50} = 0.7 \text{ nM}$) and good selectivity against $\alpha v\beta 3$ (≈ 300 -fold). The *para*-isopropoxy group is placed well for further interaction with the ($\beta 1$)-SDL (specificity-determining loop), presenting an additional hydrogen-bond acceptor to the serine residue ($\beta 1$)-Ser171.

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 $\alpha 5\beta 1$, but high affinity towards the $\alpha v\beta 3$ receptor. Considering that various known selective $\alpha v\beta 3$ 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 $\alpha v\beta 3$ integrin and only average affinity for $\alpha 5\beta 1$. In general, all synthesized β -amino acid derivatives exhibit lower $\alpha 5\beta 1$ 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 ($\beta 3$)-Arg214 or adjacent residues. A closer look at the $\alpha 5$ subunit reveals two major differences when compared with the αv subunit (see Figure 1): first, the ($\alpha 5$)-Ala159 is replaced by the (αv)-Asp150, which favors an extra hydrogen donor on the basic group opposite to the one interacting with (αv)-Asp218. Secondly, the mutation of (αv)-Thr212 to ($\alpha 5$)-Gln221, which shortens this region of the binding pocket, causes the slightly longer β -amino acids to better fit into the $\alpha v\beta 3$ binding site.

To clearly demonstrate that the smaller $\alpha 5$ binding pocket can be used to gain $\alpha v\beta 3$ 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: IC₅₀ values of integrin ligands on α5β1 and αvβ3.

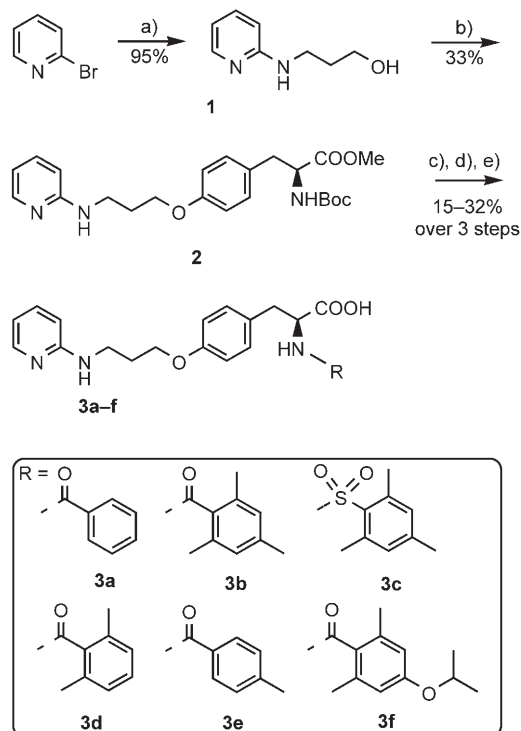
Entry	Compound	R	IC ₅₀ [nM] ^[a]	
			α5β1	αvβ3
1	3a		243	190
2	3b		2.5	703
3	3c		46	3.4
4	3d		3.1	1624
5	3e		416	318
6	3f		0.7	279
7	6a		3946	13
8	6b		215	2.5
9	6c		67	0.9
10	6d		6969	245
11	6e		264	1.2

[a] IC₅₀ 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. Intra-molecular 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 (IC₅₀ > 10 μM for **3e** and ≈ 1 μM for **6a**)

towards the platelet integrin αIIbβ3, which is crucial for developing leads for antiangiogenic cancer therapy.

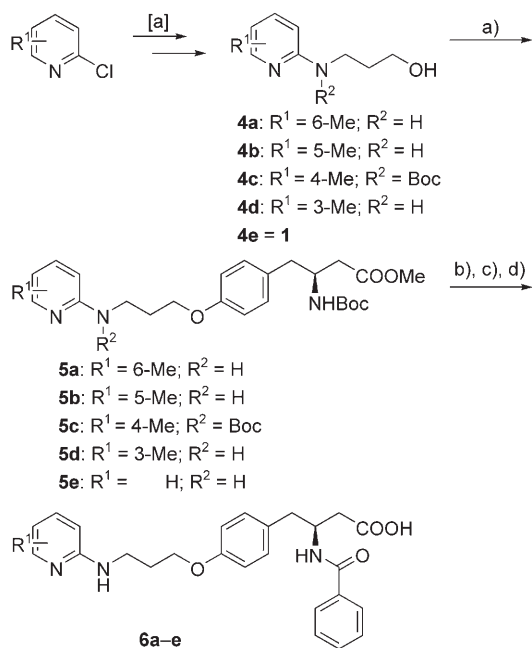
The small compound library (Table 1) was produced by a Mitsunobu reaction of protected α- or β-tyrosine esters with several basic aminopyridinyl alcohols. The aminopyridinyl alcohol **1** was synthesized by nucleophilic substitution of the commercially available 2-bromopyridine in neat 3-aminopropanol 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, PBu₃, ADDP, THF, 0 °C, 8 h; c) aqueous HCl, dioxane, 1 h; d) benzoyl chloride, NaHCO₃, 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, H₂O, HPLC purification. ADDP = azodicarboxylic dipiperidine, Boc = *tert*-butoxycarbonyl, DIPEA = *N,N*-diisopropylethylamine, DMF = *N,N*-dimethylformamide, HATU = *O*-(7-azabenzotriazol-1-yl)-*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 dipiperidide (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 aminopyridinyl 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 , ADPP, 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 NaHCO₃ 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 $\alpha 5 \beta 1$ integrin in the development of antiangiogenic drugs for cancer therapy, we herein present the small non-peptidic molecule **3f**, which selectively binds to the $\alpha 5 \beta 1$ 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 $\alpha v \beta 3$ over the $\alpha 5 \beta 1$ receptor, whereas very low affinity towards the platelet integrin $\alpha IIb \beta 3$ has been observed. On the basis of $\alpha 5 \beta 1$ 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 $\alpha 5 \beta 1$ ligands for the purpose of lead generation and biochemical studies on integrin selectivity.

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