Integrin Ligands (2)

International Edition: DOI: 10.1002/anie.201508713
German Edition: DOI: 10.1002/ange.201508713

Small Cause, Great Impact: Modification of the Guanidine Group in the RGD Motif Controls Integrin Subtype Selectivity

*Tobias G. Kapp, Maximilian Fottner, Oleg V. Maltsev, and Horst Kessler**

Abstract: *Due to its unique role as a hydrogen-bond donor and its positive charge, the guanidine group is an important pharmacophoric group and often used in synthetic ligands. The chemical modification of the guanidine group is often considered to destroy its function. Herein, we show that the N-methylation, N-alkylation, or N-acylation of the guanidine group can be used to modify the receptor subtype specificity of the integrin ligand cilengitide. Using the αvβ6/α5β1-biselective ligand c(isoDGRkphg) and the αvβ6-specific ligand c-(FRGDLAFp(NMe)K(Ac) as examples, we show that the binding affinities of the ligands can be fine-tuned by this method to enhance the selectivity for αvβ6. Furthermore, we describe a new strategy for the functionalization of integrin ligands. By introducing longer N-alkylguanidine and N-acylguanidine groups, we are able to simultaneously identify a hitherto unknown anchoring point and enhance the subtype selectivity of the ligand.*

Integrins sense and regulate cell attachment both between cells and to the extracellular matrix (inside-out signaling and outside-in signaling, respectively).[1] One common feature of the integrin family is a heterodimeric structure that consists of α and β subunits.[2] These structures form 24 different subtypes in mammals, which can be classified according to their binding partners (e.g. laminin, collagen). Eight of these subtypes form the Arg-Gly-Asp (RGD)-binding class.[3] This tripeptide sequence is present in a variety of extracellular matrix proteins such as fibronectin and vitronectin.[4] Different integrins are involved in many pathological processes such as metastasis and tumor vascularization.[5] The structural differences among the subtypes are often small because most of them share different natural RGD-containing proteins; however, they exhibit drastically different binding affinities. For instance, the subtype αvβ3 can bind to the ECM ligands vitronectin, von-Willebrand-factor, fibrinogen, osteopontin, and fibronectin.[6] This makes it challenging to develop selective compounds, especially for subtypes that are often co-expressed and present in different cellular and pathological situations. In this context, the fibronectin binding subtypes αvβ3 and α5β1 are particularly important; these subtypes both play important roles in angiogenesis and tumor development.[7]

[*] T. G. Kapp, M. Fottner, Dr. O. V. Maltsev, Prof. Dr. H. Kessler
Institute for Advanced Study and Center for Integrated Protein Science (CIPSM), Technische Universität München
Lichtenbergstrasse 4, 85747 Garching b. München (Germany)
E-mail: kessler@tum.de

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201508713.

The initial goal of early ligand development was primarily to obtain ligands possessing high αvβ3 affinity. At that time, selectivity for other integrin subtypes (except integrin αIIbβ3) was only a minor issue. The spatial screening of cyclic RGD-containing peptides yielded the pentapeptide *c*(RGDf-(*N*Me)V) (cilengitide)[8,9] as the most potent αvβ3 ligand (0.54 nM); cilengitide also has a relatively high α5β1 binding affinity (15.4 nM).[8,10] When the importance of different integrins in biology became evident, the need for integrin subtype selective peptides became clear. In a parallel approach, peptidomimetics were investigated by our group and others,[11] leading to completely selective αvβ3 and α5β1 ligands. These molecules could be functionalized for medical (e.g. molecular imaging) and biophysical applications. Determining the distinct differences in the biological functions of these two subtypes opens the door for their future applications (e.g. their use in personalized medicine).[12]

The binding mode of RGD integrin ligands was elucidated from the crystal structure of cilengitide bound to the αvβ3 extracellular domain.[13] The key elements of this binding interaction in all RGD-binding integrins include the metal-ion-dependent adhesion site (MIDAS), located in the β subunit, and the strong *side-on* bidendate H-bonding interaction (guanidine carboxylate) in the α subunit. Recently, the crystal structure of the α5β1 headpiece in complex with a peptidic ligand[14] unveiled a remarkable and important difference in the binding modes of the guanidine group in the αv- and α5-binding pockets. In the α5 subunit the guanidine group participates in two interactions: the bidendate *side-on* binding of HN_{δ} and $HN_{\omega 1}$ to Asp227, and the *end-on* binding interaction of $HN_{\omega 1}$ and $HN_{\omega 2}$ with Gln221. In the αv pocket, the only interaction of guanidine is the bidendate H-bonding to Asp218 (Figure 1).

Provided with this structural insight, we were interested in whether this small difference could be used to rationally design subtype-selective peptidic integrin ligands. Theoretically, two molecules with differently modified guanidine group in the arginine side chain and opposing selectivity should be obtainable in a model system of a biselective ligand. In one case, blocking the *end-on* interaction of the molecule with the receptor (modification of $N_{\omega 1}$) would result in higher αvβ3 selectivity since the significant binding energy contributing to the α5β1 interaction would be lost. In the opposite case, disturbing the *side-on* interaction by blocking HN_{δ} would allow only an *end-on* interaction, resulting in a higher selectivity for the subtype α5β1. As a model system, we chose the αvβ3/α5β1 biselective cyclic peptide cilengitide. To block the interaction, distinct hydrogen atoms in the guanidine group were replaced by methyl groups. This alteration prevents hydrogen bonding while introducing only minimal

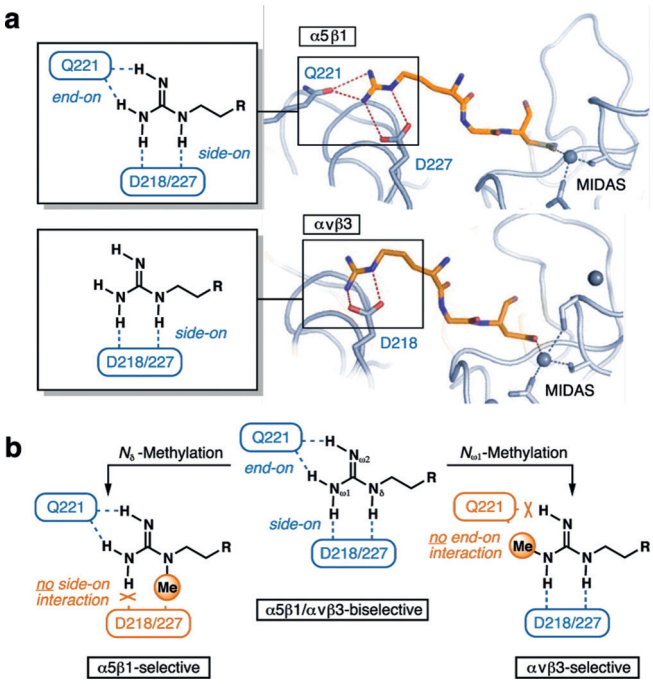

Figure 1. a) Linear RGD-ligand binding to the subtypes α5β1 (top) and αvβ3 (bottom). Differences are found in the interaction of arginine guanidine group with the α5 and αv subunit, respectively, as shown enlarged in the box. Two interactions are found for α5β1 (*side-on* and *end-on*); for αvβ3 only *side-on* is observed. b) Concept of subtype selectivity: a biselective guanidine compound (middle) is methylated on N_ω (right) → improvement of αvβ3 selectivity by blocking *end-on* interaction to Q221; N_δ-methylation (left) blocks *side-on* interaction to D218, *end-on* interaction is still possible → improvement of α5β1 selectivity.

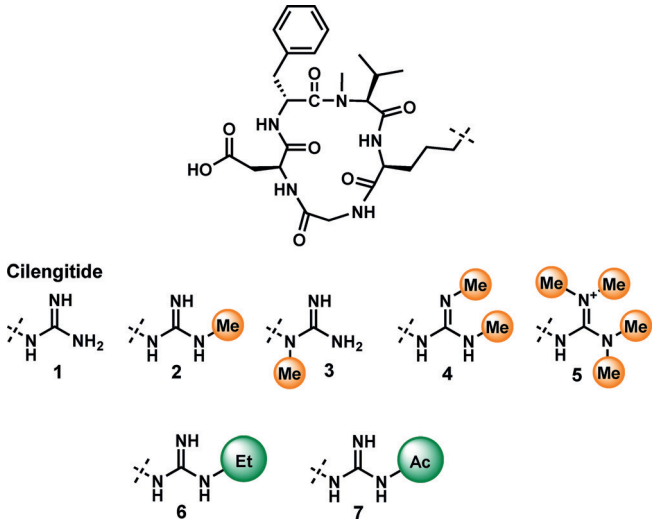

Figure 2. Cilengitide (**1**) and its substituted guanidine derivatives **2**–**7**.

steric and electronic changes into the system. To probe this hypothesis, four methylated analogues of cilengitide were synthesized, including one di- and one tetramethylated compound (Figure 2).

The syntheses of **2**, **4**, **5**, **6** and **7** began with solid-phase synthesis (SPPS) of the linear, orthogonally Dde-protected peptide H-Asp(O*t*Bu)-D-Phe-(*N*Me)Val-Orn(Dde)-Gly-OH, followed by cyclization and subsequent orthogonal Dde deprotection (Figure 3). The final compounds were obtained after guanidinylation followed by deprotection of the acid-labile side-chain protecting groups. For the guanidinylation step, two different protocols were used. The terminally monoalkylated compounds **2** and **6** were obtained after guanidinylation with alkylated *N*,*N*′-di-Boc-1*H*-pyrazole-1-carboxamidine precursors. These are obtained by Mitsunobu reaction of *N*,*N*′-di-Boc-1*H*-pyrazole-1-carboxamidine with the corresponding alcohol, for example, ethanol for the generation of an ethyl group.[15] The precursor for the acetylated guanidine **7** was synthesized starting from *S*-methylpseudothiourea (Supporting Information, SI).[16] For **4** and **5**, the ornithine side-chain amine groups were guanidinylated using the commercially available *N*,*N*′-dimethylthiourea and *N*,*N*,*N*′,*N*′-tetramethylthiourea in the presence of NEt_3 and $HgCl_2$ as base and activator; **3** was obtained after the *N*-methylation of the ornithine side chain on a solid support, cyclization, *o*-Ns deprotection in solution, and guanidinylation with *N*,*N*′-di-Boc-1*H*-pyrazole-1-carboxamidine (SI).

The results of the in vitro evaluation of the ligands correspond surprisingly well with the theoretical model described above. For **2**, which is methylated at $N_{\omega1}$, the α5β1 binding affinity vanished completely (> 1 μM), whereas the αvβ3 binding affinity decreased from 0.54 to 8.4 nM (Table 1). The terminal methyl group prevents the *end-on* binding interaction. In contrast, methylation at N_δ blocks the *side-on* binding interaction. In αv, where the latter is the only interaction, this modification leads to a total loss of binding affinity. In the α5 pocket, this *side-on* interaction is also blocked, whereas the *end-on* interaction is still possible, resulting in a α5β1-selective peptide (**3**) with reduced overall binding affinity (51 nM). Furthermore, a compound with a higher degree of methylation and, consequently, blocking of *side-on* and *end-on* binding, should possess no binding affinity at all. The biological evaluation of **4** and **5** demonstrated no affinity (> 1000 nM) for both subtypes αvβ3 and α5β1.

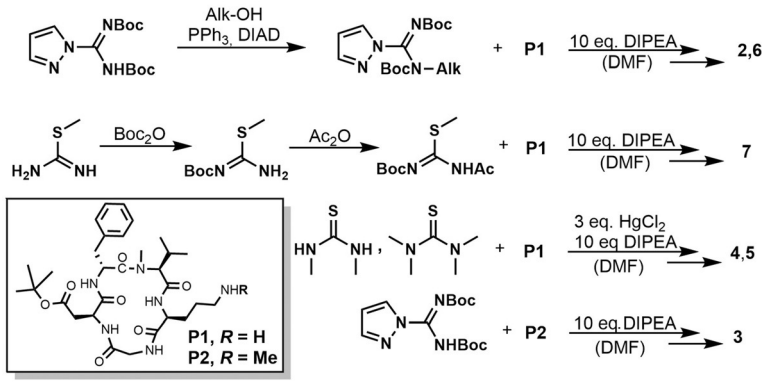

Figure 3. Synthesis of compounds **2**–**7**.

Table 1: Binding affinities of cilengitide (**1**) and its derivatives **2**–**7** for αvβ3 and α5β1.[a]

	αvβ3 [nM]	α5β1 [nM]
1[b]	0.54 ± 0.04	15.4 ± 1.7
2	8.4 ± 0.8	i.a.
3	i.a.	51 ± 4.2
4	i.a.	i.a.
5	i.a.	i.a.
6	0.93 ± 0.1	i.a.
7	6.5 ± 0.5	15.3 ± 0.9

[a] The binding affinities were determined in a solid-phase binding assay using a previously established protocol.[20] The selectivity profile for each compound can be found in the SI. [b] Cilengitide. i.a. > 1000 nM.

Table 2: Binding affinities of compounds **10**–**14** for αvβ6 and α5β1.[a] as determined in a solid phase binding assay using a previously established protocol.[20] The selectivity profile for each compound against other integrin subtypes can be found in the SI.

		αVβ6 [nM]	α5β1 [nM]
10	*c*(phg*iso*DGRk)	18 ± 2.5	8.3 ± 0.7
11	*c*(phg*iso*DGR(Me)k)[b]	13 ± 2.1	120 ± 15
12	*c*(FRGDLAFp(*N*Me)K(Ac))	0.26 ± 0.04	72 ± 9
13	*c*(FR(Me)GDLAFp(*N*Me)K(Ac))[b]	1.3 ± 0.23	i.a.
14	*c*(FR(Et)GDLAFp(*N*Me)K(Ac))[b]	0.28 ± 0.03	i.a.

[a] The binding affinities were determined in a solid-phase binding assay using a previously established protocol.[20] The selectivity profile for each compound can be found in the SI. [b] Modification of arginine on the terminal $N_{\omega1}$ is indicated with the substituent brackets following R (R(Alk)). i.a. > 1000 nM; lowercase letters (p,h,g,k) denote D-amino acids.

Having closely examined the crystal structure model of the αv binding pocket and taken into consideration the tolerance of the 6-methyl-2-aminopyridine scaffold in previously described αvβ3 peptidomimetic ligands,[17] we supposed that alkyl groups, which are larger than the methyl group in **2**, might also be tolerated. Indeed, we found that the presence of ethyl guanidine in this position resulted in a ligand with much better binding affinity than the methylated derivative. With a binding affinity of 0.93 nM, **6** retained almost full αvβ3 affinity of cilengitide while no binding could be observed for α5β1 and all other investigated subtypes. Hence, **6** shows one of the best αvβ3 binding affinities and subtype selectivity profiles ever described (see SI). It is well known that in acylguanidines,[18] the basicity is reduced by 4–5 orders of magnitude ($pK_a \approx 8$) compared to basic guanidine compounds ($pK_a \approx 13$); for alkylated compounds, the pK_a is reported to be 11–12.[19] This implies that at physiological pH, alkylguanidines are generally positively charged, whereas acylguanidines are at least partly uncharged. This reduced basicity is often used to tune the pharmacokinetic properties of bioactive molecules to, for example, improve their bioavailability. Furthermore, in both the charged and uncharged states, acylguanidines form planar systems. In addition to the *N*-alkylated molecules described above, one compound (**7**) with a terminally acetylated guanidine $N_{\omega1}$ was also investigated. Interestingly, the shift in selectivity seen for the alkylated molecules was not observed for **7**. Whereas the binding affinity for α5β1 remained unchanged (approx. 15 nM), the αvβ3 affinity was reduced from 0.54 to 6.5 nM, indicating a strong shift in selectivity towards α5β1. To explain this effect, we hypothesized that the carbonyl of the acetyl group can interact with one amide proton of Gln221 in the α5 subunit, compensating for the lost interaction between H$N_{\omega1}$ and the carbonyl group of Gln221.

Surprised by the drastic effect on selectivity caused by these rather simple modifications, we decided to attempt to apply this technique in a wider sense to tune and adjust the binding affinity profiles of integrin ligands. In particular, we wanted to investigate whether terminal alkylation could be used as a general approach to remove or significantly reduce α5β1 binding affinity by blocking the α5β1-specific interaction with Gln221. Our first system was the αvβ6/α5β1-biselective peptide *c*(*iso*DGRkphg) (**10**).[21] After methylation (**11**) of $N_{\omega1}$, binding affinity for α5β1 decreased from 8 to 120 nM, whereas αvβ6 affinity slightly increased from 18 to 13 nM (Table 2). In another example, we investigated the recently developed helix-mimicking 9-mer cyclic peptide *c*(FRGDLAFp(*N*Me)K(Ac)) (**12**) developed in our lab.[22] This peptide shows very high binding affinity (0.26 nM) for αvβ6 and a residual affinity of 72 nM for α5β1. As in the previously discussed examples, the method could be successfully applied, as demonstrated by the reduction in binding affinity for α5β1 to > 1000 nM by methylation of the $N_{\omega1}$ of the arginine guanidine group (**13**). Nevertheless, this methylation also significantly reduced the αvβ6 affinity to 1.3 nM, which could be overcome by introducing an ethyl substituent (**14**), as in the above example of cilengitide.

As demonstrated above, the substitution of guanidine allows us to influence and tune the subtype selectivity for αv- and α5-containing integrins. Considering the structural features of the binding pockets of both α subunits, it appears that space in elongation of H$N_{\omega2}$ is not restricted. A long alkyl or acyl chain on $N_{\omega2}$ would allow a new way of functionalizing integrin ligands by simultaneously interacting with the guanidine and linking out of the binding pocket.

For a proof-of-concept study, two functional compounds were synthesized: one acylated on $N_{\omega2}$ with 6-acetyl-aminohexanoic acid (**8**) and the other alkylated on $N_{\omega2}$ with 6-acetylaminohexane (**9**) (Figure 4). To synthesize **8** and **9**, the orthogonally Dde-deprotected peptide P1 was guanidinylated with the corresponding tailor-made *N*,*N*′-di-Boc-1*H*-pyra-

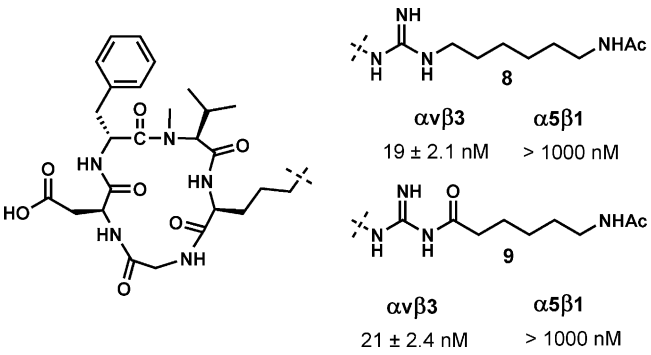

Figure 4. Functionalization of cilengitide via guanidine.

zole-1-carboxamidine (alkylation) and *N*-(*tert*-butoxycarbonyl)-*S*-methylisothiourea precursor (acylation; see SI).

The results obtained in the in vitro binding assay clearly verified our hypothesis. With both modifications, the first guanidine-functionalized integrin ligands were obtained and showed affinities in the range of approximately 20 nM for αvβ3 with complete selectivity against α5β1. The alternative interaction seen in **7,** which resulted in a good α5β1 affinity, is not possible here due to steric hindrance. Instead, the chain is oriented out of the binding pocket. The same is observed for the hexylguanidine linker. The binding affinities of this new class of functionalized molecules are comparable to that of long-established *c*(RGDfK(AhxAc)) (24 nM), the "gold standard" of functionalized integrin ligands.

In conclusion, we were able to show that simple modification of the guanidine group in the integrin ligand cilengitide can be used to tune integrin subtype selectivity in two directions, leading to two isomeric compounds varying only in the position of a methyl group but having completely opposite selectivity properties for αv and α5 integrins. Furthermore, the applicability of this procedure was shown for two more systems, significantly improving selectivity against α5β1. Finally, we were able show that by substituting the guanidine group, functionalization of molecules is also possible.

Acknowledgements

T.G.K acknowledges the International Graduate School for Science and Engineering (IGSSE) of the Technische Universität München for financial support. H.K. thanks also the CIPSM and the Deutsche Forschungsgemeinschaft for support.

Keywords: cilengitide · integrin ligands · peptide modifications · receptor subtype selectivity · RGD motif

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 1540–1543
Angew. Chem. **2016**, *128*, 1564–1568

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Received: September 17, 2015
Revised: October 26, 2015
Published online: December 9, 2015

