Selective Cell Adhesion

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Functionalizing αvβ3- or α5β1-Selective Integrin Antagonists for Surface Coating: A Method To Discriminate Integrin Subtypes In Vitro**

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Investigating the different functions of distinct surface receptors is essential to understand the complex interactions between cells and their extracellular environment. Cells use specific transmembrane receptors of the integrin family to anchor and respond to extracellular matrix (ECM) proteins. In doing so, integrins are capable of regulating cell migration, survival, cell cycle progression, and differentiation, which are essential tasks for the development of all multicellular organisms. Integrins are often classified according to their binding specificity for extracellular ligands. For instance, arginine–glycine–aspartate (RGD)-containing proteins,[1] such as fibronectin (Fn), exhibit high binding affinity to 8 of the 24 integrins expressed in mammals. Among the fibronectin-binding integrins, αvβ3 and α5β1 play crucial roles during embryogenesis, angiogenesis, and in pathology.[2–4] Despite the wealth of information on integrin biology, it is unclear how cell functions and responses are regulated by a single integrin subtype. To address this relevant issue, the design of new active molecules that are able to selectively recognize distinct integrin subtypes is essential for in vitro studies and the development of selective drugs for disease therapy (personalized medicine). Moreover, the conjugation of functional groups to such ligands without affecting their affinity and selectivity for cell surface receptors remains a difficult task to achieve.

Herein, we report a strategy to synthesize αvβ3- or α5β1-specific ligands for the functionalization of nanostructured gold surfaces[5] and demonstrate that cell adhesion can be selectively mediated by a single integrin subtype. The adhesion of either αvβ3- or α5β1-expressing cells was clearly mediated or blocked through coating of gold nanoarrays with these molecules, thus giving striking evidence for their selectivity. These compounds are a powerful tool to elucidate the difference between αvβ3 and α5β1 integrin-mediated cell adhesion.

The interaction of integrins with ECM proteins leads to the clustering of integrins and the recruitment of intracellular proteins to the integrin cytoplasmic domains.[6] The recruited proteins, collectively called the adhesome,[7] play crucial roles in transducing integrin-mediated processes and are thus also involved in biological processes, such as angiogenesis and tumor development. They are therefore interesting targets for pharmacological research.[8,9] Although the functions of integrins in cell adhesion and formation of focal contacts have been studied with unselective cyclic RGD peptides and other ECM mimetics,[10] the exact role of αvβ3 and α5β1 integrin subtypes played in these processes remains to be elucidated. Natural integrin ligands are not well suited for those studies owing to the lack of integrin subtype selectivity.[11]

Coating of surfaces with peptidic integrin ligands for biophysical cell adhesion studies, to develop implant materials, or to identify binding motifs was realized in various cases.[10,12] However, it turned out that peptides, in contrast to peptidomimetics, were not able to achieve high activity and sufficient selectivity. Nowadays, it is still challenging to functionalize selective peptidomimetics for this purpose without losing activity.

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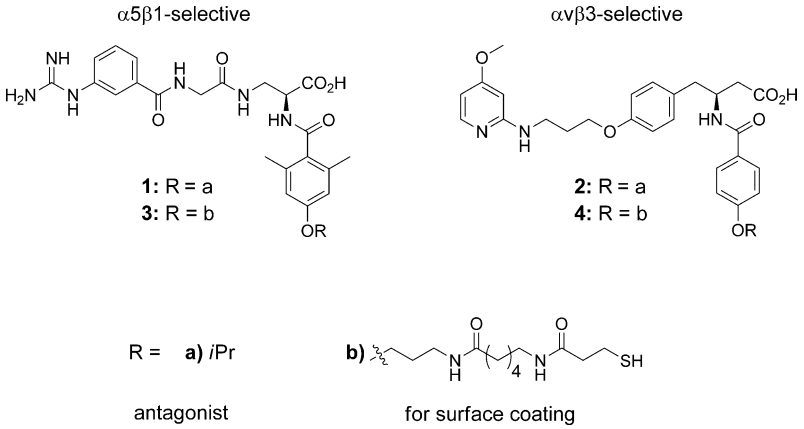
The design of integrin antagonists with the ability to visualize, block, and inhibit distinct integrin subtypes during cell adhesion has been a major goal of research over the last two decades. Since the discovery of the integrin recognition motif RGD,[1] we and others have focused on the design of integrin antagonists specifically targeting either α5β1 or αvβ3, while maintaining selectivity against the platelet integrin αIIbβ3.[13–15] This specificity is particularly important for possible future clinical use, as the inhibition of the RGD-binding αIIbβ3 integrins in platelet adhesion would have deleterious side effects in patients. By designing RGD-based peptidomimetics,[16] we recently obtained the selective antagonist **1** that specifically binds and inhibits α5β1 (Scheme 1).[15] However, the synthesis of a highly active αvβ3 antagonist that has low affinity for α5β1 remained an unmet challenge. By designing a library of β-tyrosine-based scaffolds we have now succeeded for the first time in synthesizing a subnanomolar active αvβ3 antagonist **2** (Scheme 1) with selectivity against α5β1 by two orders of magnitude (Table 1).



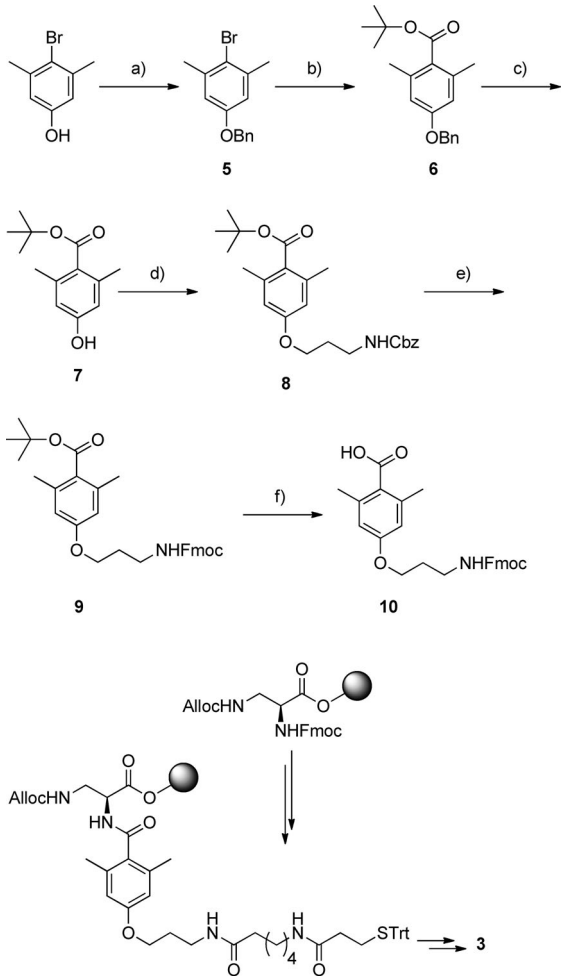
Scheme 1. α5β1-Selective (**1**) and αvβ3-selective (**2**) integrin antagonists and their thiol-functionalized analogues (**3**, **4**) for binding to gold nanoparticles.

Table 1: Selectivity profiles of the free antagonists and their thiol-functionalized derivatives in comparison to cilengitide.[13]

Compound	IC_{50}(αvβ3) [nM]	IC_{50}(α5β1) [nM]
1	3001 ± 205	2.3 ± 0.02
2	0.55 ± 0.07	120 ± 27
3	229 ± 23	1.5 ± 0.09
4	1.8 ± 0.7	130 ± 19
Cilengitide	0.20 ± 0.07	11 ± 1.2

Docking of peptidomimetics into the X-ray structure of the αvβ3 integrin[17] and into the homology model of α5β1 integrin[18] has been performed to elucidate the optimal positions for functionalizing the integrin antagonists without affecting their biological activity.[14,15] We found that the isopropoxy group of the aromatic residue in compound **1** points out of the binding pocket of both α5β1 and αvβ3, and it is thus a suitable position for functionalization. We applied this strategy also to compound **2**, as we assumed, based on the homology model for α5β1,[18] a similar binding mode as for **1**.

For the functionalization of the α5β1-selective antagonist, molecule **10** was synthesized in a six-step synthesis performed in solution (Scheme 2). This molecule is a crucial functionalization building block and offers a convenient way for a straightforward attachment of any desired moiety to the selective integrin antagonist **1** by Fmoc-based[19] (Fmoc = 9-fluorenylmethoxycarbonyl) solid-phase peptide synthesis (SPPS).[20] It should be mentioned that several previous attempts for functionalization of **1** with other building blocks failed both in solution and on solid phase. With compound **10**, the synthesis of the thiol-functionalized α5β1-antagonist **3** could be easily performed on a solid support. After loading



Scheme 2. Synthesis of building block **10** and functionalization of the α5β1-selective integrin antagonist on a solid support, yielding thiol **3**. a) NaH, 0 °C, DMF; BnBr, RT; b) *n*BuLi, −78 °C, THF; Boc_2O, RT; c) H_2, [Pd/C], MeOH; d) 1,1′-(azodicarbonyl)dipiperidine, PBu_3, THF; e) H_2, [Pd/C], MeOH; Fmoc-OSu, $NaHCO_3$, THF/H_2O; f) TFA/triisopropylsilane/H_2O 95/2.5/2.5. Alloc = allyloxycarbonyl, Bn = benzyl, Boc = *tert*-butoxycarbonyl, Cbz = carboxybenzyl, TFA = trifluoroacetic acid, Trt = trityl.

the resin with *N*-α-Fmoc-*N*-β-alloc-L-diaminopropionic acid and Fmoc deprotection, **10** is coupled to the free amine. At this point, the functionalization was performed to introduce any group of interest for further biological investigations that is compatible with Fmoc-based SPPS. Finally, the peptidomimetic backbone was built up leading to the thiolated α5β1 ligand **3** (for the detailed synthesis, see the Supporting Information). Functionalization of the αvβ3-selective antagonist was performed in a similar way.

To prove our concept of functionalization regarding biological activity, we tested the IC_{50} values of the synthesized compounds in a recently reported[21] competitive solid-phase integrin binding assay to evaluate their activity towards the integrins αvβ3 and α5β1 (see the Supporting Information for the detailed procedure). The activity of the thiolated compound **3** for α5β1 was fully retained (1.5 nM) compared to the free antagonist **1** (2.3 nM) as well as the selectivity against αvβ3. The thiol-functionalized compound **4** retained activity for αvβ3 (1.8 nM) and showed remarkable selectivity against α5β1 (130 nM). Furthermore, all compounds showed no activity for the platelet integrin αIIbβ3, which is of high importance for further use. Thus, the obtained IC_{50} values are a substantial proof to validate our concept of functionalization.

To investigate the selectivity profile of compounds **3** and **4**, we performed adhesion assays on functionalized gold nanoarrays with two different genetically modified fibroblast lines that express either αvβ3 or α5β1 integrins. In previous studies, we used gold nanoarrays presenting cyclic RGD peptides to determine the spatial requirements for integrin-mediated cell adhesion and spreading.[5] Moreover, this approach is very powerful, as quasi-hexagonally organized nanoparticles with a diameter of about 7 nm enable testing cellular adhesion, while it prevents uneven distribution of immobilized ligands and thus makes integrin-mediated cell adhesion occur more homogeneously all over the culture substrate. This approach allows the study of cell adhesion to defined binding sites for integrins, while avoiding at the same time unspecific adhesion and integrin binding. Here, gold nanoparticles with a lateral interdistance of 30 nm served as anchoring points for the terminal thiol group of the selective peptidomimetics.[5,22] After passivating the glass substrate with polyethylene glycol (PEG) and functionalization of the gold surface with the integrin-selective compounds **3** and **4**, αvβ3 or α5β1 integrin-expressing fibroblasts were seeded in low serum-containing (0.5 % FBS) medium to reduce non-specific protein adsorption onto the substrate, and, thus allowing the cells to exclusively interact with the immobilized peptidomimetics (Figure 1). The difference in the ability of the two cell lines to adhere onto surfaces functionalized with compound **3** or **4** was striking. As demonstrated in Figure 1, α5β1-expressing fibroblasts adhered after one hour exclusively onto gold surfaces coated with the α5β1-selective compound **3**. In contrast, αvβ3-expressing cells adhered and spread only on the immobilized αvβ3-specific compound **4**. Moreover, both cell lines were not able to adhere onto the other surface even after six hours. To prove that the cell adhesion is triggered specifically by the respective peptidomimetics, cell adhesion assays were performed on surfaces coated with a non-integrin binding peptide, the thiolated c(RADfK), as a negative control. No adhesion of cells could be identified in this case for both cell lines (data not shown). Additionally, we quantified the spreading area and the percentage of spread α5β1- and αvβ3-expressing cells on the selective-functionalized nanoarrays after 1 h and 6 h of incubation (Supporting Information, Figures S1 and S2).

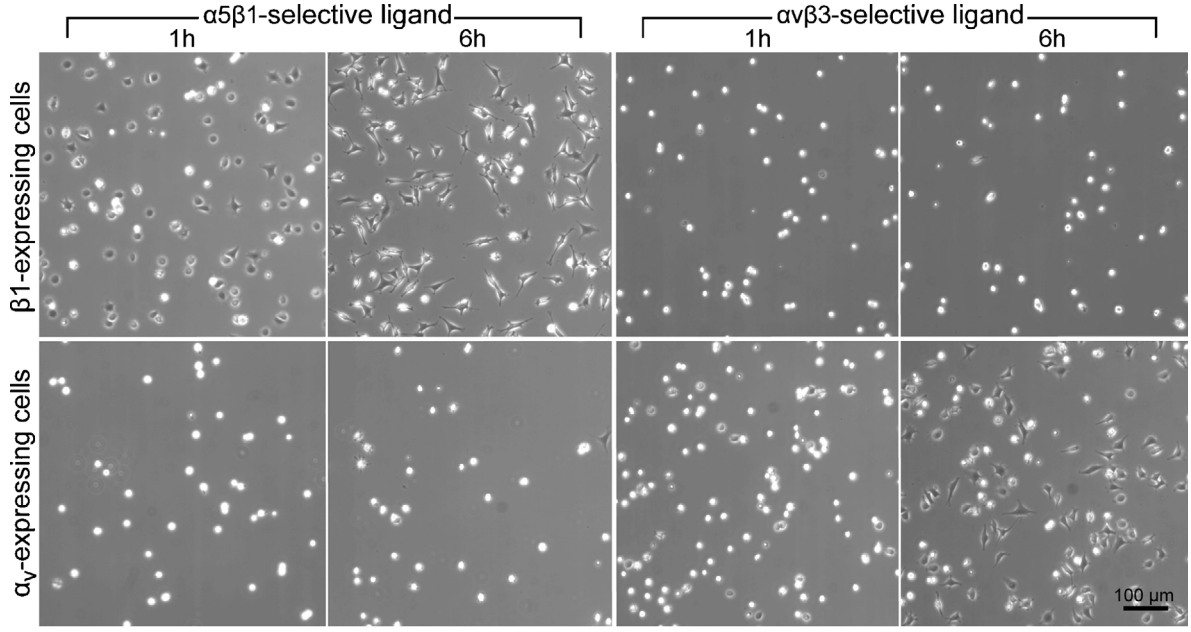


Figure 1. Cell adhesion assay. Low-magnification phase-contrast microscope images of αvβ3 or α5β1 integrin-expressing fibroblasts seeded onto gold nanoarrays and functionalized with an α5β1-selective compound (left) or an αvβ3-selective compound (right). The 100 μm scale bar applies for all of the images.

The analysis of cell spreading for each surface clearly demonstrates a selective cell adhesion mediated by the compounds **3** and **4** although the spreading of αvβ3-expressing cells occurred with a delay of about one hour when compared to the α5β1-expressing cells. The similar time periods required for α5β1- and αvβ3-expressing fibroblasts to spread on gold particle surfaces functionalized with compound **3** or **4**, respectively, and on fibronectin-coated plastic surfaces indicates that integrin subtype-mediated cell adhesion was selectively triggered by the two different integrin-binding peptidomimetics (data not shown).

In conclusion, compounds **3** and **4** are the first α5β1- or αvβ3-selective integrin antagonists functionalized with thiol groups for the surface modification of gold-based substrates. They show a remarkable difference in mediating cell adhesion of αvβ3- or α5β1-expressing fibroblasts. Furthermore, we were able to functionalize antagonists **1** and **2** in an efficient way without losing activity as well as selectivity, as demonstrated by the measured IC_{50} values. Additionally, this study gives a strong hint that such antagonists could be similarly functionalized in that position for any purpose (for example for molecular imaging) while maintaining their biological profiles. The development of these molecules provides a new method to investigate differences between α5β1- or αvβ3-mediated cell adhesive processes.

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