Published in final edited form as:

Mol Cancer Ther. 2010 October ; 9(10): 2714–2723. doi:10.1158/1535-7163.MCT-10-0308.

The use of one-bead one-compound combinatorial library technology to discover high-affinity $\alpha\text{v}\beta 3$ integrin and cancer targeting RGD ligands with a build-in handle

Wenwu Xiao¹, Yan Wang¹, Edmond Y. Lau², Juntao Luo¹, Nianhuan Yao¹, Changying Shi¹, Leah Meza¹, Harry Tseng¹, Yoshiko Maeda¹, Pappanaicken Kumaresan¹, Ruiwu Liu¹, Felice C. Lightstone², Yoshikazu Takada³, and Kit S. Lam¹

¹Department of Biochemistry and Molecular Medicine, University of California Davis, 2700 Stockton Street, Sacramento, California 95817, United States

²Physical and Life Sciences Directorate, Lawrence Livermore National Laboratory, 7000 East Avenue, Livermore, California 94550, United States

³Department of Dermatology, University of California Davis, 4501 X Street, Sacramento, California 95817, United States

Abstract

The $\alpha\text{v}\beta 3$ integrin, expressed on the surface of various normal and cancer cells, is involved in numerous physiological processes such as angiogenesis, apoptosis, and bone resorption. Because this integrin plays a key role in angiogenesis and metastasis of human tumors, $\alpha\text{v}\beta 3$ integrin ligands are of great interest to advances in targeted-therapy and cancer imaging. In this report, one-bead-one-compound (OBOC) combinatorial libraries containing the RGD motif were designed and screened against K562 myeloid leukemia cells that had been transfected with human $\alpha\text{v}\beta 3$ integrin gene. Cyclic peptide LXW7 was identified as a leading ligand with a build-in handle that binds specifically to $\alpha\text{v}\beta 3$ and showed comparable binding affinity ($\text{IC}_{50} = 0.68 \pm 0.08 \mu\text{M}$) to some of the well-known RGD “head-to-tail” cyclic pentapeptide ligands reported in the literature. The biotinylated form of LXW7 ligand showed similar binding strength as LXW7 against $\alpha\text{v}\beta 3$ integrin, whereas biotinylated RGD cyclopentapeptide ligands revealed a 2 to 8 fold weaker binding affinity than their free forms. LXW7 was able to bind to both U-87MG glioblastoma and A375M melanoma cell lines, both of which express high levels of $\alpha\text{v}\beta 3$ integrin. *In vivo* and *ex vivo* optical imaging studies with biotinylated-ligand/streptavidin-Cy5.5 complex in nude mice bearing U-87MG or A375M xenografts revealed preferential uptake of biotinylated LXW7 in tumor. When compared with biotinylated RGD cyclopentapeptide ligands, biotinylated LXW7 showed higher tumor uptake but lower liver uptake.

Keywords

one-bead one-compound (OBOC) combinatorial library; RGD ligands; $\alpha\text{v}\beta 3$ integrin; optical imaging

Corresponding author and the person to whom reprint requests should be sent: Kit S. Lam. Department of Biochemistry and Molecular Medicine, Division of Hematology & Oncology, University of California Davis, 2700 Stockton Street, Sacramento, California 95817, United States Phone:(916) 734-0910, Fax: (916) 734-4418. Kit.lam@ucdmc.ucdavis.edu.
Dr. Wenwu Xiao and Dr. Yan Wang are co-first authors.

There are no potential conflicts of interest.

INTRODUCTION

The $\alpha v\beta 3$ integrin serves as a receptor for a variety of extracellular matrix proteins displaying the arginine-glycine-aspartic acid (RGD) tripeptide sequence (1). The integrin, expressed on the surface of various normal and cancer cells, is involved in multiple physiological processes including angiogenesis, apoptosis, and bone resorption. Since this integrin plays a key role in angiogenesis and metastasis of human tumors, $\alpha v\beta 3$ integrin ligands are of great interest to advances in targeted-therapy and cancer imaging.

The RGD motif was described in early 1980's by several groups such as Michael D. Pierschbacher and Erkki Ruoslahti (2). Many RGD analogues have been designed and synthesized (3) since then and many naturally occurring snake venoms also contain a RGD motif (4). Phage-display peptide library screening has been used to identify peptide ligands targeting various integrins, thereby elucidating unique integrin or tissue targeting peptides. Using this screening methodology, the nonapeptide, CDCRGDCFC, was identified to be highly selective against αv integrins (5). Another "design approach" based upon "spatial screening" of cyclopeptides, in which conformational change is induced by variation of the ring size, amino acid chirality and retro-inverso structures, N-methylation of peptide backbone, or introduction of constraining structural elements, has led to the discovery of Cilengitide (3, 6, 7). This cyclic peptide, *cyclo*(RGDf-N(Me)V-), binds strongly and relatively selectively to $\alpha v\beta 3$ integrin, and is now in clinical trials for the treatment of several different kinds of cancers (8). However, due to intrinsic constraints of these screening processes, either only L-amino acid residues could be included in the phage display peptide libraries or the number of peptides that can be simultaneously tested in the "spatial screening" process is rather limited. Here we report on the use of one-bead one-compound (OBOC) combinatorial library method (9) to identify high affinity ligands specifically targeting $\alpha v\beta 3$ integrin. The OBOC library is synthesized on beads using the "split-mix" method such that each bead displays only one chemical entity. The resin has been grafted with polyethyleneglycol (PEG) linkers, which act as a build-in handle for library compounds. Combined with whole cell binding assays, OBOC permits rapid synthesis and high-throughput screening of millions of peptides and/or synthetic small molecules, with various structures and conformations, for their ability to bind to unique target proteins on the cancer cell surface. Using this technique, several important ligands against different tumor cell surface integrin receptors have been identified in recent years; these include LLP2A which targets $\alpha 4\beta 1$ integrin (10), OA02 (11), LXY1 (12) and LXY3 (13) which bind $\alpha 3\beta 1$ integrin. Here, OBOC combinatorial libraries bearing the RGD and related motif were designed, synthesized and screened against $\alpha v\beta 3$ integrin receptor transfected K562 myeloid leukemia cells. A novel cyclic RGD peptide, cGRGDdvc (LXW7), cyclized by a disulfide bond and with a build-in handle at the carboxyl terminus, was identified and subsequently demonstrated to possess high binding affinity and specificity against $\alpha v\beta 3$ integrin. Furthermore, *in vivo* and *ex vivo* imaging experiments indicated LXW7-biotin/streptavidin-Cy5.5 complex was able to target U-87 MG glioblastoma and A375M melanoma xenografts with high efficiency and low liver uptake.

MATERIALS AND METHODS

Synthesis of the focused OBOC libraries

The OBOC libraries were synthesized on TentaGel S NH₂ resin using the bilayer bead encoding strategy (14) and "split-mix" method (9). A typical synthetic cycle utilizing the "split-mix" approach is described as follows: (i) beads are split into aliquots as desired; (ii) each aliquot of beads is coupled with a specific Fmoc-protected amino acid in the presence of HOBt and DIC for 2 h; (iii) all aliquots of beads are mixed together and washed with DMF five times. Coupling completeness and Fmoc deprotection are monitored by the Kaiser

test. For Fmoc deprotection, beads are incubated with 20% 4-methyl piperidine solution in DMF twice (5 min, 15 min) and then thoroughly washed with DMF, MeOH, and DMF three times each, respectively. For Alloc deprotection, the beads were incubated with (Pd(PPh₃)₄) (0.2 equiv) and PhSiH₃ (20 equiv) in DCM for 30 min (twice) and washed with 0.5% diethyldithiocarbamic acid sodium salt in DMF (3 times) and DMF (10 times). The structures of Library 1-4, the used amino acids and detailed synthesis procedures for the libraries were described in Supplemental Materials and Methods (Table S1, S2 and Scheme S1).

Cells

We obtained K562, U-87MG, and A375M from American Type Culture Collection (Manassas, VA, Oct 2007- no further authentication was performed). The Human umbilical vein endothelial cells (HUVEC) were purchased from PromoCell Company (Heidelberg, Germany, Oct 2005-no further authentication was performed). The $\alpha v \beta 3$ -K562 cells were furnished by Dr. Yoshikazu Takada (May 2006-tested by immunostaining). The $\alpha IIb \beta 3$ -K562 (Jun 2009-tested by immunostaining) and $\alpha v \beta 5$ -K562 (Mar 2010-tested by immunostaining) cells were gifts from Dr. Jennifer Cochran (Stanford University)

Whole cell binding assay and bead screening

The cells were collected and cell pellets were resuspended with 10 mL growth medium in a 10 cm Petri dish. Thereafter the beads were incubated with suspended cells, and the whole dish was kept shaking at a speed of 40 rpm inside 37 °C incubator under 5% CO₂. Beads showing stronger binding were picked up, washed with 8 M Guanidine chloride, water sequentially, and finally sequenced with ABI protein sequencer (Model 494).

Docking simulation study

Conformations for LXW7 were generated from replica-exchange molecular dynamics simulation (details in Supplementary Materials and Methods) (15). The structures from dynamics studies were clustered using MMTSB (16) and representative structures were taken from the main clusters. The peptide *cyclo*(RGDfV) and LXW7 were each docked into the RGD binding site of integrin $\alpha v \beta 3$ (PDB ID 1L5G) (17) using the program Autodock 3.05 (18). Amber charges (19) were assigned to the ligand using the program Chimera (20). Kollman-united atom charges were used for the protein (21). A 80 × 80 × 90 grid was used with a spacing of 0.375 Å. The grid was centered in the middle of the binding sites. A Lamarckian algorithm was used to generate conformations for the ligand within the active site. The parameters used for the Lamarckian genetic algorithm were the same as reported by Legge *et al.* except the maximum number of energy evaluations was 250,000 (22). A total of 1000 conformers were generated for the ligand in the binding sites. The conformers were clustered using a 2.0 Å RMSD.

Synthesis of free form and biotinylated form of RGD peptides

Cyclo(RGDfE), *cyclo*(RGDyK) and *cyclo*(RGDfK) were obtained from Peptides International (Louisville, KY). Cyclic peptides were synthesized on Rink amide resin (loading 0.59 mmol/g), using 1-Hydroxybenzotriazole/1, 3-Diisopropylcarbodiimide as coupling reagents. Three fold molar excess of Fmoc-protected amino acids to resin was used for coupling. Coupling completeness and Fmoc deprotection were monitored by ninhydrin test. (Supplemental Materials and Methods)

Flow cytometry

Echistatin is a 49 amino acid peptide belonging to the family of disintegrins and binds to $\alpha v \beta 3$ integrin with high affinity, which can be competed efficiently by linear and cyclic

peptides containing the RGD sequence. For the determination of half maximum binding inhibition concentration (IC_{50}) of all the RGD peptides, peptides at different concentrations were premixed with 2 μ M Echistatin-FITC, incubated with 3×10^5 $\alpha v \beta 3$ -K562 and K562 cells separately in 100 μ L binding buffer (1 $\times$ PBS containing 10% FBS and 1 mM $MnCl_2$) for 30 min on ice, and evaluated with flow cytometry. In this assay, there was background binding from Echistatin-FITC to K562 cells. However, all these tested peptides had no inhibition effect to the background binding.

The cells staining with LXW7-Bio was conducted as following: 1 μ M LXW7-Bio was incubated with 3×10^5 cells on ice for 30 min in binding buffer. The samples were washed with 1 mL washing buffer (1 $\times$ PBS containing 1% FBS) three times and incubated with 1:500 dilution of streptavidin-PE (1 mg/mL) for 30 min on ice. After one additional wash, samples were tested by flow cytometry. To test the expression of the $\alpha v \beta 3$ integrin on U-87MG and A375M, samples were stained with 1 μ g mouse anti-human $\alpha v \beta 3$ -PE (23C6, Santa Cruz Biotechnology, INC) on ice for 30 min, washed once with washing buffer, evaluated by flow cytometry. For the blocking experiment, 10 μ g anti-human $\alpha v \beta 3$ (LM609, Chemicon) were premixed with 1 μ M LXW7-Bio and incubated with cells, followed by streptavidin-PE incubation and detection with flow cytometry.

The samples were analyzed with Coulter XL-MCL Flow Cytometry. The histogram and mean fluorescence intensity (MFI) were determined. The IC_{50} measurements were calculated using Graph Prism software (www.graphpad.com).

Tumor xenografts

Animal studies were performed according to approved protocol by IACUC of the University of California, Davis. Female athymic nude mice (nu/nu), obtained from Harlan (Indianapolis, IN) at 5-6 weeks of age were injected subcutaneously in the right flank with 5×10^6 tumor cells suspended in 200 μ L PBS. When the subcutaneous tumors reached 0.5 to 1.0 cm in diameter or 21-28 days later, the tumor-bearing mice were subjected to in vivo and ex vivo imaging studies.

In vivo and ex vivo mouse optical imaging

The mouse was anesthetized by injection of 30 μ L Nembutal (50 mg/mL) prior to optical imaging. Tetravalent peptide-biotin-streptavidin complex (1.8 nmol), prepared by mixing 7.2 nmol of biotinylated peptide with 1.8 nmol of streptavidin-Cy5.5 in PBS overnight at 4°C, was injected via the tail vein. Images were acquired with a Kodak IS2000MM Image station (Rochester, NY) with excitation filter 625/20 band pass, emission filter 700WA/35 band pass, and 150 W quartz halogen lamp light source set to maximum. Six hours post injection, a second series of images were captured. The mice were sacrificed and the organs were excised for ex vivo imaging. Data was collected and analyzed using the Kodak ID 3.6 software by drawing the region of interest (ROI) on the image.

Data processing and statistics

We calculated mean fluorescence intensities of the tumor by means of the region-of-interest function using Kodak 1D Image Analysis Software (Kodak). All the data are shown as mean $\pm$ s.d. of n independent measurements. Student's t-test was used for statistical analysis of ex vivo imaging intensity. Statistical significance was indicated by $P < 0.05$ and $P < 0.01$.

RESULTS

The screening of RGD-containing OBOC libraries against $\alpha v \beta 3$ integrin

In our initial study, human umbilical vein endothelial cells (HUVEC) were used as a neovasculature cell model (23) to identify peptide ligands against newly-formed tumor blood vessels. Two cyclic RGD containing peptide libraries, octamer Library 1 (Table 1) and nonamer Library 2 (Supplementary Data Table S1), were synthesized and screened against HUVEC using a whole cell binding assay (9). Library 1 elicited stronger binding with HUVEC than Library 2, and was therefore used for further screening analysis. As shown in Table 1, RGD was found to be the preferred motif for HUVEC binding with Library 1. However, at the x_2 , x_6 and x_7 positions, there were no obvious preferences for any specific amino acid. Considering the broad binding profile of RGD motif (24) against several known integrins, some of which are expressed on the surface of HUVEC (25), the varied sequence results obtained were therefore not particularly surprising. $\alpha v \beta 3$ integrin has been reported as the major integrin expressed in neovasculature (26, 27). We therefore hypothesize that cells expressing high levels of $\alpha v \beta 3$ integrin would be a more appropriate cell model for identifying ligands that bind specifically to neovasculature. $\alpha v \beta 3$ integrin transfected K562 leukemia cells, expressing a high level of $\alpha v \beta 3$ integrin and a baseline level of $\alpha 5 \beta 1$ integrin (expressed by the parent cell line), were used as a living cell probe to screen Library 1 (Supplementary Data Figure S1). As shown in Table 1, this experiment yielded Gly as the preferred amino acid at the x_2 position (60%) and *D*-Asp occurred with increased frequency (40%) at x_6 position. As a control, Library 1 was also screened with the parent cell line (non-transfected K562 cells), which yielded a higher frequency of *D*-Lys and *D*-Arg (57%) at the x_2 position.

We have previously demonstrated that reduction of ligand loading on the bead surface increases the screening stringency and therefore facilitates the identification of more potent ligands (28). To further explore the amino acid preferences flanking the RGD motif, Library 3, with 20% down-substitution of bead surface to facilitate an expected higher screening stringency, was synthesized and screened with $\alpha v \beta 3$ -K562 cells. Under this higher stringency screening condition, 100% of the peptides from Library 3 yielded Gly at x_2 position with an acidic amino acid *D*-Asp or *D*-Glu at either position x_6 (40%) or x_7 (20%) (Table 1). Polar amino acids *D*-Asn, *D*-Gln and *D*-Thr were also found to be predominant at x_6 position (40%). The cGRGDdvc sequence occurred twice. Other than the 20% acidic residues, there was no obvious preference in the x_7 position.

To further narrow down the amino acid preferences at x_6 and x_7 positions, we designed and synthesized Library 4, in which at “ X^K ” position, only Lys and Orn were used as building blocks. Our assumption was that by changing the RGD motif to weaker but related motif, such as KGD, the screening stringency on the x_2 , x_6 and x_7 positions would be increased. The screening results showed that Gly was still preferred in x_2 , and acidic amino acid (*D*-Asp and *D*-Glu) appeared with higher frequency (63%) at x_6 position, and more polar (including acidic residues) and hydrophobic amino acids occurred in the x_7 position. Interestingly, the sequence cGKGDdvc was found as expected and two other sequences, cGKGDsec and cGKGDdsc appeared twice independently.

Binding affinity of leading peptides

Based on the screening results shown above, LXW1-8 peptides (Supplementary Data Table S3) were selected for further binding affinity analysis (Table 2). LXW1, LXW2, LXW4, LXW6, LXW7 and LXW8 were selected directly from the data compiled in Table 1, LXW3 and LXW5 were derivatives of LXW4 and LXW6, respectively, with Lys changed to Arg at the third position. The IC_{50} measurements of the peptides inhibiting the Echistatin-FITC

binding with $\alpha v\beta 3$ -K562 were determined. The results showed that LXW7 (Figure 1a) had the highest binding affinity to $\alpha v\beta 3$ integrin with IC_{50} at $0.68 \pm 0.08 \mu M$. LXW3 showed similar affinity. The binding affinities of LXW1, LXW2 and LXW5 were found to be 2-4 times lower than that of LXW7. Changing of Arg to Lys in the “X^K” position in LXW4, LXW6 and LXW8 decreased the affinity dramatically. These findings validated the rationale for the Library 4 design and confirmed that replacement of Arg by Lys in the “X^K” position greatly increased the selection stringency of the amino acids adjacent to the RGD motif.

Binding characterization of LXW7 and biotinylated LXW7

Of the 8 peptides tested, LXW7 has the highest binding affinity against $\alpha v\beta 3$ integrin. To test its specificity, LXW7 was prepared in biotinylated form (Figure 1b) and incubated with $\alpha IIb\beta 3$, $\alpha v\beta 5$ and $\alpha v\beta 3$ transfected K562 and parent K562 cells ($\alpha 5\beta 1$), followed by incubation with streptavidin-PE. The peptide binding was tested by flow cytometry. The results showed that LXW7 bound strongly to $\alpha v\beta 3$ -K562 cells, weakly to $\alpha v\beta 5$ -K562 cells and $\alpha IIb\beta 3$ -K562 cells, and no binding to K562 cells (Figure 1c). Several other integrins such as $\alpha v\beta 1$, $\alpha v\beta 6$, $\alpha v\beta 8$ and $\alpha 8\beta 1$ have been reported to bind to ligands via RGD sequence as well (29). Further studies on LXW7 binding specificity against these integrins will be addressed in future experiments when those transfected cell lines become available. RGD peptides targeting $\alpha v\beta 3$ integrin have been studied and developed for many years. Cyclic pentapeptides such as *cyclo*(RGDFV) were found to show high binding affinity to $\alpha v\beta 3$ integrin. Furthermore, the *cyclo*(RGDF-*N(Me)V*-) analogue demonstrated even higher binding capability and selectivity against $\alpha v\beta 3$ integrin. Cyclic peptides such as *cyclo*(RGDFE), *cyclo*(RGDFK) and *cyclo*(RGDYK) were commonly used to carry optical imaging probes or radiometal chelates to target $\alpha v\beta 3$ integrin on tumor cells. The binding affinities of LXW7 and these cyclic RGD pentapeptides against $\alpha v\beta 3$ integrin were compared based on their ability to inhibit Echistatin-FITC binding against $\alpha v\beta 3$ -K562. The binding affinity of LXW7 was found to be 2-3 times lower than that of *cyclo*(RGDYK) and *cyclo*(RGDF-*N(Me)V*-) but similar to that of *cyclo*(RGDFV), *cyclo*(RGDFE) and *cyclo*(RGDFK) (Table 2).

To use these cyclic peptides for imaging and drug delivery, one will need to tether these ligands to the payload. Since LXW7 was discovered through screening OBOC combinatorial libraries with an on-bead binding assay, LXW7 already has a build-in handle at the C-terminus to which the library compound was linked to the bead. Therefore, biotinylation can be conveniently carried out at the C-terminus of the ligand. In contrast, peptide *cyclo*(RGDF-*N(Me)V*-) or *cyclo*(RGDFV) lack a handle because of head to tail cyclization. Consequently, Val was replaced with either Lys or Glu, to which the payload can be covalently linked. However, such linker can potentially affect the binding of the ligand to the integrin. We prepared *cyclo*(RGDYK), *cyclo*(RGDFK) and *cyclo*(RGDFE) with biotin attached to either Lys or Glu residues via a hydrophilic linker (Supplementary Data Figure S2). We then determined the binding affinity of each of these biotinylated forms and compared them with the corresponding non-biotinylated forms. Not unexpected, LXW7 and biotinylated LXW7 were found to have near identical binding strength to $\alpha v\beta 3$ integrin. However, biotinylation of other pentapeptides resulted in a 2-8 fold decrease in affinity (Table 2).

Docking simulation study on LXW7

To further explain the binding specificity of LXW7 against $\alpha v\beta 3$ integrin, molecular docking calculations were performed (Figure 2). The crystal structure of $\alpha v\beta 3$ integrin contains the ligand *cyclo*(RGDF-*N(Me)V*-), but for our docking simulations the analogous ligand *cyclo*(RGDFV) was used for ease of building. In the docking simulations, many different clusters of conformations are obtained and choosing the binding conformation is

not always obvious. To aid in determining the correct conformation, the ligand has to be interacting with the ion in the MIDAS site and also interact with one of the Asp in the α subunit. In all crystal structures of integrins with a ligand, these two conditions are always fulfilled. One of the lowest energy clusters of LXW7 was able to interact with the carboxylate side chain of Asp218 in the α -subunit and coordinate to the Mg^{2+} in the MIDAS site of the β -subunit (Figure 2a). Although LXW7 and *cyclo*(RGDfV) bind to the same site and the Val side-chain in LXW7 is also in an almost identical position to the phenylalanine side-chain in *cyclo*(RGDfV), the conformation of the RGDs and the ligands in general are different. Firstly, The LXW7 peptide forms a bowl-like conformation where glycine in the RGD has a kinked structure (Figure 2a) and the orientation of the Arg side-chain with Asp218 (α -subunit) is not optimal (Figure 2d) although the Asp can interact with Mg^{2+} easily (Figure 2b). Secondly, the *D*-Asp is able to form a salt bridge with Arg214 in the β -subunit (Figure 2 b, c) and the C-terminal carbonyl oxygen is able to coordinate to a Ca^{2+} in the ADMIDAS site in the β -subunit. This additional interaction probably contributes to the specific binding of LXW7. Thirdly, the calculated interaction energy for LXW7 (-18.70 kcal/mole) to $\alpha v\beta 3$ integrin is even greater than the calculated interaction energy for the well known ligand *cyclo*(RGDfV) (-15.07 kcal/mole), which indicates LXW7 bears a better conformational fit in the binding site. Lastly, having *D*-Ala instead of Gly preceding RGD did not change the docking conformation of LXW7 although experimentally it was shown Gly was highly favored. The molecular dynamics may provide a possible reason for the difference in binding preference. Measuring the alpha carbon distance between Arg and Asp of RGD within LXW7 (cGRGDdvc) and caRGDdvc shows there is a difference in the dynamics of these two peptides. The distance between the alpha carbons of Arg and Asp of cGRGDdvc were consistently larger and showed greater fluctuations than caRGDdvc (Supplementary Data Table S4). This implies that both the conformational preference and flexibility of cGRGDdvc differs from caRGDdvc.

LXW7 binds tumor cells with $\alpha v\beta 3$ integrin expression

Many different cancer cells such as glioblastoma (U-87 MG) and melanoma (A375M) express $\alpha v\beta 3$ integrin on their cell surface. LXW7 was expected to bind these cells and could be used as a targeting agent. As shown in Figure 3a and c, both tumor cells express $\alpha v\beta 3$ integrin, and the binding of biotinylated LXW7 against the integrin was blocked significantly by anti- $\alpha v\beta 3$ integrin (Figure 3b, d). The binding affinity of LXW7 against U-87MG, A375M and $\alpha v\beta 3$ -K562 cells was tested. All three cell lines were found to have similar binding constants in the range of 72-89 nM (Supplementary Data Table S5).

In vivo and *ex vivo* targeting effect of LXW7 against tumors in xenografts on nude mouse

The *in vivo* targeting effect of LXW7 was further demonstrated in nude mouse implanted subcutaneously with U-87MG and A375M xenografts. The tetravalent imaging complex LXW7-biotin-streptavidin-Cy5.5 was found to accumulate specifically in the xenografts compared with the streptavidin-Cy5.5 control (Figure 4a and 4b). Besides the high signal uptake in xenografts, the kidney was the only organ showing obvious accumulation of the imaging probe. These data indicates LXW7 has the ability to carry optical imaging dye to target tumors expressing $\alpha v\beta 3$ integrin. Confocal microscopy of U-87MG xenograft cryosections revealed that LXW7 also distributed on the neovasculature with CD31 positive expression (Supplementary Data Figure S3). This finding further validated the targeting properties of LXW7 against tumor cells and newly formed blood vessels expressing $\alpha v\beta 3$ integrin.

RGD peptides have been generally reported as the targeting ligands against $\alpha v\beta 3$ integrin. The tumor targeting ability of biotinylated LXW7 and several "head-to-tail" cyclic RGD pentapeptides were also compared in U-87 MG xenograft mouse model. *Ex vivo* imaging

showed LXW7-Bio had higher tumor uptake than *cyclo*(RGDfE)-Bio, *cyclo*(RGDfK)-Bio and *cyclo*(RGDyK)-Bio (Figure 4c, d). Furthermore, the liver uptake from LXW7-Bio was near control level (streptavidin-Cy5.5 without peptide), while the signals from the cyclic RGD pentapeptides in liver were much higher (Figure 4c, d). These results indicate the biotinylated RGD cyclopentapeptides have much higher nonspecific accumulation in the liver.

DISCUSSION

The $\alpha v \beta 3$ integrin receptor has received much clinical interest as a cancer target because the integrin expression correlates well with tumor progression and invasiveness of melanoma, glioma, lung and breast cancers (30-34). Furthermore, $\alpha v \beta 3$ integrin is over-expressed on activated endothelial cells of tumor neovasculature, but is under-expressed in quiescent endothelial cells of most normal tissues and organs (27) (35). Therefore, there is a critical need to identify ligands targeting $\alpha v \beta 3$ integrin with high binding affinity and specificity for cancer diagnosis and therapy (36-38). The RGD motif is the most common integrin binding sequence found within many extracellular matrix proteins and disintegrins (39, 40). However, different integrins recognize diverse RGD-containing proteins and peptides. The binding affinity and specificity are modulated by the amino acid residues adjacent to the RGD triad, the auxiliary binding motifs in the ligand, and to a large extent by the conformational presentation of the triad. In this report, we have demonstrated that through screening OBOC libraries with RGD and related motif against $\alpha v \beta 3$ integrin, one will be able to narrow down the possible amino acids flanking RGD and discover specific ligands with higher affinity and selectivity.

To focus our discovery effort on ligands against one specific integrin each time, we used $\alpha v \beta 3$ -transfected K562 cells and K562 parent cells as the screening probes. We designed and synthesized RGD containing OBOC combinatorial cyclic peptide libraries (Library 1 and 2) with 100% beads surface substitution for initial screening. The use of 20% down-substitution beads library (Library 3) and library without RGD motif (Library 4) further narrow down the amino acid possibility on the flanking positions of RGD triads. Finally, the amino acids in the x_2 , x_6 and x_7 positions displayed much more restrictive sequences. Gly was almost exclusively preferred at the x_2 position. The x_6 position had more acidic amino acids (*D*-Asp and *D*-Glu, 63%), which according to the docking data for LXW7, are very important for peptide-integrin binding. The x_7 position preferred polar and hydrophobic amino acids. Partly in agreement with our findings, other groups also reported the high frequency appearance of Gly in amino side of the RGD triad with phage display screening and solid-phase integrin inhibition assay, but few of them mentioned the residue preference at the carboxyl side of the triad (41-44). The requirement of Gly in the amino side of RGD in LXW7 might be due to the need for flexibility (45) to adopt many different conformations to fit in the binding site. This was further supported by the molecular dynamics study on LXW7, in which cGRGDdvc showed greater conformational preference and flexibility than caRGDdvc (Supplementary Data Table S4). Overall, our results indicate an amino acid residue preference around the RGD motif as demonstrated by selective binding of peptides with expanded lateral sequence.

Peptides with an RGD motif or peptidomimetics against $\alpha v \beta 3$ integrin (46) have been utilized as probes for tumor imaging and for monitoring therapeutic response to anti-angiogenic agents. We focused on RGD peptide ligands and compared LXW7 with several other well-known RGD ligands. The majority of peptide imaging agents have been developed based on the *cyclo*(RGDfV) template. Since it is a head-to-tail cyclic peptide and the Asp side chain is critical for receptor binding, one will need to modify the peptide in order to introduce a handle for covalent attachment of the imaging probes such as fluorophor

or radiometal chelate. *Cyclo*(RGDfK), *cyclo*(RGDfE) and *cyclo*(RGDyK) are the most common RGD cyclopentapeptides reported in the literatures in which the Val was replaced by either Lys or Glu. Using a competitive ELISA assay, we have determined that the binding affinity of LXW7 is similar to that of *cyclo*(RGDfK) and *cyclo*(RGDfE), and about 2 times lower than that of *cyclo*(RGDyK) (Table 2). Since peptide *cyclo*(RGDfV) has been optimized in size to fit the binding pocket of the $\alpha v \beta 3$ integrin, introduction of any labeling groups to the side chain of amino acids Lys or Glu (in replacement of Val) would likely be within close proximity to the integrin recognizing RGD moiety, resulting in decreased receptor binding affinity and specificity (47). In contrast, incorporation of payloads at the carboxyl end of the LXW7 peptide would avoid the potential adverse affects to receptor binding owing to the large distance between the linker and the binding region of the peptide. We compared the targeting efficacy between LXW7 and the three RGD cyclopentapeptides. In contrast to the zero interference effect from biotinylation of LXW7, the biotinylation of the three RGD cyclopentapeptides showed a 2-8 fold decrease in binding affinity when compared to their free forms and 2-8 times lower binding affinity than biotinylated LXW7 (Table 2). These findings were further supported by optical imaging studies on U-87MG nude mouse xenograft model, in which the biotinylated LXW7 exhibited better tumor targeting than the three biotinylated cyclopentapeptides.

Furthermore, the undesirable liver uptake of biotinylated LXW7 was significantly lower than other cyclopentapeptides. There have been several reports on high uptake of radiolabeled *cyclo*(RGDfE), *cyclo*(RGDfK) or *cyclo*(RGDyK) into the liver (47-49). The mechanism underlying the different liver uptake profiles between biotinylated LXW7 and the biotinylated cyclopentapeptides have yet to be determined. The enhanced hydrophilicity of LXW7 as a result of an extra *D*-Asp adjacent to the RGD motif may perhaps partly decrease the hepatobiliary clearance of the peptide. Further bio-distribution studies based upon LXW7 radioconjugates should help clarify this issue.

In conclusion, the OBOC combinatorial library method has again lended itself as a powerful tool to identify and optimize specific ligands against cell surface receptors. In this report, a novel peptide ligand, LXW7 with an RGD motif, was identified using OBOC combinatorial chemistry. The positions flanking the RGD motif were restricted to certain amino acids specific for binding with the $\alpha v \beta 3$ integrin. LXW7 demonstrated high targeting efficacy and specificity for the $\alpha v \beta 3$ integrin expressed in tumor cells and neovasculature. Moreover, LXW7 permits easy functionalization for payload conjugation without attenuating the binding affinity of the peptide. For these reasons, LXW7 has great potential as a highly efficient peptide ligand for targeted imaging and drug delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Mr. Jared Townsend and Mrs. Mary Saunders for editorial assistance.

This work was supported by NIH R33CA-86364, R33CA-99136, U19CA113298, R01CA115483 (Dr. Kit S. Lam), NIH 1R21CA135345 (Dr. Ruiwu Liu), DOD CML064046 and Children Miracle Net Work at UC Davis (CMNKP-06) (Dr. Pappanaicken Kumaresan). Part of this work performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344 (Drs. Edmund Lau and Felice Lightstone).

References

1. Jin H, Varner J. Integrins: roles in cancer development and as treatment targets. *Br J Cancer*. 2004; 90:561–5. [PubMed: 14760364]
2. Pierschbacher MD, Ruoslahti E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature*. 1984; 309:30–3. [PubMed: 6325925]
3. Haubner R, Gratias R, Diefenbach B, Goodman SL, Jonczyk A, Kessler H. Structural and functional aspects of RGD-containing cyclic pentapeptides as highly potent and selective integrin $\alpha v \beta 3$ antagonists. *J Am Chem Soc*. 1996; 118:13.
4. Markland FS. Snake venoms and the hemostatic system. *Toxicon*. 1998; 36:1749–800. [PubMed: 9839663]
5. Koivunen E, Wang B, Ruoslahti E. Phage libraries displaying cyclic peptides with different ring sizes: ligand specificities of the RGD-directed integrins. *Biotechnology (N Y)*. 1995; 13:265–70. [PubMed: 9634769]
6. Kessler H, Gratias R, Hessler G, Gurrath M, Muller G. Conformation of cyclic peptides. Principle concepts and the design of selectivity and superactivity in bioactive sequences by ‘spatial screening’. *Pure & Appl Chem*. 1996; 68:5.
7. Dechantsreiter MA, Planker E, Matha B, et al. N-Methylated cyclic RGD peptides as highly active and selective $\alpha (V) \beta (3)$ integrin antagonists. *Journal of medicinal chemistry*. 1999; 42:3033–40. [PubMed: 10447947]
8. Desgrosellier JS, Cheresh DA. Integrins in cancer: biological implications and therapeutic opportunities. *Nature reviews*. 2010; 10:9–22.
9. Lam KS, Salmon SE, Hersh EM, Hruby VJ, Kazmierski WM, Knapp RJ. A new type of synthetic peptide library for identifying ligand-binding activity. *Nature*. 1991; 354:82–4. [PubMed: 1944576]
10. Peng L, Liu R, Marik J, Wang X, Takada Y, Lam KS. Combinatorial chemistry identifies high-affinity peptidomimetics against $\alpha 4 \beta 1$ integrin for in vivo tumor imaging. *Nat Chem Biol*. 2006; 2:381–9. [PubMed: 16767086]
11. Aina OH, Marik J, Gandour-Edwards R, Lam KS. Near-infrared optical imaging of ovarian cancer xenografts with novel $\alpha 3$ -integrin binding peptide “OA02”. *Mol Imaging*. 2005; 4:439–47. [PubMed: 16285906]
12. Xiao W, Yao N, Peng L, Liu R, Lam KS. Near-infrared optical imaging in glioblastoma xenograft with ligand-targeting $\alpha 3$ integrin. *Eur J Nucl Med Mol Imaging*. 2009; 36:94–103. [PubMed: 18712382]
13. Yao N, Xiao W, Wang X, et al. Discovery of targeting ligands for breast cancer cells using the one-bead one-compound combinatorial method. *Journal of medicinal chemistry*. 2009; 52:126–33. [PubMed: 19055415]
14. Liu R, Marik J, Lam KS. A novel peptide-based encoding system for “one-bead one-compound” peptidomimetic and small molecule combinatorial libraries. *J Am Chem Soc*. 2002; 124:7678–80. [PubMed: 12083920]
15. Sugita Y, Okamoto Y. Replica-exchange molecular dynamics method for protein folding. *Chemical Physics Letters*. 1999; 314:141–51.
16. Feig M, Karanicolas J, Brooks CL 3rd. MMTSB Tool Set: enhanced sampling and multiscale modeling methods for applications in structural biology. *J Mol Graph Model*. 2004; 22:377–95. [PubMed: 15099834]
17. Xiong JP, Stehle T, Zhang R, et al. Crystal structure of the extracellular segment of integrin $\alpha V \beta 3$ in complex with an Arg-Gly-Asp ligand. *Science*. 2002; 296:151–5. [PubMed: 11884718]
18. Morris GM, Goodsell DS, Halliday RS, et al. Automated docking using Lamarckian genetic algorithm and an empirical binding free energy function. *J Comp Chem*. 1998; 19:1639–62.
19. Cornell WD, Cieplak P, Bayly CI, et al. A second generation force field for the simulation of proteins, nucleic acids and organic molecules. *J Am Chem Soc*. 1995; 117:5179–97.
20. Pettersen EF, Goddard TD, Huang CC, et al. UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem*. 2004; 25:1605–12. [PubMed: 15264254]
21. Weiner SJ, Kollman PA, Case DA, et al. A new force field for molecular mechanical simulations of nucleic acids and proteins. *J Am Chem Soc*. 1984; 106:765–84.

22. Legge GB, Morris GM, Sanner MF, Takada Y, Olson AJ, Grynszpan F. Model of the alphaLbeta2 integrin I-domain/ICAM-1 DI interface suggests that subtle changes in loop orientation determine ligand specificity. *Proteins*. 2002; 48:151–60. [PubMed: 12112684]
23. Skovseth DK, Kuchler AM, Haraldsen G. The HUVEC/Matrigel assay: an in vivo assay of human angiogenesis suitable for drug validation. *Methods in molecular biology*. 2007; 360:253–68. [PubMed: 17172733]
24. Ruoslahti E. RGD and other recognition sequences for integrins. *Annu Rev Cell Dev Biol*. 1996; 12:697–715. [PubMed: 8970741]
25. Lusinskas FW, Lawler J. Integrins as dynamic regulators of vascular function. *Faseb J*. 1994; 8:929–38. [PubMed: 7522194]
26. Max R, Gerritsen RR, Nooijen PT, et al. Immunohistochemical analysis of integrin alpha v beta3 expression on tumor-associated vessels of human carcinomas. *Int J Cancer*. 1997; 71:320–4. [PubMed: 9139861]
27. Brooks PC, Montgomery AM, Rosenfeld M, et al. Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell*. 1994; 79:1157–64. [PubMed: 7528107]
28. Wang X, Peng L, Liu R, Xu B, Lam KS. Applications of topologically segregated bilayer beads in 'one-bead one-compound' combinatorial libraries. *J Pept Res*. 2005; 65:130–8. [PubMed: 15686543]
29. Goodman SL, Holzemann G, Sulyok GA, Kessler H. Nanomolar small molecule inhibitors for alphav(beta)6, alphav(beta)5, and alphav(beta)3 integrins. *Journal of medicinal chemistry*. 2002; 45:1045–51. [PubMed: 11855984]
30. Pasqualini R, Koivunen E, Ruoslahti E. Alpha v integrins as receptors for tumor targeting by circulating ligands. *Nat Biotechnol*. 1997; 15:542–6. [PubMed: 9181576]
31. Albelda SM, Mette SA, Elder DE, et al. Integrin distribution in malignant melanoma: association of the beta 3 subunit with tumor progression. *Cancer Res*. 1990; 50:6757–64. [PubMed: 2208139]
32. Bello L, Francolini M, Marthyn P, et al. Alpha(v)beta3 and alpha(v)beta5 integrin expression in glioma periphery. *Neurosurgery*. 2001; 49:380–9. [PubMed: 11504114]
33. Falcioni R, Cimino L, Gentileschi MP, et al. Expression of beta 1, beta 3, beta 4, and beta 5 integrins by human lung carcinoma cells of different histotypes. *Exp Cell Res*. 1994; 210:113–22. [PubMed: 7505746]
34. Sengupta S, Chattopadhyay N, Mitra A, Ray S, Dasgupta S, Chatterjee A. Role of alphavbeta3 integrin receptors in breast tumor. *J Exp Clin Cancer Res*. 2001; 20:585–90. [PubMed: 11876555]
35. Brooks PC, Clark RA, Cheresh DA. Requirement of vascular integrin alpha v beta 3 for angiogenesis. *Science*. 1994; 264:569–71. [PubMed: 7512751]
36. Curley GP, Blum H, Humphries MJ. Integrin antagonists. *Cell Mol Life Sci*. 1999; 56:427–41. [PubMed: 11212296]
37. Tucker GC. Integrins: molecular targets in cancer therapy. *Curr Oncol Rep*. 2006; 8:96–103. [PubMed: 16507218]
38. Meyer A, Auernheimer J, Modlinger A, Kessler H. Targeting RGD recognizing integrins: drug development, biomaterial research, tumor imaging and targeting. *Curr Pharm Des*. 2006; 12:2723–47. [PubMed: 16918408]
39. Ruoslahti E, Pierschbacher MD. Arg-Gly-Asp: a versatile cell recognition signal. *Cell*. 1986; 44:517–8. [PubMed: 2418980]
40. D'Souza SE, Ginsberg MH, Plow EF. Arginyl-glycyl-aspartic acid (RGD): a cell adhesion motif. *Trends Biochem Sci*. 1991; 16:246–50. [PubMed: 1926332]
41. Pfaff M, Tangemann K, Muller B, et al. Selective recognition of cyclic RGD peptides of NMR defined conformation by alpha IIb beta 3, alpha V beta 3, and alpha 5 beta 1 integrins. *The Journal of biological chemistry*. 1994; 269:20233–8. [PubMed: 8051114]
42. Li R, Hoess RH, Bennett JS, DeGrado WF. Use of phage display to probe the evolution of binding specificity and affinity in integrins. *Protein Eng*. 2003; 16:65–72. [PubMed: 12646694]
43. Richards J, Miller M, Abend J, Koide A, Koide S, Dewhurst S. Engineered fibronectin type III domain with a RGDWXE sequence binds with enhanced affinity and specificity to human alphavbeta3 integrin. *J Mol Biol*. 2003; 326:1475–88. [PubMed: 12595259]

44. Silverman AP, Levin AM, Lahti JL, Cochran JR. Engineered cystine-knot peptides that bind $\alpha(v)\beta(3)$ integrin with antibody-like affinities. *J Mol Biol.* 2009; 385:1064–75. [PubMed: 19038268]
45. Richardson JS. The anatomy and taxonomy of protein structure. *Adv Protein Chem.* 1981; 34:167–339. [PubMed: 7020376]
46. Harris TD, Kalogeropoulos S, Nguyen T, et al. Structure-activity relationships of ^{111}In - and $^{99\text{m}}\text{Tc}$ -labeled quinolin-4-one peptidomimetics as ligands for the vitronectin receptor: potential tumor imaging agents. *Bioconjugate chemistry.* 2006; 17:1294–313. [PubMed: 16984141]
47. Chen X, Park R, Tohme M, Shahinian AH, Bading JR, Conti PS. MicroPET and autoradiographic imaging of breast cancer αv -integrin expression using ^{18}F - and ^{64}Cu -labeled RGD peptide. *Bioconjugate chemistry.* 2004; 15:41–9. [PubMed: 14733582]
48. Dijkgraaf I, Kruijtz JA, Liu S, et al. Improved targeting of the $\alpha(v)\beta(3)$ integrin by multimerisation of RGD peptides. *Eur J Nucl Med Mol Imaging.* 2007; 34:267–73. [PubMed: 16909226]
49. Chen X, Park R, Hou Y, et al. microPET and autoradiographic imaging of GRP receptor expression with ^{64}Cu -DOTA-[Lys3]bombesin in human prostate adenocarcinoma xenografts. *J Nucl Med.* 2004; 45:1390–7. [PubMed: 15299066]

Abbreviations

Natural amino acids were designated by the standard three-letter code. “D” stands for D-configuration.

Aad	2-aminohexanedioic acid
All-OSu	N-allyloxycarbonyloxy succinimide
HATU	2 - (7 - Aza - 1H - benzotriazole - 1 - yl) - 1,1,3,3 - tetramethyluronium hexafluorophosphate
Bec	(R)-2-amino-3-(2-carboxyethylsulfanyl)propanoic acid
Boc2O	Di-t-butyl dicarbonate
DIC	1, 3-Diisopropylcarbodiimide
DIPEA	N, N'-diisopropylethylamine
EDT	1, 2-ethanedithiol
Equiv	Equivalent
Fmoc-OSu	N-(9-fluorenylmethyloxycarbonyloxy)-succinimide
HBTU	(O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate
HOAt	1 - Hydroxy - 7 - azabenzotriazole
HOBt	1-Hydroxybenzotriazole
MeOH	Methanol
DCM	Dichloromethane
DMF	N, N-Dimethylformamide
TFA	Trifluoro acetic acid
Tis	Triisopropylsilane
Orn	Ornithine

Figure 1.

a: Structure of LXW7 b: Structure of biotinylated LXW7 c: Series of integrin transfected K562 cells were stained with LXW7. Samples with black curves were treated with 1 μ M LXW7-Biotin and detected with Flow Cytometry. Red curves depict samples without treatment of LXW7-Biotin and as negative controls. LXW7 showed remarkable positive binding with α v β 3, very weak cross-reactivity with α v β 5 and α IIb β 3, no binding with α 5 β 1.

Figure 2. Docking simulation studies on LXW7. Panel a displays the docked conformation of *cyclo*(RGDfV) and LXW7 (yellow and cyan, respectively) on the surface of αvβ3 integrin. Panel b indicates an interaction between the carboxylate side chain of Asp of both ligands with the Mg²⁺ within the MIDAS domain of the β-subunit. The salt bridge formed between *D*-Asp of LXW7 and Arg214 of the β-subunit is shown in Panel c. Panel d illustrates the salt bridge formed between Arg of LXW7 and Asp218 of the α-subunit. The guanidinium side chain of Arg in *cyclo*(RGDfV) is able to interact with both carboxylate oxygens while the side chain of Arg in LXW7 only interacts with one of the carboxylate oxygens.

Figure 3.

The bindings of LXW7 to U-87 MG and A375M cells were blocked by anti- $\alpha v \beta 3$ antibody (LM609). Both glioblastoma U-87MG (a) and melanoma A375M (c) displayed expression of $\alpha v \beta 3$ integrin. LXW7 bound to both tumor cells, and the binding was markedly blocked by anti-human $\alpha v \beta 3$ antibody (b, d).

Figure 4.

In vivo and *ex vivo* near infra red fluorescent imaging on nude mice implanted with xenografts. Six hours after tail vein injection with (1) Streptavidin-Cy5.5 alone or (2) Biotinylated LXW7-Streptavidin-Cy5.5 complex, Kidney uptake was high in both mice but preferential uptake into the U-87MG tumor (a) and A375M (b) were noted in mice given biotinylated LXW7-Streptavidin-Cy5.5 complex. The images shown represent one of three groups. *Ex vivo* imaging of U-87MG xenograft bearing nude mice after injection of Streptavidin-Cy5.5-biotinylated LXW7 and cyclic RGD pentapeptides (*cyclo*(RGDfE), *cyclo*(RGDfK) and *cyclo*(RGDyK)) was shown in (c). LXW7 exhibited high tumor uptake with low liver uptake (c). The reverse was true for the other three cyclic RGD pentapeptides. Relative fluorescence uptake by tumor and liver (c) was quantified in (d). Streptavidin-Cy5.5 alone without peptide was utilized as control.

Table 1

Sequences of positive beads selected from the libraries screening with cells.

Library 1 ^a			Library 3 ^a			Library 4 ^a		
HUVEC	K562(α5β1)	αvβ3-K562	αvβ3-K562		αvβ3-K562		αvβ3-K562	
ckRGDdnc	crRGDqnc	cGRGDyhc	cGRGDdvc (2) ^b		cGKGDdsc(2) ^b			
ckRGDdyc	crRGDmnc	cGRGDsfc	cGRGDdfc		cGKGDdvc			
ckRGDric	ckRGDfqc	cGRGDmec	cGRGDdnc		cGKGDdqc			
ckRGDyqc	ckRGDfdc	cGRGDqic	cGRGDdic		cGKGDeyc			
crRGDdac	cmRGDhmc	cGRGDfvc	cGRGDehc		cGKGDefc			
crRGDdwc	cmRGDhdc	cGRGDdfc	cGRGDetc		ctKGDdyc			
caRGDhac	cqRGDymc	ckRGDdic	cGRGDnpc		cGKGDsec(2) ^b			
caRGDhmc		ctRGDdfc	cGRGDnyc		cGKGDnyc			
caRGDrdc		caRGDdgc	cGRGDnhc		cGKGDnwc			
cGRGDlgc		cqRGDyfc	cGRGDnec					
cGRGDnic			cGRGDhdc					
csRGDfde			cGRGDhgc					
csRGDqgc			cGRGDhpc					
cyRGDyec			cGRGDqdc					
clRGDqgc			cGRGDtyc					
cpRGDwnc								
cqRGDwpc								
cdRGDhgc								

^aLibrary 1 (8mer) was synthesized on TentaGel beads using “split-mix” synthetic method. Library 3 and 4 were constructed using topological segregated bilayer beads, in which 80% of the outer layer (16% of total loading) is blocked by acetylated glycine; then the library is constructed on the rest of 20% of the outer layer (4% of total loading). The building blocks on different positions are:

X⁺: R, K and Orn. X⁻: Asp, Glu, Aad and Bec. X^K: K and Orn. x₂, x₆, and x₇: 19 D-amino acids (excluding D-cysteine).

^bThe number in the parenthesis indicates the appearance frequency of the peptide sequence.

Note: Small letters indicate D-amino acids.

Table 2

*IC*₅₀ of selected peptides and biotinylated peptides inhibiting the binding of Echistatin- FITC to αvβ3-K562 cells

Peptide	<i>IC</i> ₅₀ (μM)
cGRGDsfc (LXW1)	2.55±0.18
cGRGDdfc (LXW2)	1.7±0.12
cGRGDsec (LXW3)	0.89±0.09
cGKGDsec (LXW4)	>20
cGRGDdsc (LXW5)	1.23±0.10
cGKGDdsc (LXW6)	>20
cGRGDdvc (LXW7)	0.68±0.08
LXW7-Bio	0.62±0.07
cGKGDdvc (LXW8)	>20
<i>cyclo</i> (RGDfE)	0.60±0.07
<i>cyclo</i> (RGDfE)-Bio	4.7±0.8
<i>cyclo</i> (RGDfK)	0.65±0.08
<i>cyclo</i> (RGDfK)-Bio	1.37±0.2
<i>cyclo</i> (RGDyK)	0.34±0.04
<i>cyclo</i> (RGDyK)-Bio	1.35±0.09
<i>cyclo</i> (RGDfV)	0.54±0.05
<i>cyclo</i> (RGDf-N(me)V-)	0.27±0.03