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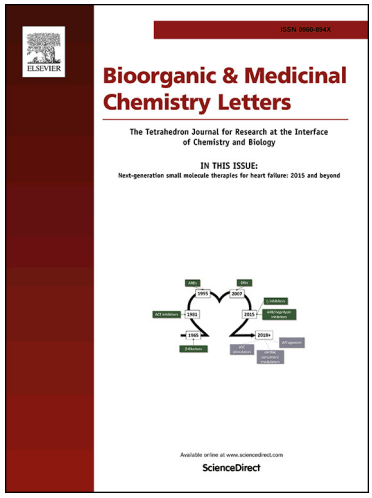

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Design and synthesis of novel dual-cyclic RGD peptides for $\alpha_v\beta_3$ integrin targeting

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ABSTRACT

The specific binding of RGD cyclic peptide with integrin $\alpha_v\beta_3$ attracts great research interest for tumor-targeting drug delivery. Herein, we designed and synthesized a series of dual-ring RGD-peptide derivatives as a drug carrier for $\alpha_v\beta_3$ targeting. Three novel peptides showed excellent cell adhesion inhibition effect, in which, P3 exhibited 7-fold enhancement in IC_{50} compared with cyclo(RGDfK). Drug-loaded cytotoxicity experiment and imaging experiment indicated that such dual-cyclic RGD peptides have good tumor targeting effects. This work provides a new strategy for the design of novel RGD peptides.

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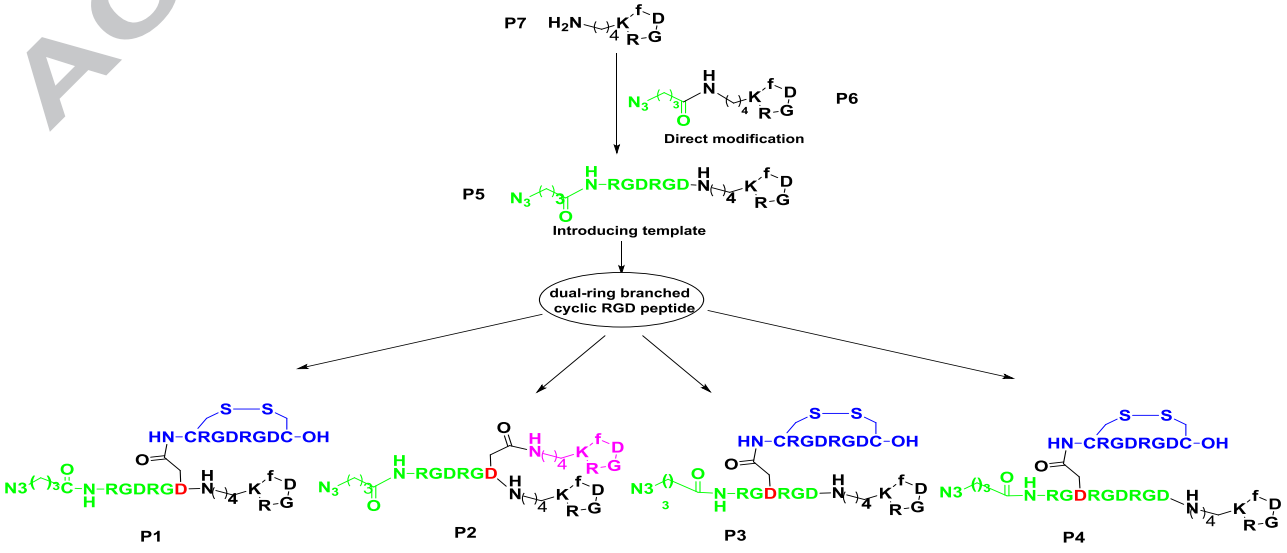
In the past decade, peptides and their derivatives have attracted great attention in the field of drug development.[1, 2] Among them, the arginyglycylaspartic acid (RGD) peptide as a recognized ligand of integrin has a good application prospect in tumor diagnosis and targeted therapy. Integrin $\alpha_v\beta_3$ is highly over-expressed on the surface of various tumor cells, but it is expressed much less in mature vascular endothelial cells and normal organ systems.[3] Therefore, the specific binding of the RGD peptide with the integrin $\alpha_v\beta_3$ provides a promising strategy for tumor-targeting delivery of therapeutic molecules which could dramatically reduce the damage to normal tissues or organs during tumor treatment[4] and also directly inhibit the interaction of integrin with extracellular matrix protein to induce apoptosis of tumor cells. In the previous work, RGD peptide has demonstrated prospective application as a tumor-targeting moiety to reduce adverse effects and enhance anti-tumor activity. Meanwhile, development of novel RGD peptides against integrin $\alpha_v\beta_3$ with higher affinity, better specificity, and enhanced stability represents a new trend in this field.

Compared to linear peptides, cyclic peptides exhibit increased structural stability which is useful in peptide drug design for optimized properties, including enhanced *in vivo* stability, prolonged half-life, and improved biological activity.[5-8] A typical subtype of cyclic peptides consists of a disulfide bond between side-chain Cystine motifs to form a loop peptide which indicates potentials in drug development.[9] It is also reported that the cyclic RGD peptides has better binding affinity with integrin on the cell membrane compared with the linear RGD,[1, 7, 10] as well as better tumor-targeting and anti-cancer effect.

Cyclo(RGDfK), originally designed and synthesized by the Kessler's group,[7] is a cyclic RGD peptide with good affinity and selective inhibition against integrin $\alpha_v\beta_3$.[11] The Lys moiety of this peptide can serve as a conjugation site since its side-chain amino group may react with a small-molecule payload to achieve drug-RGD conjugates.[12] A major concern on this strategy is that the integrin binding affinity of cyclo(RGDfK) might decrease after drug conjugation. In previous study, two moieties of cyclo(RGDfK) were employed to attain a dimeric RGD peptide E[c(RGDfK)]$_2$ with a proper distance linker(Gly-Gly-Gly,PEG$_4$ et al.) [13,14] to fit the distance between two integrins, which enhance the interaction in a "bivalent" manner.[15] RGD peptides have been extensively used in conjugation with nanoparticles, radionuclides and near-infrared fluorescent dyes, providing a wide application prospect in the corresponding tumor treatments such as the tracing of therapeutic agents or targeted drug delivery.[15,16,17]

On the other hand, using cyclo(RGDfK) as a carrier for drug conjugation and integrin-targeting delivery encounters practical issues such as its relatively lower binding affinity with integrin compared with the natural cyclic peptide Cilengitide and the decreased integrin interaction after modifications. Herein, we sought to design a series of novel dual-cyclic RGD peptides, which could improve and maintain the integrin binding while conjugating with small-molecule drugs. We tend to assemble two different cyclic RGD motifs onto the C-terminus and side chain of a linear RGD peptide template, forming a unique branched cyclic RGD peptide system for optimizing the integrin targeting effect. Branched cyclic peptides naturally exist in the biosystems, such as "Lassopeptide"[18] and the recently reported branched cyclic peptide Lassomycin with a selective antibacterial activity against *Mycobacterium tuberculosis*. Branched cyclic peptide is also a kind of biomolecule with great potential for drug discovery and development.[19]



Scheme 1: The designed compounds of dual-cyclic RGD peptide P1-P4.

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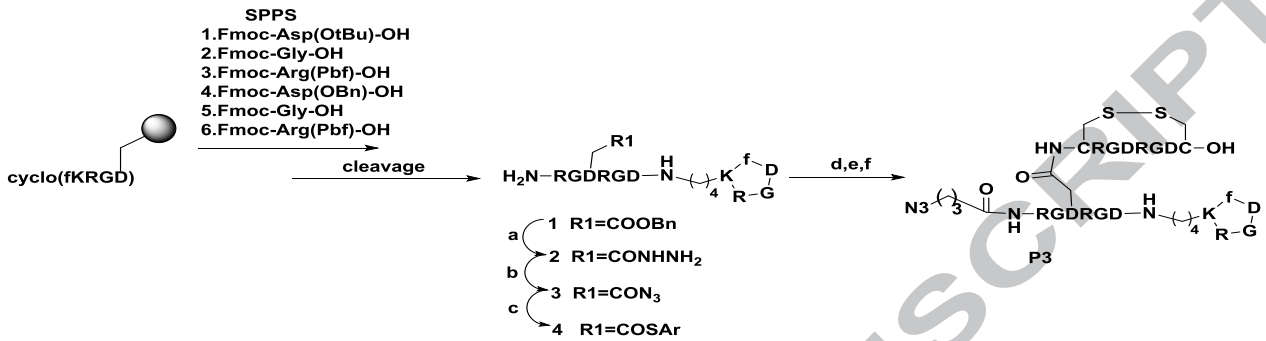
In this work, we used the linear RGD hexapeptide or nonapeptide as the templates to introduce two kinds of cyclic RGD peptides, cyclo(RGDfK) and cylco(CRGDRGDC), onto the C-terminus and side chains respectively. These linear RGD peptide templates were expected to partially contribute to integrin interaction and enhance the affinity of the full structure. For cyclic RGD peptides, we sought to assemble cyclo(RGDfK) at the C-terminus and introduce cyclo(RGDfK) or cyclo(CRGDRGDC) onto the side-chain Asp of the RGD



amino group of cyclo(RGDfK). After cleavage, the mono-cyclic RGD peptide comprising the Asp side chain benzyl ester was obtained, and benzyl ester was hydrazinolyzed by a 5% hydrazine hydrate in DMF solution to obtain the corresponding peptide hydrazide. Successively, it was treated with $NaNO_2$ at a pH 2-3 to give the carbonyl azide, then the solution was adjusted to pH 5.5 and MPAA(4-Mercaptophenylacetic acid) was added to form a thioester. The side-chain thioester reacted with peptide CRGDRGDC via native chemical ligation and the resulted branched peptide was oxidized with air to give the dual-cyclic RGD peptide. The N-terminus of the peptide was modified with the active ester $N_3(CH_2)_3CO_2Su$ (Su=succinimidyl) to carry an azido tag for conjugation with a small-molecule drug or a fluorescent dye (SI, Fig. S9.). In order to facilitate the comparison of integrin binding activity, we also synthesized an azide-modified cyclic peptide P6 and a monocyclic peptide P5 comprising a linear RGD hexapeptide (SI. Fig. S1).

Scheme 2: Synthesis of compound P3. *Reaction conditions*: (a) 5% NH_2NH_2 in DMF, r.t., 30 min,95%; (b) $NaNO_2$, pH 2, –10 °C, 15 min, 95%; (c)MPAA, pH 5.5, –10 °C, 15 min, 95%; (d) CRGDRGDC (5 equiv), pH 6.0, 4 °C, 12 h, 80%; (e) 20% DMSO, air, r.t., 4 h, 95%; (f) $N_3(CH2)_3COOSu$ (2-5 equiv), pH 7.5, r.t., 15 min, 95%.

templates to investigate their activities. For late-stage conjugation with small-molecule drugs or fluorescent dyes, we assembled an azido group at the N-terminus of these dual-cyclic RGD peptides. As shown in Scheme 1, a series of novel dual-cyclic peptide derivatives were designed and synthesized to understand the SAR of different branch sites and cyclic RGD motif combination.

The synthesis of the target peptides followed our previously published method for branched cyclic peptides[20,21] with significant optimization. Side-chain benzyl ester on Asp was the precursor that was readily converted to a hydrazide and an acyl azide successively. Then, the corresponding cyclic peptide moieties were assembled by direct amidation or side-chain native chemical ligation. Peptides P1 and P2 were synthesized according to our previous method (SI, Figs. S3, S11.).[21] The synthesis of P3 and P4 was dramatically improved as shown in Scheme 2 and Figure 1. In the new procedure, we synthesized the cyclo(fKRGD) on the solid support that avoided the twice amidation with acyl azide or thioester in the previous method. Thereafter, the RGD linear peptide was elongated on side-chain

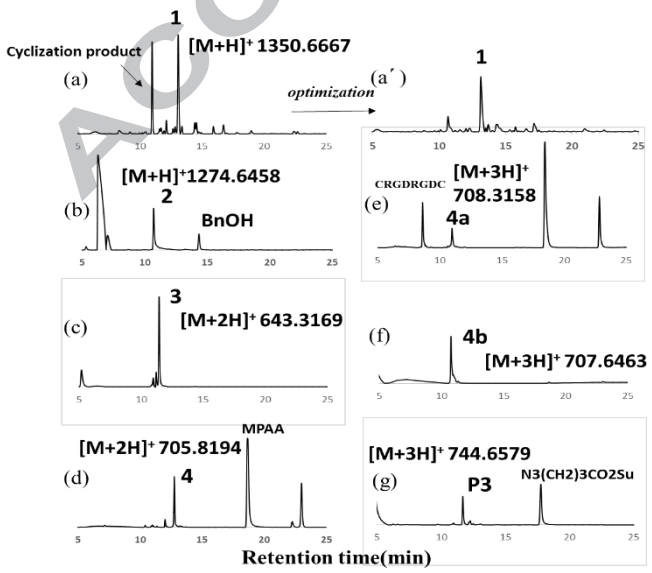


Figure 1: HPLC profiles of procedures in the synthesis of P3. (a) *In situ* RGD peptide **1** by SPPS before optimization; (a') *In situ* RGD peptide **1** by SPPS after optimization; (b) *in situ* carbonyl hydrazide peptide **2**; (c) *in situ* carbonyl azide peptide **3**; (d) *in situ* thioester peptide **4**; (e) side-chain ligation of thioester **4** with CRGDRGDC for 12 h; (f) *in situ* dual-ring branched cyclic RGD peptide **4a** after disulfide-bond formation; (g) azide labeling of dual-ring branched peptide **4b** with $N_3(CH_2)_3COOSu$.

In this new procedure, on-resin synthesis of the C-terminal cyclic peptide cyclo(RGDfK) simplified the late-stage processes by avoiding the introduction of two different cyclic peptides at the C-terminus and the side chain via an orthogonal design on both reactive sites. Meanwhile, consumption of multiple equivalents of cyclic peptide motif is also avoided, which is more economical. In addition, we also optimized the process to reduce the cyclization side reaction on side-chain Asp. During the deprotection of the Fmoc with 20% piperidine for 10 minutes, the side-chain benzyl ester may partially be removed under this basic condition that lead to the cyclization of the side-chain Asp carboxyl group with the adjacent amide nitrogen atom (Fig. 1, Panel a). In an optimized condition, we conducted the de-Fmoc by 3-minutes treatment of 20% piperidine, effectively reducing the formation of cyclized by-products to a limit level (Fig. 1, Panel a').

With the target RGD dual-ring peptides in hands, we sought to investigate their integrin binding properties. An integrin-mediated cell adhesion assay model was utilized to evaluate the integrin-targeting activity of RGD peptides, using the tumor cell line SKOV-3 which indicated over-expression of integrin $\alpha_v\beta_3$. Fibrinogen is the natural ligand of $\alpha_v\beta_3$ integrin and is involved in cell adhesion. Firstly, we coated fibrinogen on a plate overnight, and then human ovary cancer cells (SKOV-3) and our synthetic RGD peptide compound was added successively after washing (No RGD peptide as a control). Since the RGD peptides competed with fibrinogen for binding to the integrin receptor $\alpha_v\beta_3$ on SKOV-3 cells, tumor cells that bound to fibrinogen can remain on the plate, while bound to RGD peptides that inhibited adhesion would be eluted. Then stained it by MTT method, the OD value was measured, and finally the statistical data (*Inhibition rate* = (*Control group - Experimental group) / Control group*). For comparison purpose, Cilengitide was used as

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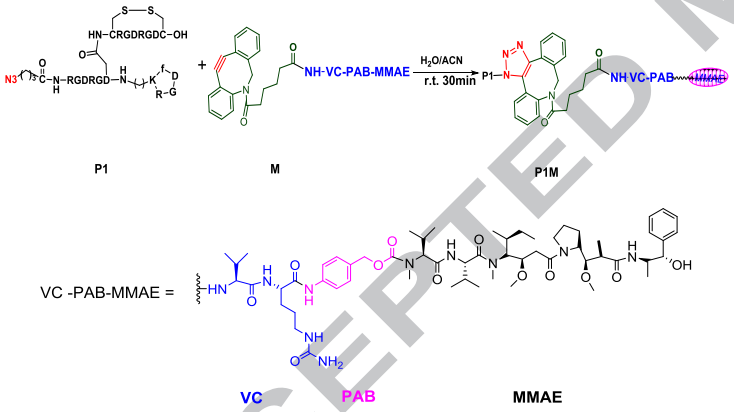
a positive control[22] in all these tests (SI, Fig. S16.). The adhesion inhibition IC_{50} was obtained as shown in Table 1.

The results showed that peptide P6 with azido modification for the amino group of cyclo(RGDfK) significantly reduced the activity, and its IC_{50} was 109.6 μM compared to 14.8 μM of cyclo(RGDfK). This data clearly demonstrated that direct conjugation on cyclic RGD peptide led to weaker binding affinity towards integrin and more sophisticated design is required for RGD-based drug carrier. Peptide P5, bearing a linear RGD hexapeptide template, indicated an increased activity (IC_{50} of 25.1 μM) compared with P6, that validated the effectiveness of the RGD template in structural design. Base on the comparison of the activities of P2 (IC_{50} = 8.9 μM) and P7 (IC_{50} = 14.8 μM), it proved that the modification of branched dual-cyclic peptide facilitated the enhancement to the activity of the cyclo(RGDfK). From the activity comparison result between P1 (IC_{50}=4.2μM) and P2, it demonstrated that the cyclo(CRGDRGDC) introduction to the side chain lead to 2-fold higher activity than cyclo(RGDfK). Further comparison of the IC_{50} values of P1, P3 and P4 indicated the modification position of cylco(CRGDRGDC) at side chain also has an important effect on the activity. When the interval between these two RGD cyclic peptides is 3-4 amino acids (compound P3), the inhibitory activity is the best, which is 7-fold higher than cyclo(RGDfK). In all, we introduced the cyclic RGD motifs to the azido-tagged RGD peptide templates to obtain a series of dual-ring RGD peptide derivatives (P1-P4), which indicated 5- to 50-fold more potent than P6. These data exhibited that the dual-cyclic RGD peptide derivatives effectively enhance the integrin targeting ability and can tolerate more structural modifications, and has the potential to serve as a tumor-targeting drug carrier.

Table 1. Cell adhesion inhibition activity of peptide compounds in SKOV-3 cell line (mean ±SD, n=3.)

Compound	Molecular formula	M. wt.	IC_{50}(μM) in SKOV-3 Cells
Cilengitide	$C_{27}H_{40}N_8O_7$	588.66	0.23 ± 0.01
P1	$C_{85}H_{134}N_{38}O_{30}S_2$	2230.95	4.2 ± 0.11
P2	$C_{82}H_{125}N_{33}O_{24}$	1955.95	8.9 ± 0.18
P3	$C_{85}H_{134}N_{38}O_{30}S_2$	2230.95	2.13 ± 0.09
P4	$C_{97}H_{154}N_{44}O_{35}S_2$	2559.10	24.5 ± 0.64
P5	$C_{55}H_{86}N_{24}O_{18}$	1370.65	25.1 ± 0.68
P6	$C_{31}H_{46}N_{12}O_8$	714.35	109.6 ± 2.86
Cyclo(RGDfK) P7	$C_{27}H_{41}N_9O_7$	603.31	14.8 ± 0.67



Scheme 3: The reaction of dual-cyclic RGD peptide derivative drug-loading.

Table 2. Cytotoxicity assays of MMAE derivatives on $\alpha_v\beta_3$-negative MCF-7 cells and $\alpha_v\beta_3$-positive SKOV-3 cells (mean ±SD, n=3.)

Compound	IC_{50}(μM) in MCF-7 Cells	IC_{50}(μM) in SKOV-3 Cells
M	22.3 ± 1.30	1.0 ± 0.03
P6M	93.2 ± 5.03	4.5 ± 0.38
P1M	19.9 ± 1.48	0.44 ± 0.06

Next, we chose peptides **P1** and **P6** for drug conjugation on their N-terminal azide. A dibenzocyclooctyne (DBCO)-tagged toxin, DBCO-VC-PAB-MMAE (**M**),[23] was loaded onto **P1** or **P6** by click reaction, giving the RGD-drug conjugate product P1-DBCO-VC-PAB-MMAE (**P1M**) (Scheme 3) and **P6M** respectively. MMAE (MonoMethyl atristatin E) is a cytotoxic drug acting on microtubules. We expect that the novel RGD dual-cyclic peptide could enhance the tumor targeting effect of MMAE.

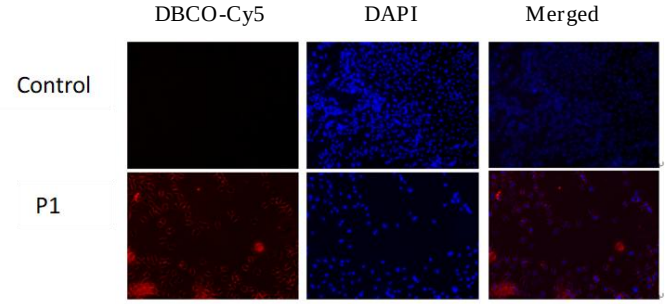


Figure 2. Dual-ring RGD peptide P1 enables cell imaging via integrin interaction. Control group: without treatment of P1 peptide.

The cytotoxicity assays of the compounds **M**, **P1M** and **P6M** against $\alpha_v\beta_3$-negative MCF-7 cells and $\alpha_v\beta_3$-positive SKOV-3 cells were conducted respectively (Table 2). The results exhibited that there was no significant difference in the cytotoxicity of **M** and **P1M** on $\alpha_v\beta_3$-negative MCF-7 cell line (IC_{50}=22.3μM VS 19.9μM), suggesting that dual RGD cyclic peptide did not contribute to the anti-tumor activity against $\alpha_v\beta_3$-negative cells. **P6M** indicated reduced activities in both MCF-7 and SKOV-3 cell line compared with **M**, that may be due to the rigid structure of monocyclic peptide which is too close to the loading drug and inferences the pharmacophore of MMAE. And the cytotoxicity of **P1M** was 2-fold higher than **M**, indicating the targeting effect of the RGD peptide. MMAE is a potent toxin against various tumor cell lines. It seems the DBCO-tagged MMAE (compound **M**) only indicated modest anti-tumor activity on both MCF-7 and SKOV-3 cells that is probably due to the DBCO-modification

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reduce its activity. Meanwhile, the response sensitivity of these two cell lines to the toxin is also different that led to about 20-fold difference in IC_{50} values. However, the integrin-targeting effect of **P1M** is still clear and promising. In the future, using these novel RGD dual-cyclic peptides to design nano-particle system bearing multivalent RGD and toxin, will provide a new option for integrin-targeting drug delivery.

In addition, to validate the tumor targeting effect of RGD dual-cyclic peptides, we performed cell imaging assay to trace the RGD attachment on tumor cell membrane. We incubated P1 with $\alpha_v\beta_3$-positive SKOV-3 cells. After washing, we used DBCO-tagged Cy5 to stain the cell-surface attached P1 via click reaction. Fluorescence microscopy imaging results were shown in Figure 2. The cells were able to be observed the staining effect of Cy5 after P1 adhesion, implicating its good bind affinity to cell-surface integrin $\alpha_v\beta_3$ and its tumor-targeting effect.

In summary, we designed and synthesized a novel class of RGD dual-cyclic peptide derivatives for integrin targeting. Different cyclic RGD peptides are introduced at their C-terminus and side chain of Asp by RGD chain peptide templates, and azide functional groups are modified at their N-terminus for potential drug delivery applications. In chemical synthesis, we have optimized the process to avoid cyclization side reactions caused by benzyl protection shedding. In the inhibition of integrin $\alpha_v\beta_3$-mediated cell adhesion experiment, we found the dual-cyclic RGD peptides P1, P2, and P3 based on this template can effectively enhance the binding of integrin $\alpha_v\beta_3$, and the distance between the two RGD cyclic peptides are also proved to be able to influence integrin binding. In cell imaging experiment, we validated the targeting effect of compound P1 to integrin $\alpha_v\beta_3$ on overexpressed tumor cells. In addition, the conjugate of P1 with the cytotoxic drug MMAE also showed tumor targeting activity. This work explored a novel RGD peptide design strategy, which provided new ideas for integrin-based tumor-targeted drug delivery and diagnosis.

Acknowledgments

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Supplementary Material

Supplementary material is available online.

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Graphical Abstract

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