Integrins

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Selective Imaging of the Angiogenic Relevant Integrins α5β1 and αvβ3**

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The binding of αvβ3 and α5β1 integrins to extracellular matrix (ECM) proteins is essential for many biological processes including tumor angiogenesis and tumor development.[1] Currently, there is significant disagreement in the literature as to how and what extent these RGD-binding[2] integrins exert their functions in normal and pathological angiogenesis.[3] Furthermore, it is unclear how inhibition of distinct integrins is influencing tumor growth. Herein, we present selective RGD-based peptidomimetics for specific in vivo targeting and blocking of α5β1 or αvβ3 integrins. These small molecules were functionalized for molecular imaging by positron emission tomography (PET) to specifically target integrins in vivo. Our studies demonstrate the first successful α5β1-based PET imaging using these different peptidomimetics and give a striking demonstration of their integrin subtype selectivity by showing that they can differentiate between two tumor types with different patterns of integrin α5β1 and αvβ3 expression. Additionally, we show that bFGF and VEGF stimulated angiogenesis in rat aortic ring (RAR) assays is specifically blocked by α5β1- or αvβ3-selective inhibition of blood-vessel sprouting with these integrin ligands. Moreover, both ligands exert significant antitumor effects in vivo in a fibrosarcoma model in mice.

The relevance of integrins during tumor angiogenesis and tumor development is well established.[4] This is also reflected by differential integrin expression patterns in normal versus tumor tissue as well as in different stages of tumor progression.[5] Although the functions of integrins have been extensively studied during development and postnatal hemostasis, the exact role of these distinct integrin subtypes in tumor angiogenesis, tumor cell growth, and tumor cell dissemination remains to be fully elucidated. The high expression of αvβ3 integrins on the tumor vasculature led to the development of inhibitory antibodies[6] and small molecules for antitumor therapy.[7] Whereas the RGD peptide Cilengitide[7] attenuates tumor angiogenesis and tumor growth in distinct preclinical models, genetic ablation of αvβ3 integrin or αvβ3 and αvβ5 integrins in mice leads to enhanced tumor angiogenesis, pointing to a complex role of these integrins in this context.[8] One potential explanation for the apparent inconsistency might be a dose dependency of integrin inhibitors, causing either stimulation or blocking of angiogenesis.[3b,c] Moreover, certain integrin-directed antibodies have been shown to induce integrin clustering thereby enhancing tumor angiogenesis.[9] Furthermore, the specificity of antibodies may not be sufficient, since they often do not target the binding pocket, but rather other integrin domains. The role of α5β1 in angiogenesis is not fully established either. Its ability to co-traffic with the epidermal growth factor receptor (EGFR) suggests a tumor-promoting role.[10] Other reports point to a context-dependent function with a promoting role in certain tumors and an inhibitory function in others. Hence, small molecules that recognize these different integrin subtypes selectively in vitro as well as in vivo would enable exploration of different integrin functions.

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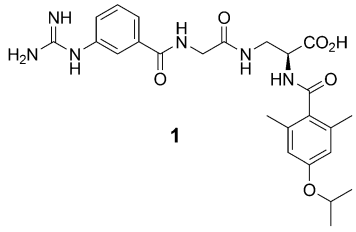
Recently, we have designed RGD-based integrin antagonists specifically targeting either α5β1 or αvβ3, while maintaining no activity for the platelet integrin αIIbβ3.[11] These peptidomimetics (**1** and **2**) were functionalized with 1-((1,3-dicarboxy)propyl)-4,7-(carboxymethyl)-1,4,7-triazacyclononane (NODAGA), a commonly used bifunctional chelator,[12] to yield the α5β1-selective compound **3** and the αvβ3-selective compound **4** (Table 1). Determination of their IC_{50} values in a recently reported competitive solid-phase integrin binding assay[13] pointed out that incorporation of the NODAGA chelator was achieved without affecting activity or selectivity in comparison to free ligands (Table 1). Thus, **3** and **4** were used to verify their subtype selectivity in vivo and to assess their potential as agents for PET imaging after labeling with $^{68}Ga^{3+}$.

Table 1: Structures and selectivity profiles of α5β1- and αvβ3-selective antagonists (**1** and **2**) and the functionalized derivatives (**3** and **4**) for PET imaging. All IC_{50} values are referenced to Cilengitide.

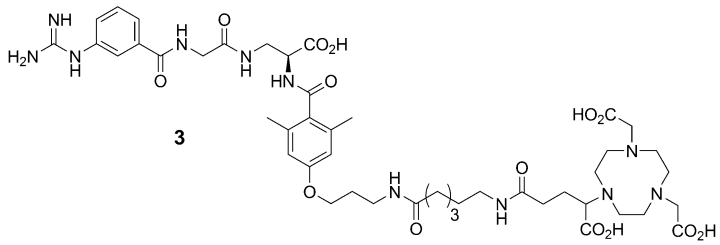
Compound	IC_{50}(αvβ3) [nM]	IC_{50}(α5β1) [nM]
1[a]	>1000	2.3 ± 0.02
2[a]	0.55 ± 0.07	120 ± 27
3	>1000	1.3 ± 0.08
4	1.5 ± 0.6	118 ± 7.1
Cilengitide[a]	0.20 ± 0.07	11 ± 1.2

[a] These values have been reported previously.[11c]

α5β1-selective:

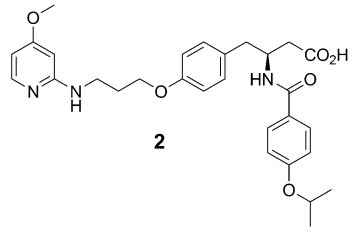


1



3

αvβ3-selective:



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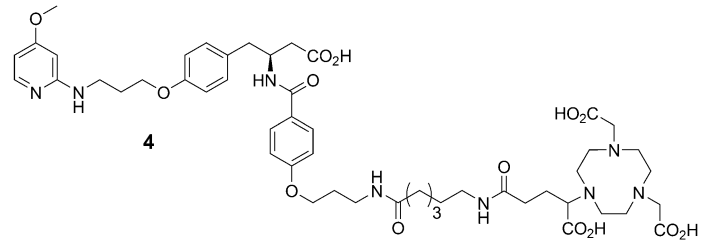

4

To confirm the selective tumor accessibility of the compounds, we investigated the ^{68}Ga-labeled integrin antagonists **3** and **4** in small-animal PET imaging experiments (Figure 1). To this end, female BALB/c nude mice were inoculated with a highly α5β1-expressing tumor xenograft (human colon carcinoma, RKO) in the right shoulder and a highly αvβ3-expressing tumor xenograft (human melanoma, M21) in the left shoulder, with only low endogenous α5β1-expression.

The α5β1-selective compound **3** specifically accessed the α5β1-expressing tumor in the right shoulder of the mouse (Figure 1 A). In addition to the strong uptake into the tumor displaying α5β1, we also observed some uptake in the αvβ3-expressing tumor (left shoulder). This is a result of α5β1 integrin co-expression at low level in this cell line, as demonstrated by FACS analysis (Supporting Information, Figure S1). To verify the specificity of tracer binding to α5β1, we performed a blocking study by injecting 50 μg of unlabeled compound **3** 10 min prior to tracer injection (Figure 1 B). Uptake of **3** into the α5β1-positive tumor was efficiently blocked, and uptake found in all other organs was negligible. Activity accumulation was only found in kidneys and bladder, owing to the renal excretion of the tracer. In the complementary experiment, the αvβ3-selective compound **4** showed selective uptake in the αvβ3-expressing tumor grown in the left shoulder and no uptake at all in the α5β1-displaying tumor (Figure 1 C). The higher uptake in the liver and intestine of compound **4** compared to compound **3** may result from the lower hydrophilic properties of compound **4** compared to the α5β1 antagonist. It is evident that for future studies and clinical translation, the pharmacokinetic properties of this radiotracer certainly have to be optimized by increasing its hydrophilic properties. However, for investigating the selectivity profile and targeting distinct integrin subtypes, which is the focus of this proof of principle study, the PET imaging characteristics of compound **4** were fully sufficient. Thus, the imaging fits exactly to the integrin profiles of the tumor cell lines as demonstrated by FACS analysis, which verified high α5β1 expression and lack of αvβ3 expression in the RKO cells, and high αvβ3 expression in M21 cells, with only low α5β1 expression (Figure S1). Moreover, immunohistochemistry and autoradiography experiments confirmed the in vivo PET imaging results and provided evidence for the high selectivity and specificity of compounds **3** and **4** in imaging the α5β1 and αvβ3 integrins, respectively (Figure S2).

Furthermore, we investigated the anti-angiogenic effects of **1**, **2**, and Cilengitide (6 μM or 60 μM) on spontaneous capillary sprouting from fresh rat aorta rings cultured in

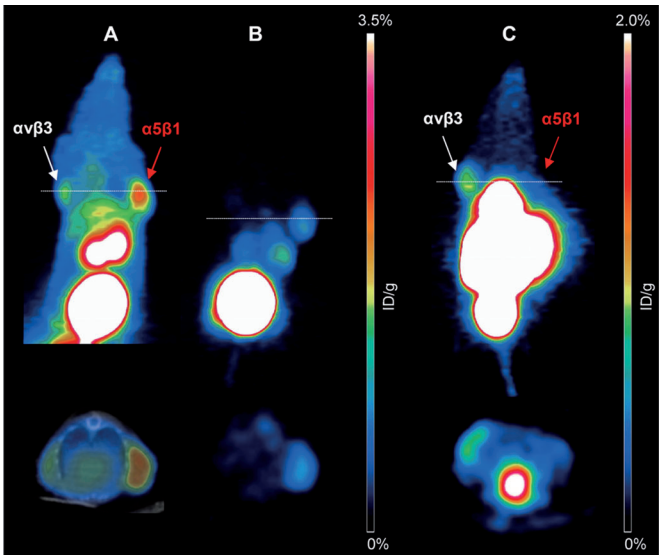

Figure 1. Maximum intensity projection (MIP) images of microPET scans. Upper row: mice bearing RKO and M21 tumor xenografts on right and left shoulder, respectively. MIP comprises data recorded 70–90 min after injection of radiotracer (^{68}Ga-**3** or ^{68}Ga-**4**). White arrow: M21; red arrow: RKO. Lower row: axial slices corresponding to the white line in upper row MIP images. A) Injection of ^{68}Ga-**3**. B) ^{68}Ga-**3** blocking experiment (injection of 50 μg (2.5 mg kg^{-1} body weight) of unlabeled **3** 10 min prior to tracer injection). C) Injection of ^{68}Ga-**4**.

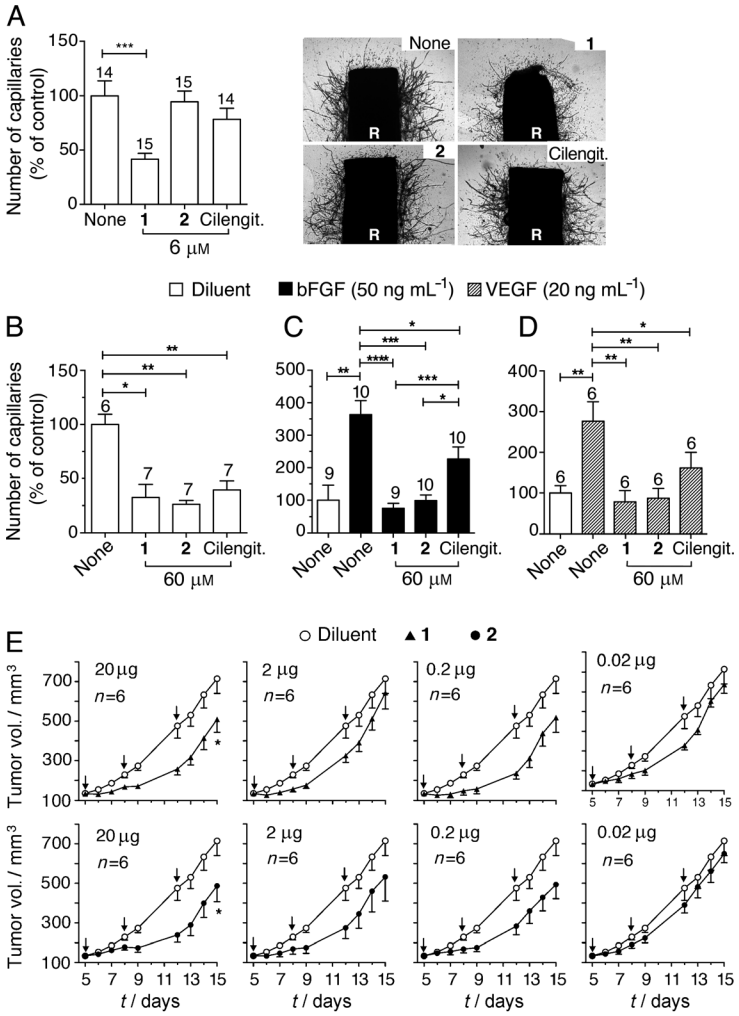

Figure 2. Effect of **1**, **2**, and Cilengitide on spontaneous, bFGF- or VEGF-induced capillary sprouting from rat aortic rings cultured in collagen gel. Rings of rat aorta were embedded in collagen gels immediately after excision from the animal (fresh rings; A and B) or after incubation in culture medium for 10–14 days and washing (3-times per week) to reduce endogenous pro-angiogenic factors (quiescent rings; C and D). Bars represent the number of capillary-like structures emerging from the aorta rings after 4–5 days, expressed as percentage of control (means ± standard error). The number of rings used is indicated in each panel. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; Mann Whitney test. E) Effect of **1** and **2** on WEHI-164 fibrosarcoma growth in mice. Antitumor activity of peptidomimetics administered (i.p.) at indicated doses. Arrows indicate time of treatment. Tumor volumes (mean ± standard deviation, 6 mice/group). *$p < 0.05$, two-tailed t-test.

collagen gel. Compound **1**, but not **2** or Cilengitide, significantly inhibited capillary formations when administered at a concentration of 6 μM, whereas all compounds displayed significant inhibitory effects at a dose of 60 μM (Figure 2A,B). Significant inhibitory effects were obtained with all compounds (60 μM) on bFGF- or VEGF-induced capillary sprouting from quiescent rings (Figure 2C,D). In these assays Cilengitide induced a lower anti-angiogenic effect than the peptidomimetics **1** and **2**, both of which clearly showed strong anti-angiogenic properties pointing towards their potential to target angiogenesis.

In addition, we investigated the effect of systemic administration of various doses of **1** and **2** in mice bearing syngeneic subcutaneous WEHI-164 fibrosarcomas. Both ligands induced comparable delays of tumor growth, even at low doses (Figure 2E), suggesting that selective inhibition of α5β1 and αvβ3 can induce similar antitumor effects. Of note the antitumor effects were comparable to those exerted by Cilengitide administered in the same in vivo model,[14] suggesting that combined integrin blockade does not induce synergistic or additive antitumor effects.

In conclusion, we functionalized the α5β1- and αvβ3-selective antagonists **1** and **2** with NODAGA and gave a striking demonstration of in vivo targeting and of discrimination between tumor cells based on their different integrin pattern. In this regard, compound **3** is the first α5β1-selective integrin antagonist that enables specific molecular imaging by PET. Furthermore, with compounds **1** and **2** we were able to selectively address the angiogenic relevant integrins α5β1 and αvβ3, respectively, and to delay tumor growth in vivo. The development of these integrin-selective compounds will be of high medical relevance not only for diagnostic imaging and disease monitoring purposes (e.g. in patients afflicted with cancer), but also for therapeutic interventions by selective targeting of specific integrin species (personalized medicine), which are involved in a plethora of pathophysiological conditions, including cancer, arthritis, and cardiovascular disorders.

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