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ORIGINAL ARTICLE

Integrin $\alpha_v\beta_3$ targeting activity study of different retro-inverso sequences of RGD and their potentiality in the designing of tumor targeting peptides

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Abstract Retro-inverso peptide represented the isomer of a parent peptide in which the direction of the sequence was reversed and the chirality of each amino acid residue was inverted. Generally, retro-inverso peptides possessed equal or even higher activities compared to the original peptide. RGD was a commonly used ligand for tumor and vascular targeting due to its affinity to integrin $\alpha_v\beta_3$ receptors. The biological activity study of the isomers of RGD would indeed provide useful suggestions for the design of tumor targeting peptides. Therefore, the tumor targeting activities of octa-arginine which was modified with different retro-inverso sequences of RGD peptide were investigated in this study. Three different tandem peptides (R8-GDGR, R8-GdGr and R8-GdGR) were designed on the basis of R8-GRGD. The tumor targeting activities of these tandem peptides were evaluated both in vitro and in vivo. Finally, R8-GdGR displayed selective binding affinity to integrin $\alpha_v\beta_3$ at the cellular level, and exhibited efficient tumor homing and penetrating capabilities in vivo. Meanwhile, R8-GdGR also showed stronger neovessel targeting ability compared to the others. In conclusion, all the results demonstrated that dGR possessed similar biological activity to RGD and was a potential ligand for further designing of tumor targeting peptides.

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Introduction

Integrins are a class of cell adhesion receptors which play an important role in cell–cell and cell–matrix interactions during developmental and pathological processes (Barczyk et al. 2010; Winograd-Katz et al. 2014; Shattil et al. 2010). By forming physical connections between the inside and outside of a cell, integrins trigger a series of cellular responses including cell adhesion, migration, proliferation, survival and differentiation (Seguin et al. 2015; Desgrosellier and Cheresh 2010; Goel and Mercurio 2012). The integrin receptors are known to be glycoprotein heterodimers composed of non-covalently associated α and β subunits (Barczyk et al. 2010; Trabocchi et al. 2010). Among all the 24 different isoforms of integrin receptors, integrin $\alpha_v\beta_3$ is found overexpressed on both angiogenic endothelial cells and different malignant tumor cells such as melanomas, gliomas and oophoromas (Kibria et al. 2011; Magnon et al. 2005; Guo et al. 2014), and is considered to be highly correlated with angiogenesis, tumorigenesis, metastasis and drug resistance (Seguin et al. 2015; Leblanc et al. 2014; Contois et al. 2015). Therefore, the three amino acid sequence RGD motif (arginine–glycine–aspartic acid), which showed specific binding affinity to integrin α_v family (especially to integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$), had been widely investigated as an active targeting ligand for tumor homing drug delivery (Kibria et al. 2011; Vachutinsky et al. 2011; Graf et al. 2012; Yan et al. 2011; Zheng et al. 2014; Mingozzi et al. 2014).

The biological activities of peptides are mostly dependent upon the primary and secondary structures (Abraham

et al. 2014; Goodman and Chorev 1979; Bernstein et al. 2015; Staykova et al. 2015). In 1978, Goodman had proposed the concept of retro-inverso peptide, which represented the isomer of a parent peptide in which the direction of the sequence was reversed and the chirality of each amino acid residue was inverted (Goodman and Chorev 1979; Wermuth et al. 1997; Gentilucci et al. 2009). Theoretically, the retro-inverso peptide presents an orientation of the side chains similar to that of the parent peptides; thus, this kind of isomer may exhibited equal or even higher bioactivities compared to the original structures (Li et al. 2013; Wang et al. 2014). Recently, many researches had proved the retained activity and enhanced stability of retro-inverso isomers of the parent peptides (Li et al. 2013; Wang et al. 2014; Taylor et al. 2000; Lim et al. 2010).

In our previous study, we had designed a tandem peptide R8-RGD by conjugating a specific targeting cyclic RGD motif to a cell penetrating peptide octa-arginine, so that the formed tandem peptide combined both advantages of tumor homing peptide and cell penetrating peptide, and possessed both high binding affinity and efficient internalization properties to tumor cells (Liu et al. 2014). On the other hand, it is reported that when an R/KXXR/K sequence was exposed at the C-terminal of a peptide, this peptide would specifically bind to the cell surface receptor neuropilin-1 (NRP-1), and undergo a receptor-mediated cell, vascular and tissue penetration process (Teesalu et al. 2009). This phenomenon was termed as C-end Rule. On this basis, we found that when a reversed sequence DGR was directly conjugated to octa-arginine, the formed peptide would possess an RDGR motif at the C-terminal. Thus, we hypothesized the tandem peptide R8-DGR might exhibited stronger tumor targeting and penetrating properties due to the combination of integrin targeting and C-end Rule mediated penetration. Therefore, the tumor targeting activity investigation of the reversed DGR sequence is of great importance. In this study, we mainly focused on the integrin targeting capability investigation of several retro-inverso isomers of a tandem peptide R8-GRGD (RRRRRRRRG RGD) which would provide preliminary supporting for the further designing of an efficient tumor targeting and penetrating peptide. A glycine was utilized herein to serve DGR motif apart from R8 so that the existence of Cend Rule sequence was temporarily avoided to ensure the integrin binding activity. Several FITC-labeled tandem peptides with inversed sequence and different chirality of R8-GRGD were synthesized. Cellular uptake and competitive inhibition study were utilized to evaluate the tumor targeting properties in vitro, and in vivo biodistribution of these peptides was also carried out in order to identify the influence on the tumor homing and neovascular targeting activities of the tandem peptide when the RGD domain transformed to several different retro-inverso isomers.

Materials and methods

Materials and animals

Fluorescein isothiocyanate (FITC)-labeled peptides (R8-GRGD, R8-GDGR, R8-GdGr, R8-GdGR and R8-G) were synthesized according to the standard solid phase peptide synthesis by Chinapeptides Co. Ltd. (Shanghai, China). 4′-6-diamidino-2-pheylindole (DAPI) was purchased from Beyotime Institute Biotechnology (Haimen, China). Rabbit anti-mouse CD31 primary antibody was purchased from Epitomics, Abcam (California, USA). Alexa Fluor 594 Donkey anti-Rabbit IgG antibody was purchased from Jackson Immunoresearch Laboratories, Inc. (USA). Plastic cell culture dishes and plates were purchased from Wuxi NEST Biotechnology Co. (Wuxi, China). The other chemicals were obtained from commercial sources.

Balb/c mice were purchased from West China animal center of Sichuan University (Sichuan, China). All animal procedures for this study were approved by the Experiment Animal Administrative Committee of Sichuan University.

Cell culture

Murine glioma cells (C6) and human cervical carcinoma cells (Hela) were cultured in RPMI-1640 and DMEM medium, respectively, at 37 °C in a 5 % CO_2 humidified environment incubator (Thermo Scientific, USA). Both medium contained 10 % FBS, 100 U/mL penicillin and 100 μg/mL streptomycin.

Cellular uptake evaluation

For quantitative study, C6 cells and Hela cells were plated into six-well plates at a density of 5×10^5 cells per well and cultured for 24 h. FITC-labeled peptides were dissolved in ultrapure water and the fluorescence intensity among different groups was adjusted. Then different peptides were added into the plates at a final concentration of 0.5 μM for 4 h incubation at 37 °C. The cells were washed three times, trypsinized and then resuspended in 0.5 mL PBS. Finally the fluorescence intensity of different cells was measured by flow cytometer (Cytomics FC 500, Beckman Coulter, USA).

For qualitative study, gelatin-coated cover slips were placed in six-well plates beforehand. C6 cells and Hela cells were plated on the cover slips at a density of 1×10^5 cells per well and cultured for 24 h. FITC-labeled peptide solution was added at a final concentration of 0.5 μM. After 4 h incubation, cells were washed three times with cold PBS, fixed with 4 % paraformaldehyde for 30 min at room temperature, and finally stained with DAPI for 5 min.

Then the cells were imaged by a confocal microscope (FV1000, Olympus, USA).

As for the competitive inhibition study, free RGD peptide was pre-incubated with the C6 cells for 30 min at 37 °C before the adding of FITC-labeled peptides. Then the cells were treated as described above and imaged by a confocal microscope (FV1000, Olympus, USA).

Tumor bearing mice models

Balb/c mice weighing 20–25 g were anesthetized with 5 % chloral hydrate and inoculated subcutaneously with 1×10^6 C6 cells in the left flank. These mice were raised under standard condition and were used for experiments when the diameter of tumor reached about 10 mm.

Ex vivo imaging

FITC-labeled peptides were dissolved in ultrapure water and injected into C6 xenograft tumor-bearing mice via the tail vein at a dose of 500 μg FITC/kg. 4 h later, the mice were killed after heart perfusion with saline and 4 % paraformaldehyde. The whole tumors were collected and washed with cold PBS. Then the tumors were imaged with IVI Spectrum system (Caliper, Hopkington, MA, USA).

Tumor penetration and immunofluorescence analysis

The collected tumors were then fixed in 4 % paraformaldehyde, dehydrated in 10 and 30 % sucrose solution, and sectioned at a thickness of 10 μm. The tumor sections were incubated with rabbit anti-CD31 antibody overnight, followed by 1 h incubation with Alexa Fluor 594 Donkey anti-Rabbit IgG antibody for labeling blood vessels. The sections were then treated with DAPI for nuclei staining and imaged by a confocal microscopy (FV1000, Olympus, USA).

Results and discussions

Quantitative and qualitative evaluation of cellular uptake in vitro

On the basis of the parent peptide R8-GRGD, we designed three peptides with different retro-inverso sequences of the RGD domain including R8-GDGR, R8-GdGr and R8-GdGR (peptide sequences were listed in Table 1). R8-G without an active targeting motif was used as negative controlled peptide. The tumor targeting activities of these three tandem peptides were evaluated using flow cytometer on two cell lines in vitro. Murine glioma C6 cells and human cervical carcinoma Hela cells were chosen as integrin $\alpha_v\beta_3$ positively and negatively expressed cell lines, respectively, as reported (Liu et al. 2014; Dearling et al. 2013). According to the quantitative results (Fig. 1), on Hela cells which expressed less integrin receptors, no significant difference was observed among all the five peptides. However, R8-GRGD showed an enhanced cellular uptake to almost 3-fold higher than R8-G on C6 cells, implying that the addition of RGD domain to the sequence endued selective integrin $\alpha_v\beta_3$ targeting affinity to cell penetrating peptide. Among the other three tandem peptides, R8-GdGR exhibited similar targeting activity to R8-GRGD, while the uptake amount of R8-GDGR and R8-GdGr was obviously decreased compared to the parent peptide. Confocal images of the cellular uptake provided similar results to the quantitative data. Compared to R8-G, significant stronger green fluorescence was observed in the R8-GRGD group on C6 cells, indicating more FITC-labeled peptide was internalized into the cells (Fig. 2a). Similarly, the fluorescence intensity of R8-GdGR was nearly equal to R8-GRGD while that of R8-GDGR and R8-GdGr was relatively weaker than the positive control. And the uptake level of these five different FITC-labeled peptides was almost the same on Hela cells (Fig. 2b).

The difference of the cellular uptake on C6 cells among these three tandem peptides demonstrated that a tiny change of one amino acid residue would greatly influence

Table 1 List of peptide sequences (lower case letter represents D-amino acid residue)

Peptide	Sequence
R8-GRGD	FITC-RRRRRRRR**GRGD**
R8-GDGR	FITC-RRRRRRRR**GDGR**
R8-GdGr	FITC-RRRRRRRR**GdGr**
R8-GdGR	FITC-RRRRRRRR**GdGR**
R8-G	FITC-RRRRRRRR**G**

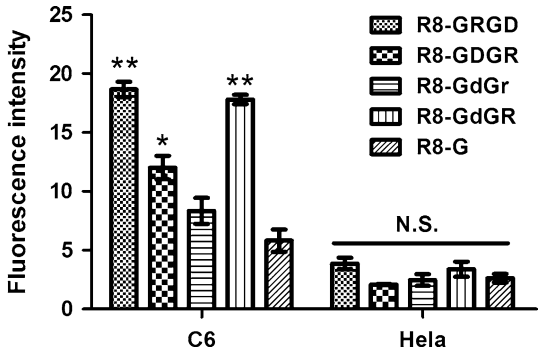

Fig. 1 Cellular uptake of FITC-labeled peptides on C6 cells and Hela cells measured by a flow cytometer ($n = 3$, mean ± SD), * and ** indicate $p < 0.05$ and $p < 0.01$ versus R8-G group

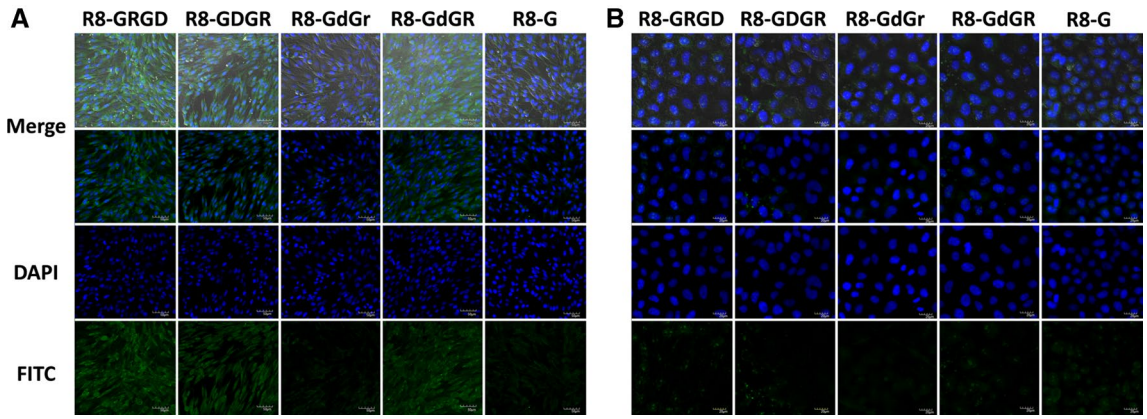

Fig. 2 Confocal images of cellular uptake on C6 cells (**a**) and Hela cells (**b**) 4 h after incubation with FITC-labeled peptides

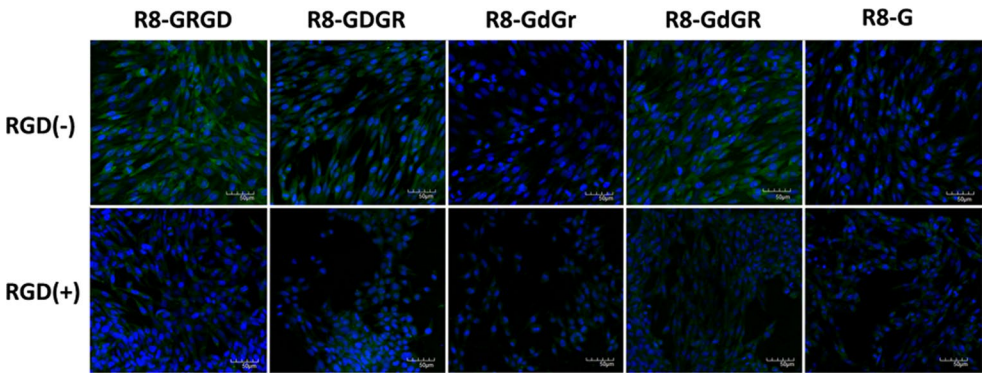

Fig. 3 Cellular uptake of FITC-labeled peptides on C6 cells without (*top*) or in the presence of free RGD (*bottom*) imaged by confocal laser scanning microscopy

the biological activity of peptides. Actually, it is commonly considered that retro-inverso isomers should reverse the sequence and invert the chirality of all the amino acid residues so that to form a similar spatial structure to the parent peptide (Taylor et al. 2000; Li et al. 2013). However, the complete retro-inverso isomer dGr exhibited the minimal cellular uptake efficiency on integrin overexpressed cells while dGR maintained the specific targeting affinity compared to RGD. Therefore, it is necessary to investigate the tumor targeting activity of different isomers of RGD both in vitro and in vivo.

On the other hand, a glycine (G) was utilized as the linker between octa-arginine and the RGD motif or its retro-inverso isomers to get more credible results. If dGR was directly conjugated to R8, then the enhanced cellular internalization property might come from the C-end Rule effect instead of binding to integrin $\alpha_v\beta_3$. As the C-end Rule phenomenon was proved to be strictly sequence-dependent and its efficient penetration effect was confirmed in many researches (Teesalu et al. 2009; Sugahara et al. 2009; Alberici et al. 2013; Wang et al. 2014; Pang et al. 2014), we used the glycine to serve DGR motif apart from R8 so that it could be concluded that dGR indeed maintained the integrin targeting activity of RGD in vitro.

Competitive inhibition study

To further ensure the enhanced internalization ability of these tandem peptides, a competitive inhibition study was carried out. Pre-incubation with competitive inhibitors could specifically block the receptors prior to the active targeting cargoes and inhibit receptor-mediated endocytosis (YoungáChoi et al. 2009; Choi et al. 2010). Cellular uptake on C6 cells without or in the presence of free RGD peptide was observed using confocal laser scanning microscope. As shown in Fig. 3, although the green fluorescence of these five peptides internalized into C6 cells displayed significant differences, the presence of free RGD successfully inhibited the cellular uptake of the tandem peptides to almost the same as that of R8-G, especially for R8-GRGD and R8-GdGR. This result confirmed that all these tandem peptides possessed enhanced internalization abilities through binding to integrin $\alpha_v\beta_3$ receptors and also suggested the similar biological activity of dGR to RGD.

Tumor targeting property in vivo

C6 xenograft tumor bearing Balb/c mice were utilized to evaluate the tumor targeting property of these five peptides

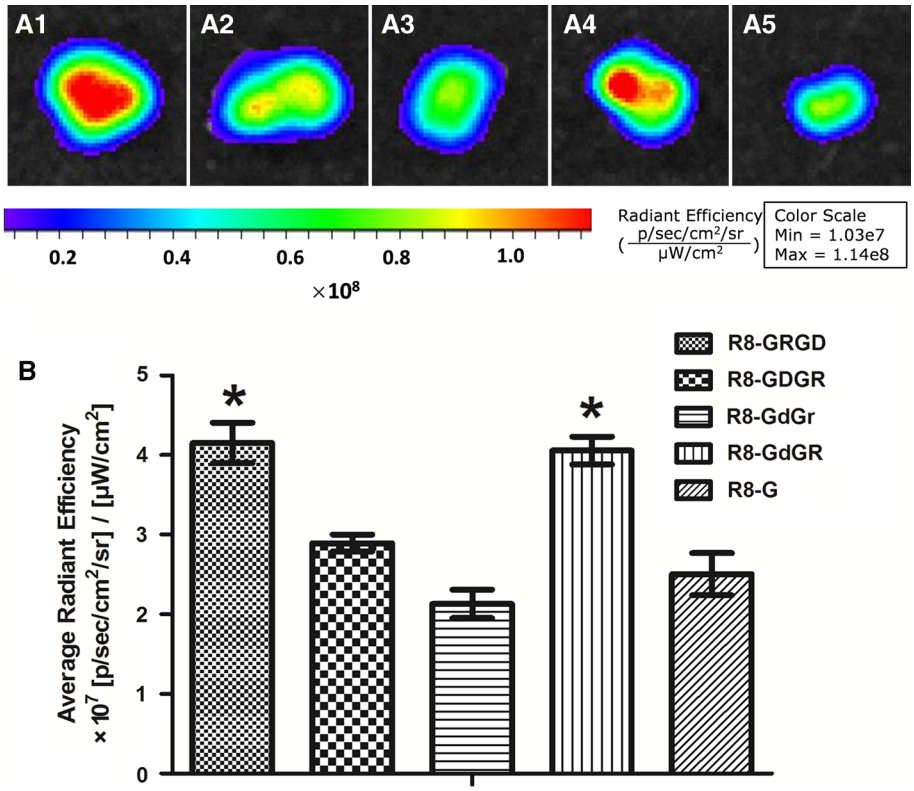

Fig. 4 Ex vivo imaging of tumors 4 h after systemic administration of FITC-labeled peptides. **a1** R8-GRGD, **a2** R8-GDGR, **a3** R8-GdGr, **a4** R8-GdGR, **a5** R8-G. **b** Relative fluorescence intensity of the tumors ($n = 3$, mean ± SD), $^*p < 0.05$ versus R8-G group

in vivo. Unlike nanocarrier-based drug delivery systems which could passively accumulate in the tumor through enhanced permeability and retention (EPR) effect, peptides could only achieve the tumor foci by actively recognizing the receptors overexpressed on tumor cells (Maeda 2015). Positively charged cell penetrating peptides such as octa-arginine in this study, would strongly accumulate and penetrate into their first-met vascular beds: the lungs (Teesalu et al. 2009; Zhang et al. 2013; Qin et al. 2011), thus R8-G exhibited relative weak accumulation in the tumor (Fig. 4a5). However, when a selective targeting motif RGD was conjugated to R8, the amount of peptide accumulated in the tumor was significantly increased (Fig. 4a1). R8-GdGR still displayed similar enhanced tumor accumulation property as R8-GRGD, while the tumor targeting activities of R8-GDGR and R8-GdGr were obviously weaker (Fig. 4a2–a4). The semiquantitative data in Fig. 4b demonstrated the same results. Therefore, dGR was proved to show the most efficient tumor targeting activity in vivo among these three isomers.

Tumor penetration property

To evaluate the tumor penetration property of R8-GRGD and its isomers, immunofluorescence staining was carried out. CD31 was utilized to stain the neovessels. As shown in Fig. 5, although the fluorescence signal of R8-G was relative weak, it could be seen that green signal in R8-G group was all around the tumor cells, indicating that octa-arginine only induced cell and tissue penetrating but could not target tumor vessels. However, when RGD was added to the sequence of the peptide, the tumor penetrating ability was significantly enhanced with stronger green signals being observed. Meanwhile, R8-GRGD also displayed neovessels targeting ability of which yellow fluorescence signal representing accurate co-localization of peptide and tumor vessels was detected. Integrin $\alpha_v\beta_3$ was reported to be highly expressed on tumor neovessels and targeting neovessels would benefit the chemotherapeutics (Kiugel et al. 2014; Gao et al. 2014; Nie et al. 2014; Yang et al. 2014). R8-GdGR, which exhibited efficient integrin $\alpha_v\beta_3$ targeting ability both in vitro and in vivo, also displayed similar penetration and targeting capabilities to R8-GRGD in the experiment. On the other hand, R8-GDGR and R8-GdGr both exhibited weaker tumor penetration properties compared to R8-GdGR.

Conclusions

In this study, we have designed three isomers of a tandem peptide R8-GRGD by conjugating different retro-inverso sequences of RGD to octa-arginine and investigated the tumor targeting activity of these peptides. Among these

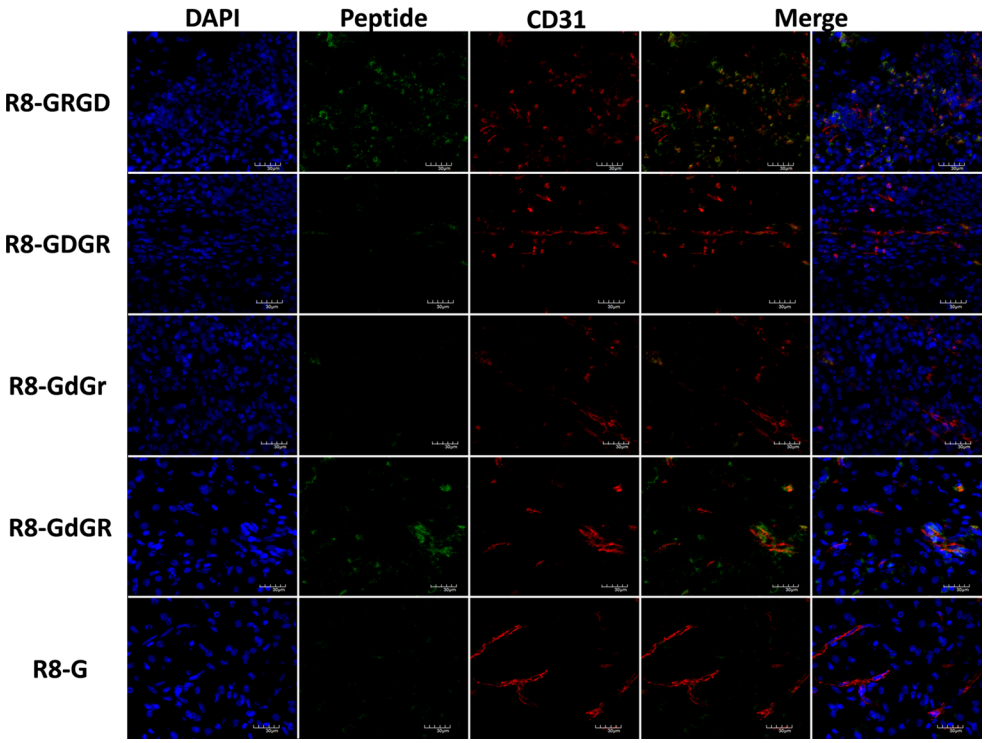

Fig. 5 Confocal images of tumor sections from C6 xenograft tumors 4 h after systemic administration of FITC-labeled peptides (*green*). The sections were stained with CD31 antibody (*red*) for tumor blood vessel and DAPI (*blue*) was used for nuclei staining (color figure online)

three tandem peptides, R8-GdGR displayed the most efficient tumor and neovascular targeting capabilities through binding to integrin $\alpha_v\beta_3$ receptors both in vitro and in vivo. In conclusion, all the results suggested that dGR was a potential ligand for further designing of tumor targeting peptide which possessed similar biological activity to RGD.

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Conflict of interest We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work and that there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in the manuscript.

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