DOI: 10.1002/cbic.200800045

Rational Design of Highly Active and Selective Ligands for the α5β1 Integrin Receptor

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The inhibition of integrin function is a major challenge in medicinal chemistry. Potent ligands are currently in different stages of clinical trials for the antiangiogenic therapy of cancer and age-related macula degeneration (AMD). The subtype α5β1 has recently been drawn into the focus of research because of its genuine role in angiogenesis. In our previous work we could demonstrate that the lack of structural information about the receptor could be overcome by a homology model based on the X-ray structure of the αvβ3 integrin subtype and the sequence similarities between both receptors. In this work, we describe the rational design and synthesis of high affinity α5β1 binders, and the optimisation of selectivity against αvβ3 by means of extensive SAR studies and docking experiments. A first series of compounds based on the tyrosine scaffold resulted in affinities in the low and even subnanomolar range and selectivities of 400-fold against αvβ3. The insights about the structure–activity relationship gained from tyrosine-based ligands could be successfully transferred to ligands that bear an aza-glycine scaffold to yield α5β1 ligands with affinities of ~1 nm and selectivities that exceed 10^4-fold. The ligands have already been successfully employed as selective α5β1 ligands in biological research and might serve as lead structures for antiangiogenic cancer therapy.

Introduction

Integrins constitute a family of heterodimeric, transmembrane cell-adhesion receptors, which connect cells to the scaffolding proteins of the extracellular matrix.[1–3] The pioneering observations that αvβ3, αvβ5 and α5β1 integrin subtypes are essential for tumour angiogenesis and can be successfully inhibited by small-molecule ligands has turned them into attractive targets for pharmaceutical research.[4–9] Up to now, a large number of peptidic[10–14] and nonpeptidic[15–25] ligands have been developed, which are all related to the common recognition motif RGD (Arg-Gly-Asp).[26] The cyclic, *N*-methylated peptide *cyclo*(-RGDf*N*MeVal-), known as Cilengitide,[14] has recently entered phase III clinical investigation for patients with glioblastoma multiforme. However, observations that mice that lack αv integrins show extensive angiogenesis, whereas mice that lack β3 or β5 integrins show no significant effects seriously challenged the status of αvβ3 and αvβ5 as proangiogenic integrins.[27–29] In contrast, its unambiguously proangiogenic role drew α5β1[30,31] into the focus of research and led to an increasing demand for selective α5β1 ligands. However, the design of α5β1 ligands is hampered by the lack of structural information about the receptor. The X-ray structure of αvβ3[32,33] in complex with Cilengitide and the high sequence similarity (>50%) between αvβ3 and α5β1 encouraged us to create a homology model of α5β1[34] and to use this model as a platform for rational design.[22] First results with ligands based on the tyrosine scaffold proved that the structural information provided by the homology model was accurate enough to design highly active α5β1 ligands with 300-fold selectivity against αvβ3 as well as ligands with reverse selectivity.[24,25] Herein, we describe the development of α5β1 ligands and the induction of selectivity by means of extensive structure–activity relationship (SAR) studies and docking experiments based on our homology model. Furthermore, the structure–activity information provided by the tyrosine-based compound library was used to transform aza-glycine-based ligands, which were initially developed in our group as αvβ3 binders,[21–23] into α5β1 ligands with selectivities that exceed 6000-fold. These ligands, with activities in the low nanomolar or subnanomolar range are by now among the most α5β1 selective compounds known and could serve as lead structures for antiangiogenic therapy of cancer and age-related macular degeneration (AMD).[35,36]

Ligand design

The tyrosine scaffold has already been successfully employed to design ligands for the platelet receptor αIIbβ3 (Tirofiban®),[37,38] and was chosen because of its easy accessibility and high variability. The essential functionalities of ligands that target the RGD-binding site are also present in our ligands: the carboxylate of the tyrosine scaffold acts as mimetic for the aspartate in RGD, whereas tyrosine provides the scaffold to maintain the orientation of the ligand's basic moiety in the proper

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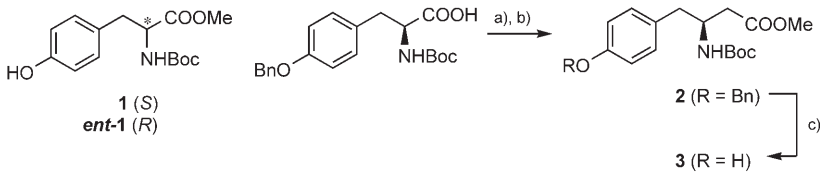
distance of ~13 Å to the carboxylate. Guanidinium groups and basic heterocycles are common arginine mimetics and thus widely used in the integrin field.[18,39] Additionally, hydrophobic moieties, especially aromatic systems, in the vicinity of the carboxyl group are known to enhance binding affinity in αv integrins. Figure 1 shows the binding mode of our ligands **34e** and **34g** docked into the α5β1 homology model and the most important mutations that discriminate αvβ3 from α5β1: the carboxylate function of the ligand coordinates a bivalent metal cation located at the MIDAS (metal-ion dependent adhesion site) whereas the aminopyridine group is engaged in a bidentate salt bridge with the highly conserved (α5)Asp227. Mutation of (αv)Asp150 to (α5)Ala159 decreases the acidity of the α5 subunit, whereas mutation of (αv)Thr212 to (α5)Gln221 slightly shortens the α5β1 binding site with respect to αvβ3. In the β subunit, (β3)Arg214 and (β3)Arg216 are mutated in β1 to the smaller residues Gly and Leu, respectively, where they open up a hydrophobic cleft that can be addressed by bulky aromatic residues. A library of tyrosine-based compounds was synthesised to probe the effects of aromatic substitution, ligand length and different basic moieties on α5β1 affinity and selectivity against αvβ3. Furthermore, the 2-carboxy-7-hydroxy-1,2,3,4-tetrahydroisochinoline was evaluated as a representative of a class of constrained tyrosine ligands.

Ligand synthesis

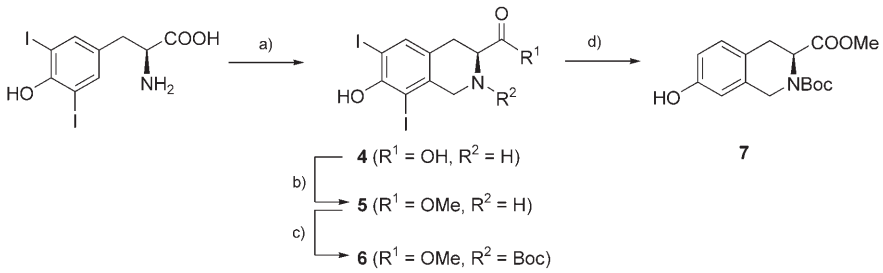
Synthesis of tyrosine-based ligands: The major building block of tyrosine-based ligands was the commercially available Boc-protected tyrosine methyl ester. The homologue β-amino acid was prepared from Boc-Tyr(Bn)-OH by Arndt–Eistert homologisation (Scheme 1).[40]

The tetrahydroisochinoline building block **7** was synthesised from the commercially available 2,5-diiodotyrosine by a Pictet–Spengler reaction according to the literature procedure (Scheme 2).[41]

To enable a Mitsunobu-type alkylation of the tyrosine, the basic moiety has to be synthesised as an aminoalcohol; this was achieved by nucleophilic aromatic substitution of the corresponding 2-bromopyridine, 2-chloropyridazine or chloropyrimidine (Scheme 3).[42] To enhance the yields of the Mitsunobu reaction, the aminopyridines were further *N*-Boc protected. As first attempts to selectively protect the nitrogen failed because of its reduced nucleophilicity, the alcohol function was first TBDPS protected.[43] Boc protection was achieved by using Boc-anhydride triethylamine with catalytic amounts of DMAP in DCM.[44] Desilylation with TBAF gave the pure pyridinylaminoalcohols in good yields.



Scheme 1. Different building blocks used as scaffold. a) 1. ethyl chloroformiate, *N*-methylmorpholine, THF, −30 °C; 2. CH_2N_2, Et_2O, −30 °C–room temperature, 99%; b) Ag^+PhCOO^-, MeOH, 90%; c) Pd/C, 1 atm H_2, MeOH, 71%.



Scheme 2. Constrained tyrosine derivative used as a scaffold. a) HCHO, $HCl_{(aq)}$/DME, reflux, 50%; b) $SOCl_2$, MeOH, room temperature, 70%, c) Boc_2O, TEA, THF, 94%; d) Pd/C, 1 atm H_2, room temperature, 85%.

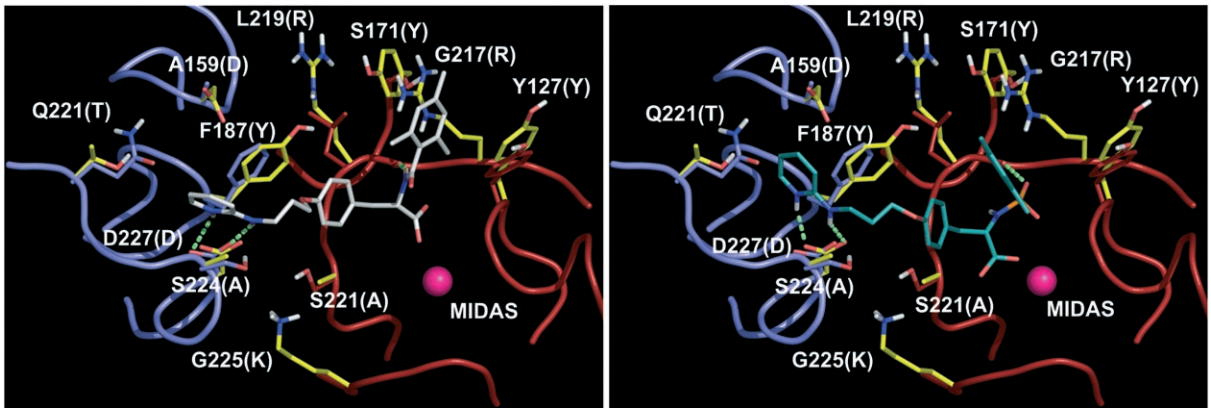
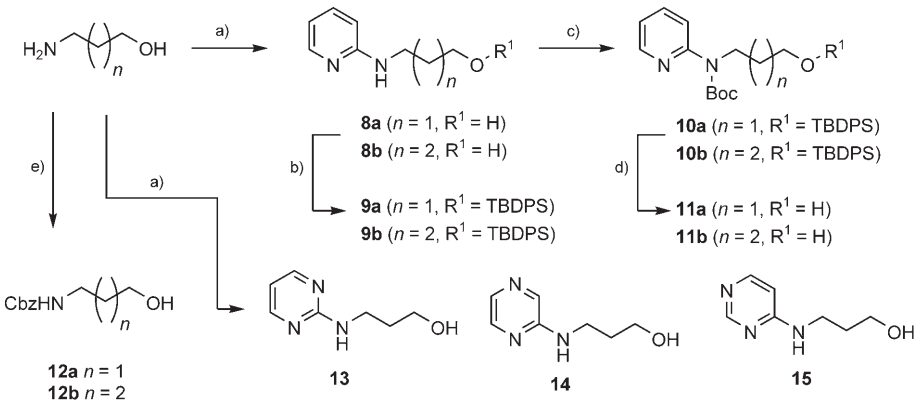
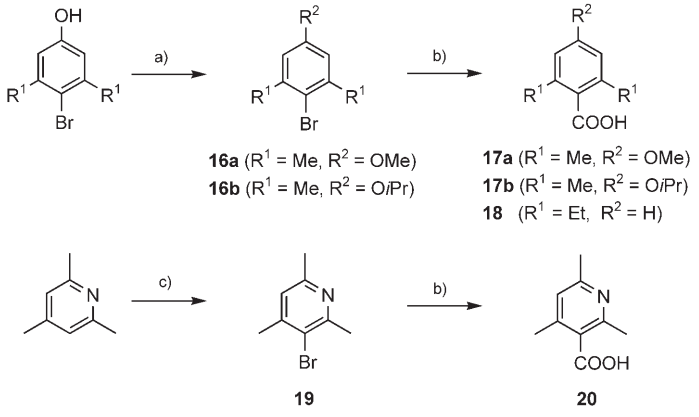


Figure 1. Comparison of the binding modes of ligand **34e** (left) and **34g** (right). Integrin α5β1 is shown as a ribbon drawing with the α5 subunit in blue and the β1 subunit in red. The MIDAS metal ion is represented as a magenta sphere and H bonds are shown as dotted lines. Relevant receptor side chains are highlighted and the corresponding residues in αvβ3 are shown in yellow and labelled in parentheses.



Scheme 3. Synthesis of various aminoalcohols as building blocks for the basic moiety of integrin ligands. a) Heteroaryl chloride/bromide, neat aminoalcohol, 150 °C (71–99%); b) TBDPSCl, imidazole, DCM (66–88%); c) Boc_2O, TEA, DMAP, THF (77–90%); d) TBAF, THF (63–64%); e) Cbz-OSu, THF, TEA (76–88%).

The aromatic carboxylic acids—those not commercially available—were synthesised by bromine–lithium exchange and trapping of the organolithium species with carbon dioxide (Scheme 4).[45] For the synthesis the alkoxybenzoic acids (**17**), 4-bromo-3,5-dimethylphenole was alkylated either with dimethylsulfate or isopropylbromide, whereas in the case of the trimethylnicotinic acid (**20**), bromine was introduced with NBS in trifluoracetic acid.[46]

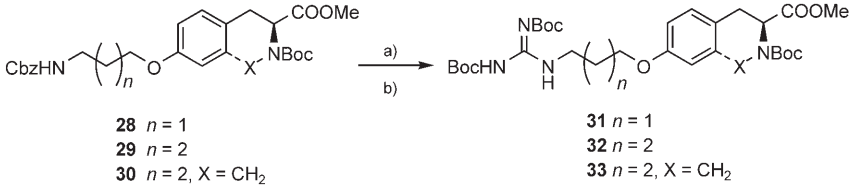


Scheme 4. Synthesis of aromatic acids. a) Me_2SO_4, K_2CO_3, THF, reflux, 84% or *i*PrBr, K_2CO_3, DMF, reflux, 70%; b) 1. *n*BuLi, THF, −78 °C; 2. CO_2, −78 °C–room temperature, 32–65%; c) NBS, TFA, H_2SO_4, room temperature, 93%.

The key step of the synthesis was the Mitsunobu-type alkylation of the tyrosine hydroxyl group (Table 1).[47,48] Although originally published with triphenylphosphine and DEAD as reagents, tributylphosphine and azodicarboxylic dipiperidide (ADDP) have been found to be the reagents of choice for the alkylation of less acidic compounds, such as phenols.[49,50] It turned out that a slow addition of diluted ADDP solution in THF at 0 °C was important for a successful reaction. However, basic molecules, such as aminopyridines, gave yields of only 15–20%, Boc-protected aminopyridines yielded 40–50%, whereas less basic aminopyrimidines, pyridazines and benzyl carbamates gave excellent yields from 75–95%. Compound **8b** could not be used for Mitsunobu reaction as it was prone to cyclisation under formation of pyridine-2-yl-pyrrol. This problem could be overcome by *N*-Boc protection (**11b**).

Table 1 shows the products and yields of the Mitsunobu reactions that were employed as ligand precursors. In the case of precursor molecules **28**–**30**, the Cbz group was hydrogenolytically cleaved and the guanidinium group introduced by using bis-Boc-thiourea and $HgCl_2$ in dry methanol (Scheme 5).[51]

In the next step, the compounds **21**–**27** were first Boc deprotected by using aqueous hydrochloric acid in dioxane, then acylated; before the last step the methyl ester was cleaved and the crude ligand purified by using reversed-phase HPLC techniques (Scheme 6). A summary of the synthesised ligands, yields and biological activity is given in Tables 2–6.

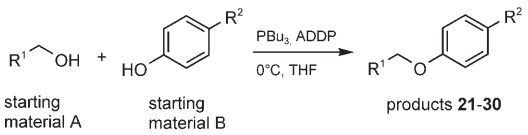


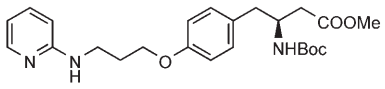
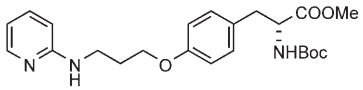
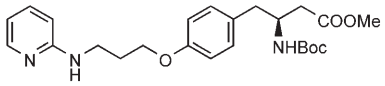
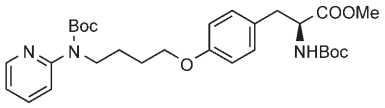
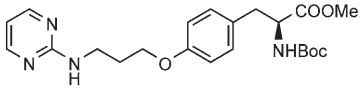
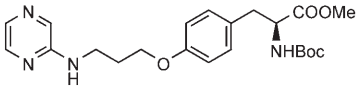
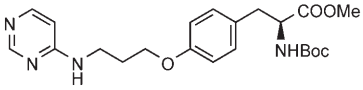
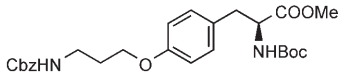
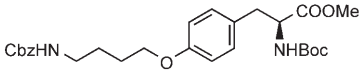
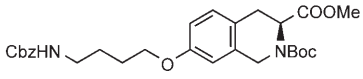
Scheme 5. Synthesis of guanidinylated ligand precursors. a) Pd/C, 1 atm H_2, MeOH; b) $(BocNH)_2CS$, $HgCl_2$, MeOH, TEA, 0 °C–room temperature, 25–33%.

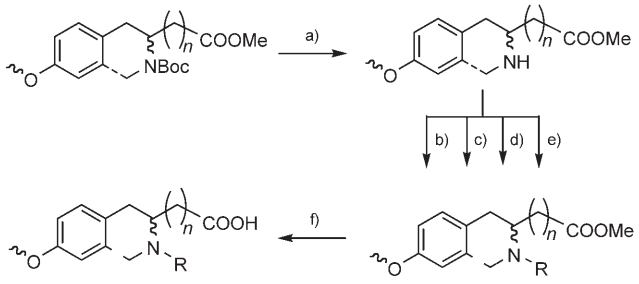
Synthesis of aza-glycine ligands on solid phase

The first approaches made in our group to design peptidomimetic αvβ3 ligands involved the incorporation of turn mimetics,[11,52] reduced amide bonds[53] and aza-peptides.[21–23,54,55] The rational for the use of aza-glycine was the structure determination of αvβ3 that bind RGD peptides, in which the sequence forms a kink around the glycine.[56a,b] The synthesis of compound libraries afforded a large variety of peptidomimetic integrin ligands that displayed a variable selectivity profile among the αv integrins and a good selectivity against the platelet receptor αIIbβ3 (Scheme 7).[21–23] This raised the question of whether the insights into the α5β1 receptor gained from homology modelling and extensive SAR studies with tyrosine-based ligands could be used to modify the aza-glycine ligands for α5β1 selectivity.

Table 1. Synthesis of ligand precursors by Mitsunobu reaction of aminoalcohols with tyrosine derivatives.



Compound	Structure	Starting material A	Starting material B	Yield [%]
21		**11a**	**1**	40
22		**8a**	*ent*-**1**	26
23		**8a**	**3**	30
24		**11b**	**1**	49
25		**13**	**1**	89
26		**14**	**1**	86
27		**15**	**1**	66
28		**12a**	**1**	91
29		**12b**	**1**	71
30		**12b**	**7**	93



Scheme 6. General synthesis of acylated integrin ligands. a) $HCl_{(aq)}$/dioxane, 0.5 h, room temperature; b) 1.2 equiv Ar-COCl or Alk-COCl, 3 equiv $NaHCO_3$, dioxane/water; c) 2 equiv Ar-COOH, 2 equiv HATU, 5 equiv DIEA, DMF, room temperature; d) 1.5 equiv Ar-SO_2Cl, 5 equiv TEA, DCM, room temperature; e) PhNCO, 1 equiv TEA, dioxane; f) 5 equiv LiOH, MeOH/water, HPLC purification.

The synthesis of the novel aza-glycine-based ligands was carried out by using TCP resin loaded with orthogonally protected 2-(*S*),3-diaminopropanoic acid (Scheme 8). This provided the essential carboxylic function and the α-amido-substitution that was found to be crucial for α5β1 selectivity. After Fmoc deprotection, the amino function was acylated with those 2,6-disubstituted aromatic acids, which gave the best results within the tyrosine-based ligand series. After Pd-catalysed Alloc deprotection, the aza-glycine was introduced via building block **40**, which had been freshly prepared from Fmoc-hydrazine and phosgene according to literature procedure.[22] The spacer unit was then coupled to the unprotected hydrazine and the guanidinium group introduced via *N,N'*-di-Boc-1*H*-pyrazole-1-carboxamidine. Although used in large excess and being costly, *N,N'*-di-Boc-1*H*-pyrazole-1-carboxamidine was the reagent of choice for guadinylation as the procedure that involved *N,N'*-di-Boc-thiourea and $HgCl_2$ was unfavourable for solid-phase synthesis because of the formation of a mercury sulfide precipitate and a general incompatibility of methanol with most resins. Most of the excess reagent could be recovered by evaporation of the solvent and recrystallisation from

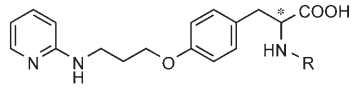
ethyl acetate/hexane. Cleavage and Boc deprotection afforded the aza-glycine ligands in satisfying yields.

Results and Discussion

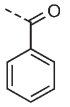
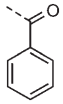
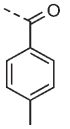
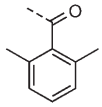
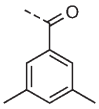
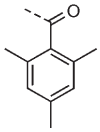
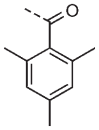
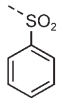
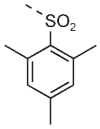
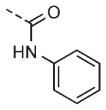
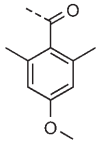
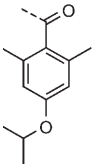
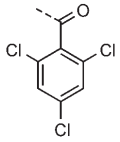
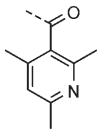
Ligands based on the tyrosine scaffold

The design and synthesis of tyrosine-based ligands afforded a series of 37 compounds, which was evaluated for binding affinity towards $\alpha5\beta1$ and $\alpha v\beta3$ by using a competitive enzyme-linked immunosorbent assay (ELISA). Screening of various aromatic residues on the scaffolds **21**/**22** helped to elucidate the essential features for $\alpha5\beta1$ selectivity (Table 2). The activity on $\alpha5\beta1$ and the selectivity against $\alpha v\beta3$ seems to be strongly connected to the substitution pattern on the aromatic moiety as well as its spatial orientation. The orientation provided by the *S* configuration was found to be essential for the affinity on both integrins. The two *R* enantiomers *ent*-**34a** and *ent*-**34e** both displayed a reduced binding affinity as well as a decreased selectivity for $\alpha5\beta1$ in the case of **34e**. The difference between amides **34a**, **34e** and the corresponding sulfonamides **34f**, **34g** was especially striking. The mesitylene sulfona-

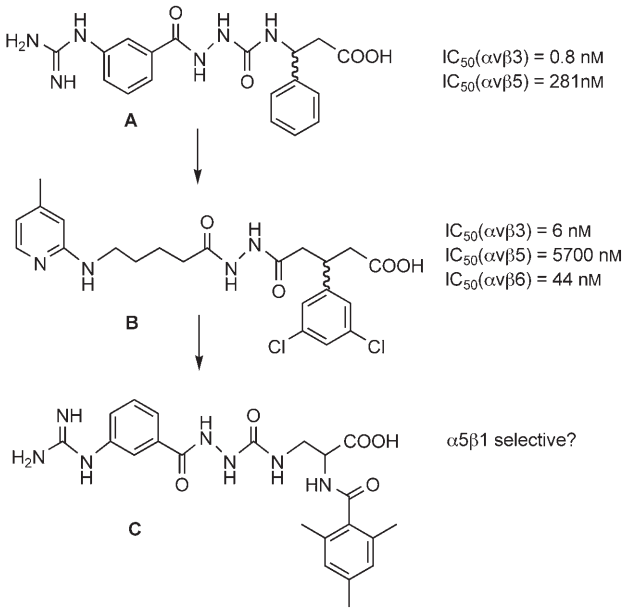
Table 2. Comparison of different aryl substituents and their effect on $\alpha5\beta1$ selectivity.



34

Compound[a]	R	Reagent, conditions[b]	Yield [%]	IC_{50} ($\alpha5\beta1$) [nM]	IC_{50} ($\alpha v\beta3$) [nM]	Compound[a]	R	Reagent, conditions[b]	Yield [%]	IC_{50} ($\alpha5\beta1$) [nM]	IC_{50} ($\alpha v\beta3$) [nM]
34a		PhCOCl, b)	11	243	190	*ent*-**34a**		PhCOCl, b)	25	6700	1030
34b		4-methyl-benzoic acid, c)	28	416	318	**34c**		2,6-dimethyl-benzoic acid, c)	17	3.1	1624
34d		3,5-dimethyl-benzoic acid, c)	15	706	509	**34e**		2,4,6-trimethyl-benzoic acid, c)	24	2.5	703
ent-**34e**		2, 4, 6-trimethyl-benzoic acid, c)	21	150	14700	**34f**		$PhSO_2Cl$, d)	16	284	1.9
34g		mesitylsulfonic acid chloride, d)	15	46	3.8	**34h**		PhNCO, e)	41	1094	37
34i		*t*BuCOCl, b)	45	34	260	**34j**		**17a**, c)	27	1	188
34k		**17b**, c)	28	0.7	279	**34l**		$C_6H_2Cl_3COCl$, b)	63	2.8	41
34m		**20**, c)	20	8.9	188						

[a] Compounds represent the *S* enantiomers; *R* enantiomers are marked with the prefix "-*ent*". The synthesis was performed from ligand precursors **21**/**22**. [b] Reagent and procedure refer to Scheme 6.



Scheme 7. Transformation of αvβ3-selective compounds, **A** and **B**, towards α5β1 selectivity.

mide moiety, which has already been employed in other highly α5β1 active compounds, resulted in a rather biselective ligand, whereas its replacement by an amide induced a selectivity of around 300-fold against αvβ3. The reason for this selectivity gain lies in the arrangement of the bulky mesitylene ring, which in **34e** is oriented towards a hydrophobic cleft in the β1 subunit, which in β3 is occupied by (β3)Arg216 (Figure 1). As for **34g**, the aromatic ring is folded back towards the interface between the two subunits, where no sterical repulsion is present in both integrins. In the case of the phenyl urea **34h**, we observed a dramatic loss of α5β1 affinity (and a slight increase for αvβ3 compared to **34a**); this hints at an unfavourable orientation of the phenyl substituent. Furthermore, **34c** demonstrates that the *para*-methyl group is redundant whereas the low affinities of **34b** and **d** point to the importance of a 2,6-disubstitution pattern for α5β1 selectivity. The 2,6-disubstitution twists the aromatic ring out of the plane of the amide bond and enables an ideal orientation of the aromatic moiety inside the hydrophobic pocket of the β1 subunit.[56–58] In β3, this binding mode is not allowed, which forces the bulky ring to point out of the binding pocket. This alternative binding mode is possible, but exposes a hydrophobic moiety to the surrounding aqueous environment, which decreases binding affinity for αvβ3. Remarkably, substitution of the methyl groups by chlorine atoms resulted in a substantial loss of selectivity due to more favourable binding to αvβ3, whereas the use of the pyridine analogue of mesitylenecarboxylic acid (**20**) decreased selectivity by a loss of α5β1 affinity. This supports the hypothesis that only hydrophobic aryl amides have a strong positive effect on α5β1 binding. Although we found that the 4-methyl substitution is not necessary, the methyl group points in the direction of (β1)-Ser517, which could be engaged in an additional interaction with a hydrogen-bond acceptor at this position. This was observed for the 4-methoxy and 4-isopropoxy compounds **34j** and **34k** (Figure 2). They displayed a very high α5β1 activity of one nanomolar and below, whereas the selectivity against αvβ3 was not affected. To test whether aryl substituents can be replaced by aliphatic groups, we synthesised the pivalyl amide **34i**, which exhibited decreased α5β1 binding compared to **34c** or **34e**, but still showed a substantial amount of selectivity.

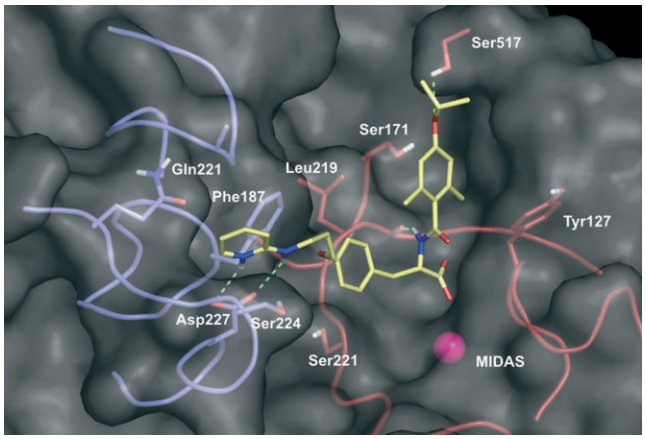
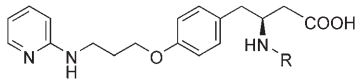


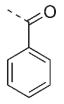
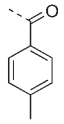
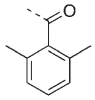
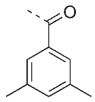
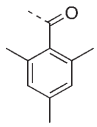
Figure 2. Superposition of the Connolly surface and the ribbon drawing (α subunit: blue, β subunit: red) of the α5β1 binding pocket with ligand **34k** docked into it. Relevant residues are highlighted and major H bonds are shown as dotted lines. The MIDAS cation is represented as a magenta sphere.

Analysis of the homology model of the α5β1 receptor revealed a mutation in the α subunit: (αv)-Thr was mutated to (α5)-Gln. This slightly shortens the binding pocket of α5β1 and should favour shorter ligands. Hence, the ligands of the **34** series are already short with respect to most ligands published for αvβ3 (ten bonds between the carboxylate and the pyridylamine versus 11–12 for other ligands). We synthesised a series of elongated ligands; the elongation could be achieved either by β^3-homotyrosine scaffolds (**35**, Table 3) or by the use of longer spacer molecules (**36**, Table 4). Elongated spacers were also used in ligands that carried guanidinium groups as basic moieties (**38**, Table 5). Comparison of the β^3-homotyrosine ligands **35a–e** with the corresponding ligands of the **34** series revealed a striking preference for the αvβ3 integrin. This is not surprising as many αvβ3 ligands are somehow substituted in the β position to the carboxylate.[18,22] The overall affinity towards α5β1 was reduced, which is partly due to the total length of the ligand but mostly because the bulky, selectivity-inducing aromatic system is not able to properly address the hydrophobic pocket in the β1 subunit. Therefore, no selectivity for α5β1 was observed for the 2,6-disubstituted benzamides **35c** and **e**. In contrast to the α-tyrosine-based ligands, the elongated α-tyrosines showed a three- to fourfold decrease in affinity, which is the result of the slightly shorter binding pocket of α5β1 (**34a**, **e**, **k** versus **36a**, **b**, **c**). Comparison between **34e** and **k** with **36b** and **c** further showed a small increase in selectivity for the elongated ligands of the **36** series. Additionally, two more 2,6-disubstituted aromatic acids were

Table 3. Comparison of different aryl substituents on elongated ligands based on the β-homotyrosine scaffold.

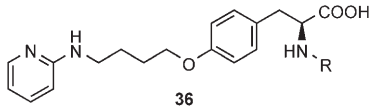


35

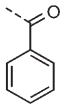
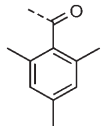
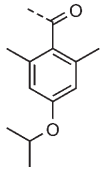
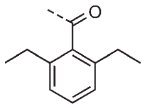
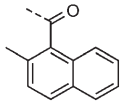
Compound	Reagent, conditions[a]	R	Yield [%]	IC_{50} (α5β1) [nM]	IC_{50} (αvβ3) [nM]
35a	PhCOCl, b)		18	264	1.2
35b	4-methyl-benzoic acid, c)		13	292	2.2
35c	2,6-dimethyl-benzoic acid, c)		11	142	2.9
35d	3,5-dimethyl-benzoic acid, c)		16	91	5.2
35e	2,4,6-dimethyl-benzoic acid, c)		22	140	20

[a] Synthesis from **23** according to Scheme 6.

Table 4. Comparison of different aryl substituents on a tyrosine scaffold with elongated linker.

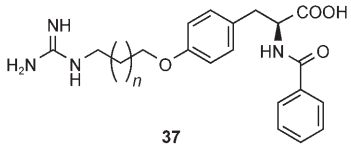


36

Compound	Reagent, conditions[a]	R	Yield [%]	IC_{50} (α5β1) [nM]	IC_{50} (αvβ3) [nM]
36a	PhCOCl, b)		12	996	111
36b	2,4,6-trimethyl-benzoic acid, c)		18	7.2	2685
36c	**17b**, c)		34	2	1455
36d	**18**, c)		32	13.5	16800
36e	2-methyl-naphthyl-carboxylic acid, c)		45	6	1412

[a] Synthesis from **24** according to Scheme 6.

Table 5. Ligands bearing guanidine groups and spacer units of different length.



37

Compound[a]	*n*	SM[b] [%]	Yield	IC_{50} (α5β1) [nM]	IC_{50} (αvβ3) [nM]
37a	1	**31**	21	60	131
37b	2	**32**	22	66	39

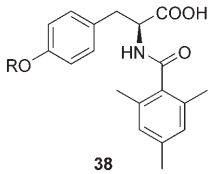
[a] Synthesis from scaffolds **31**/**32** and PhCOCl according to Scheme 6; [b] starting material.

coupled to the elongated scaffold **24**; the 2-methylnaphthyl-1-amide **36e** had very similar properties to the corresponding mesitylamide **36b**. However, replacement of the methyl substitution by sterically more demanding ethyl groups resulted in a small decrease of α5β1 affinity, but strongly increased selectivity to 1400-fold. This is the best selectivity that has been achieved with tyrosine-based ligands to date.

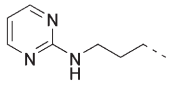
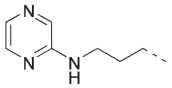
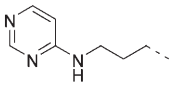
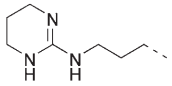
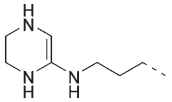
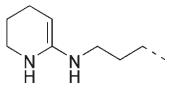
To test how important the impact of the total ligand length on α5β1 affinity is, two unselective compounds of different lengths that had a guanidinium group (**37a**, **b**) were synthesised as references (Table 5).

The results showed that there is no length-dependent affinity change for α5β1, and only a slight preference of αvβ3 for the elongated ligand. The discrepancy between these results and the increase in α5β1 selectivity observed for the **36** series compared with the **34** series can be explained by a close look at the receptor. The mutation responsible for the shortened binding pocket of α5β1 is located directly at the binding site of the basic moiety. The effect of the mutation from (αv)Thr212 to (α5)Gln221 should only affect basic groups with a higher sterical demand in the direction of (α5)Gln221, such as aminopyridines. In fact, methyl substitution at the 6-position of the aminopyridine could substantially decrease α5β1 affinity and lead to selective αvβ3 ligands, which have been published previously.[24] Another hotspot where αv differs from α5, is the lack of (αv)Asp150, which is mutated into an alanine in α5. Whereas the guanidinium group of a ligand is able to form salt bridges to both aspartates (αv)Asp218 and (αv)Asp150 in αvβ3, α5β1 has only (α5)Asp227 for this essential interaction. To probe whether this mutation can be utilised to gain selectivity, the aminopyridine moiety of the α5β1 selective ligand **34e** was replaced by a number of related basic heterocycles (Table 6). As a general observation, the affinity towards α5β1 seems to be correlated to the basicity of the heterocycle: whereas the guanidinium-like tetrahydropyrimidine **38d** gave excellent activities, the tetrahydropyridazine **38e** as well as the

Table 6. Comparison of different basic moieties on $\alpha5\beta1$ affinity and selectivity.



38

Compound[a]	Starting material	R	Yield [%]	IC_{50} ($\alpha5\beta1$) [nM]	IC_{50} ($\alpha v\beta3$) [nM]
38a	**25**		18	73	n.d.[b]
38b	**26**		17	99	n.d.[b]
38c	**27**		10	126	>20000
38d	**38a**		83	1.8	221
38e	**38b**		91	54	11082
38f	**34e**		86	42	5375

[a] Compounds **38a–c** were synthesised from the ligand precursors **25–27** and 2,4,6-trimethylbenzoic acid according to Scheme 6, procedure c). Compounds **38d–f** were prepared from the assigned starting materials by reduction with H_2 and a Pd/C catalyst; [b] not determined.

pyridazin **38b** and the two pyrimidines **38a**, **38c**, each of which are less basic than an aminopyridine, displayed reduced activity. Compound **38c**, the pyrimidin-6-yl-amino moiety of which can establish an additional interaction with ($\alpha5$)-Gln221 did not have a satisfactory $\alpha5\beta1$ affinity. It could not be determined whether this additional interaction is not present or if it is simply overruled by the drop of basicity that is associated with the substitution of pyridine by pyrimidine. Overall, it was not possible to exploit the structural differences in the α subunits to increase selectivity for $\alpha5\beta1$, whereas reverse selectivity could be induced by modification of the pyridine ring.[24]

Flexible ligands are able to adopt a variety of conformations, which often hampers selectivity. The introduction of constraints, for example, the cyclisation of tyrosine to 1,2,3,4-tetrahydroisochinolines is a popular approach to gain selectivity by conformational restriction.[41,60,61] The ligand precursor **33** was prepared by deprotection and guanidinylation of **30** (Scheme 5). After Boc deprotection, the amine (**39a**) was acylated with benzoyl chloride (**39b**), mesitylsulfonyl chloride (**39c**) or acetic acid anhydride (Scheme 9).

The results in Table 7 indicate that constriction by cyclisation positions the amino function in an unfavourable location for $\alpha5\beta1$. It can be assumed that an α substitution is generally unfavourable as the results are independent of the nature and size of the substituent. The sulfonamide, which is normally biselective for $\alpha5\beta1$ and $\alpha v\beta3$, showed a substantially reduced affinity for $\alpha v\beta3$; this hints at a general incompatibility of this scaffold with integrin affinity.

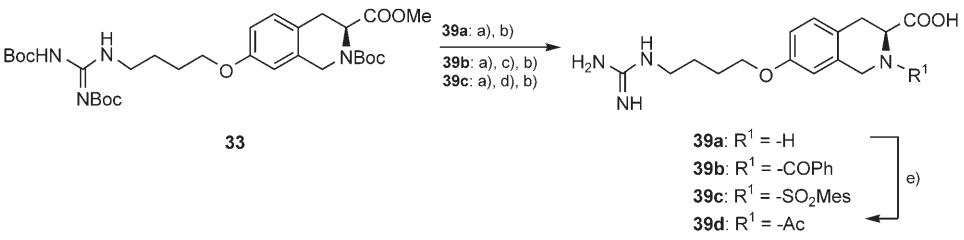
Ligands based on the azaglycine scaffold

The ligands of this series showed all the features responsible for $\alpha5\beta1$ selectivity, which were determined in the SAR study with the tyrosine-based ligands: a *S* configured, α-amino acid as carrier of the carboxyl moiety and an aromatic amide with an *ortho*-dimethyl-substitution pattern. Both mesityl groups and the slightly more potent 2,6-dimethyl-4-isopropoxyphenyl group were employed as aromatic groups whereas the basic moiety was left unchanged compared to the previously developed $\alpha v\beta3$ ligands (Scheme 7). Biological evalua-

Table 7. Biological evaluation of constrained tyrosine ligands **39**.

Compound	Yield [%]	IC_{50} ($\alpha5\beta1$) [μM]	IC_{50} ($\alpha v\beta3$) [nM]
39a	65	>10	n.d.[a]
39b	12	>10	n.d.[a]
39c	22	15.9	330
39d	88	>10	n.d.[a]

[a] Not determined.



33

Scheme 9. Synthesis of constrained tyrosine ligands **39**. a) $HCl_{(aq)}$/dioxane; b) LiOH, methanol/water; c) PhCOCl, $NaHCO_3$, dioxane/water; d) mesitylsulfonyl chloride, TEA, DCM; e) Ac_2O, $NaHCO_3$, dioxane/water.

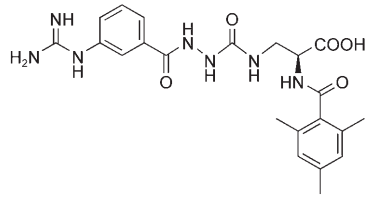
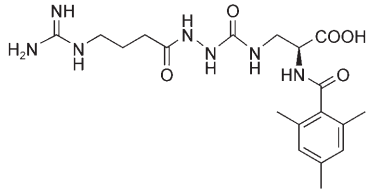
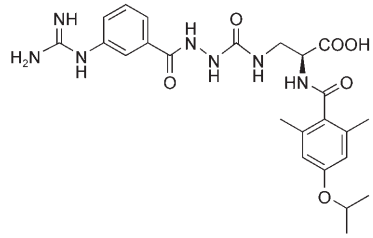
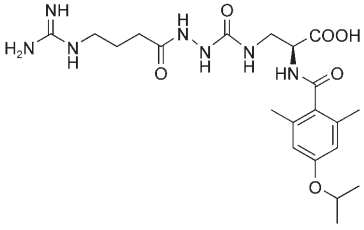
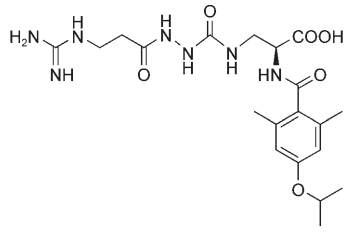
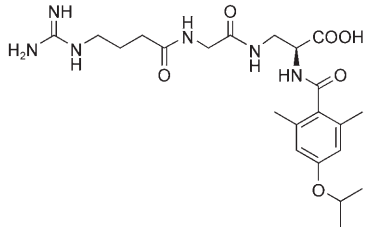
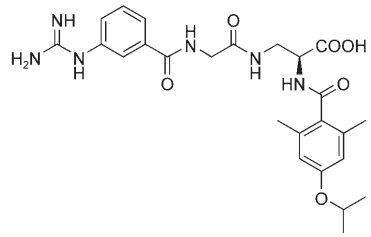
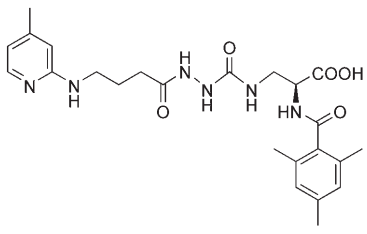
tions of the aza-glycine scaffold revealed high $\alpha5\beta1$ affinities together with a dramatic increase in selectivity compared to the tyrosine scaffold. The selectivities of 6000 and higher are due to the rigidity of the diacylhydrazone scaffold compared to the rather flexible tyrosine. The lack of degrees in freedom strongly disfavours alternative binding modes when the mesitylene moiety is oriented outside the $\alpha v\beta3$ pocket. Table 8 shows representatives of the class of diacylhydrazones optimised for $\alpha5\beta1$ selectivity.

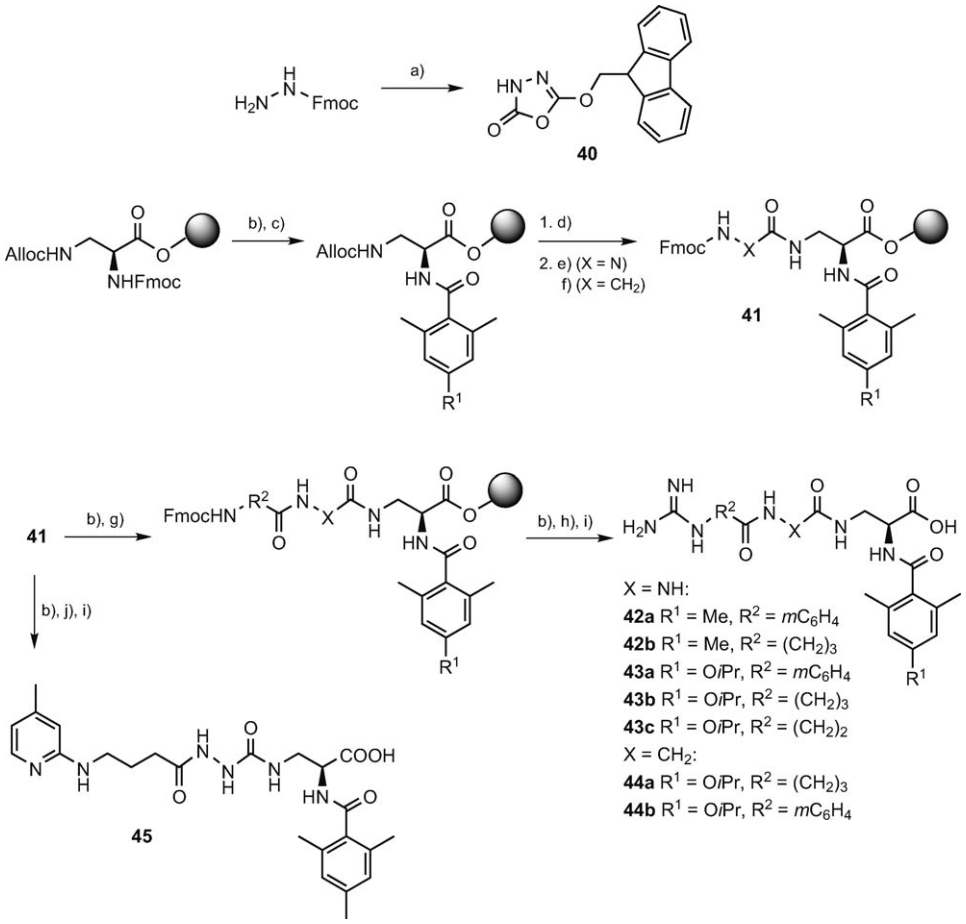
Both arylguanidyl and alkylguanidyl groups (**42a, 43a**/**42b, 43b**) showed a comparable selectivity against $\alpha v\beta3$; this once again points out that only the C-terminal part of the molecule is responsible for the selectivity. The additional hydrogen-bond acceptor (-O*i*Pr) in **43** gave a reduction of the IC_{50} value comparable with the tyrosine ligands **34j**, **k**. The approximately tenfold loss of $\alpha5\beta1$ affinity of **43c** compared to **43b** shows that the total length of the aza-glycine cannot be reduced. Furthermore, substitution of aza-glycine with glycine (**44b**) has little effect on the high affinities. The use of aminopyridines as guanidine mimetics, which afforded very high affinity in both $\alpha5\beta1$ and $\alpha v\beta3$ ligands, resulted in compound **45**, which had subnanomolar affinity for $\alpha5\beta1$ and a selectivity of $\sim10^4$-fold. Figure 3 demonstrates the fit of compound **43a** into the $\alpha5\beta1$ receptor and the main interactions, which are identical to the results obtained from docking studies with tyrosine-based ligands. Compared to the tyrosine ligands, the high selectivities of the aza-glycine ligands should be the result of an enhanced rigidity of the scaffold, which strongly disfavours the placement of the aromatic amide outside the receptor in $\alpha v\beta3$.

Conclusions and Outlook

In the face of the growing importance of selective $\alpha5\beta1$ ligands, we report herein the synthesis of a series of compounds based on the tyrosine scaffold with activities in the subnanomolar range and selectivities of ~300-fold. The ligands were optimised by docking studies with a homology model that was established in our group. A careful evaluation of ligand length, basic moieties and differently substituted aromatic moieties demonstrated the relevance of the model, and enabled us to achieve an increase in selectivity to 1200-fold and still preserve a low nanomolar activity. Furthermore, the information extracted from the extensive structure–activity relationship studies allowed the induction of $\alpha5\beta1$ selectivity in

Table 8. Biological activities of ligands based on the aza-glycine and glycine scaffolds.

	Compound	IC_{50} ($\alpha5\beta1$) [nM]	IC_{50} ($\alpha v\beta3$) [nM]		Compound	IC_{50} ($\alpha5\beta1$) [nM]	IC_{50} ($\alpha v\beta3$) [nM]
42a		1.1	6500	**42b**		6.3	>50000
43a		0.96	>4750	**43b**		3.3	>20000
43c		34	>20000	**44a**		1.5	>20000
44b		0.86	9600	**45**		0.65	6000



Scheme 8. Synthesis of aza-glycine ligands on TCP resin. a) DCM/$NaHCO_{3(aq)}$, $COCl_2$/toluene (90%); b) 20% piperidine/NMP; c) aromatic acid, HATU, DIEA, NMP; d) 0.25 equiv $Pd(PPh_3)_4$, $PhSiH_3$, DCM; e) **40**, DCM; f) Fmoc-glycine, HOBt, TBTU, DIEA, DMF; g) 3-Fmoc-aminobenzoic acid, HATU, DIEA, NMP or 4-Fmoc-aminobutanoic acid or 3-Fmoc-aminopropanoic acid, TBTU, DIEA, NMP; h) Bis-Boc-guanidinylpyrazole, $CHCl_3$, 50 °C; i) 25% TFA in DCM; j) 3-(4-methylpyridin-2-yl)aminopropanoic acid, TBTU, DIEA, NMP.

different integrin ligands by means of minor modifications, which was demonstrated for the aza-glycine/glycine scaffold. The resulting compounds displayed affinities in the low nanomolar range and below. The selectivity against $\alpha v\beta 3$ exceeded 10^4-fold, which is comparable with the level of the most selective $\alpha 5\beta 1$ ligands reported so far. Integrin $\alpha 5\beta 1$ antagonists represent promising lead structures for antiangiogenic therapy of cancer and age-related macular degeneration. The selective $\alpha 5\beta 1$ ligands have already been successfully employed in biological systems and are subject of further research on integrin function.[62]

Experimental Section

Detailed outline of the molecular docking, synthesis and analysis procedures can be found in the Supporting Information.

Acknowledgement

The authors would like to thank Burghard Cordes, Maria Kranawetter and Mona Wolf for technical support, Thorsten Lanz for biological testing, Dr. Werner Spahl for the high-resolution mass spectra, and the International Graduate School for Science and Engineering (IGSSE).

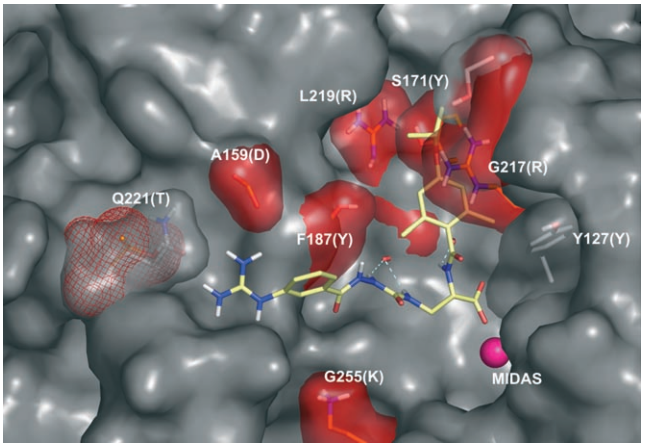


Figure 3. Superposition of the Connolly surface of the binding pocket of $\alpha 5\beta 1$ (grey) and $\alpha v\beta 3$ (red) with the aza-glycine ligand **43a** docked into it. Important receptor side chains and their corresponding residues in $\alpha v\beta 3$ are highlighted and labelled in parentheses. The MIDAS metal is represented as a magenta sphere and H bonds are shown as dotted lines.

Keywords: antiangiogenic · antitumor agents · integrins · rational design · structure–activity relationships

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Received: January 23, 2008
Published online on May 15, 2008

