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Discovery of Targeting Ligands for Breast Cancer Cells Using the One-Bead One-Compound Combinatorial Method

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Abstract

Four “One-bead one-compound” (OBOC) combinatorial libraries were designed, synthesized, and screened against MDA-MB-231 breast cancer cells. A novel cyclic peptide **1** (LXY1) with high binding specificity to $\alpha 3$ integrin, was identified. Molecular interactions between $\alpha 3$ integrin and **1** were characterized by using a series of K562 cells transfected with various mutant $\alpha 3$ integrins. Using analytic flow cytometry, the binding affinity (Kd) of **1** to $\alpha 3$ integrin on MDA-MB-231 breast cancer cells was determined to be approximately 0.4 μ M. Based on the established structure-activity relationship (SAR) study, two highly focused cyclic peptide libraries were further designed, synthesized, and screened against MDA-MB-231 breast cancer cells under stringent conditions. A novel cyclic peptide **2** (LXY3) with a high binding affinity (IC₅₀=57 nM) was identified. Moreover, the targeting efficiency and specificity of **2** to the breast adenocarcinoma tumors in mouse xenografts was further confirmed by *in vivo* and *ex vivo* near infra-red fluorescence optical imaging.

Introduction

Breast cancer is the most common malignant tumor among women, accounting for an estimated 24% of all cancer cases.¹ Despite the intensity of treatment with chemotherapy, radiation therapy and surgery, breast cancer remains the second most lethal cancer for women, accounting for 18% of all cancer deaths because of its propensity to metastasize even before the disease can be detected clinically or by screening mammography.² Hormonal therapy is useful for the medical management of estrogen receptor (ER)/progesterone receptor (PR) positive breast cancers. ER/PR negative breast cancers tend to be more aggressive, and both taxane-based chemotherapy and radiation therapy remain the main medical components of treatment. Anti-HER-2 monoclonal antibody (mAb) or Herceptin® is useful for the treatment of HER-2/Neu expressing breast cancers, although these account for only about 20% of all breast cancers.³ Radioimmunotherapy using an [¹³¹I]-labeled monoclonal antibody against tumor cells has shown some promise in early clinical studies.^{4–8} This agent, however, has limitations including, (i) its relatively large size (160 kD)⁹ and (ii) nonspecific uptake of the antibody molecules by the reticuloendothelial system in sites such as the liver, spleen, and bone marrow, leading to dose-limiting toxicities.¹⁰ Many groups have attempted to overcome these two limitations by using genetically-engineered antibody fragments with varying success.^{11, 12} An alternative approach to circumvent the problem of radioimmunoconjugates or immunotoxins is to develop peptide-based cancer cell surface or tumor neovascular cell surface

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Supporting Information Available: Synthetic procedures, Characterization of NMR and MS, Sequences of positive beads, as well as Biological data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

targeting agents to deliver radionuclides, toxins, or cytotoxic agents to the tumor site.¹³ These peptides are usually derived from binding motifs of known proteins or from phage-displayed peptide libraries, both of which are limited to L-amino acids, and thus are susceptible to proteolysis.^{14–16} Optimization of these ligands to render them resistant to proteolysis is possible but time-consuming. We have developed the “one-bead one-compound” (OBOC) combinatorial library method^{17, 18} and whole cell binding assay^{19, 20} to synthesize and rapidly identify D-amino acid-containing cancer cell targeting ligands. Peptides containing D-amino acids are generally more resistant to proteolysis. In OBOC libraries, each resin bead displays a unique peptide and millions of library beads can be screened in parallel against the biological target of interest.¹⁸ The positive beads are then physically isolated for structural determination by microsequencing using automatic Edman degradation. Direct microsequencing of the library compounds requires that the peptides consist solely of α -amino acids with free *N*-terminals, thus limiting the flexibility of peptide library design. To circumvent this, we have developed new methods to topologically segregate bilayer beads in which the library compounds are displayed on the bead surface and the encoding tags reside on the bead interior.²¹ With this approach, one can easily design and prepare encoded OBOC libraries with peptides blocked at the *N*-terminal, cyclic peptides, branched peptides, peptides with otherwise unsequenceable building blocks, peptides with free *C*-terminals, peptidomimetics, small molecules and macrocyclic molecules.^{22, 23} In this report, we describe the use of OBOC combinatorial chemistry and high throughput on-bead cell binding assays with high stringency to identify novel breast cancer targeting ligands. Through antibody blocking and analysis by flow cytometry, the cell surface receptor for these cyclic peptide ligands was determined to be $\alpha 3$ integrin. *In vivo* and *ex vivo* near infra red (NIR) optical imaging studies indicated that the novel cyclic peptide ligand **2** is an excellent candidate for the development of radio-targeting agents for breast cancer.

Results and Discussions

Design, synthesis and screen of “one-bead one-compound” peptide libraries

In our initial study, several random linear and cyclic OBOC peptide libraries were synthesized and screened against an ER-negative breast cancer cell line (MDA-MB-231) using a cell-growth-on-bead assay.^{19, 20} A cyclic peptide with the sequence cEGLGEWc was shown to bind to the MDA-MB-231 breast cancer cells. “Alanine scan” analysis on the peptide (cEGLGEWc) performed by sequentially replacing each amino acid with alanine clearly demonstrated that residues Cys-1, Glu-2, Gly-3, Gly-5 and Cys-8 were critical for cell binding. Based on this result, an OBOC cyclic peptide library (Supplementary Scheme 1) with a cX₁GX₃GX₅X₆c motif (Library **1**) was designed and synthesized. In this library, the X₁ position was diversified with 42 natural and unnatural amino acids including D-amino acids (Supplementary Table 1). To differentiate D-amino acids from L-amino acids, 20% Norleucine (Nle^a) was added to encode D-amino acids. Each position of X₃, X₅ and X₆ contained one of 41 natural and unnatural L-amino acids (Supplementary Table 1). This library was screened against MDA-MB-231 breast cancer cells using a standard whole-cell binding assay.^{19, 20} About 3×10⁵ beads (400μL of settled beads) were screened and 19 positive beads were isolated for microsequencing. The amino acid sequences of these peptides are shown in Supplementary Table 2. Strong consensus in sequence was observed in: the X₁ position with D-aspartic acid

^aAbbreviations: Abbreviations used for amino acids and designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, 247, 977-983. The following additional abbreviations are used: Nle, Norleucine; Cha, cyclohexylalanine; Tyr(3-NO₂), 3-Nitro-tyrosine; HoSer, Homoserine; 4-Pal, 3-(4-pyridyl)alanine; HoPhe, Homophenylalanine; Hyp, Hydroxy praline; Fmoc, fluorenylmethoxycarbonyl; ESI, electrospray ionization; NMR, nuclear magnetic resonance; HMQC, (¹H-detected) heteronuclear multiple-quantum coherence; HMBC, (¹H-detected) heteronuclear multiple-bond coherence; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser enhancement; HOBt, 1-hydroxy-6-chlorobenzotriazole; DIC, N, N'-diisopropylcarbodiimide; RP-HPLC, reverse-phase high performance liquid chromatography; TFA, trifluoroacetic acid; DMF, N,N-dimethylformamide.

(80%) and L-aspartic acid (15%), the X₅ position with proline and hydroxyproline (65%), the X₃ position with leucine analogues (55%) and phenylalanine analogues (20%), and in the X₆ position with polar amino acids such as asparagine (31%), and threonine (16%).

Since D-amino acids containing peptides are more resistant to proteolysis, three focused OBOC combinatorial cyclic peptide libraries (Supplementary Scheme 1) containing D-amino acids were further designed and synthesized. Based on the fact that D-aspartic acid was found in the x₁ position in 80% of the isolated peptides, we fixed the x₁ position with D-aspartic acid and randomized the other three residues (x₃, x₅, and x₆) using 19 D-amino acids in library **2**; D-Cys-OH was excluded from these residues. In library **3**, Fmoc-4-Nitro-L-Phenylalanine was introduced to the x₃ position to provide a potential amino group through the reduction of a nitro group with tin chloride. A branched random tri-peptide (x₃x₂x₁) was then coupled to the aniline. In library **4**, 44 different carboxylic acids (R₄) (Supplementary Table 3) were attached to the *N*-terminal amino group of an OBOC cyclic peptide library. To encode the carboxylic acid group of the library compound on the bead outer layer, Boc-Lys(Fmoc)-OH was coupled to the peptide chain on the bead interior. After Fmoc-deprotection, the carboxylic acids were coupled to the *N*-terminus of the library compound on the outer layer, and ϵ -amino group of lysine on the bead interior, leaving the *N*-terminal α -amino group free for decoding with standard Edman microsequencing. Since Edman degradation is slow and expensive, the bilayer bead approach²¹ was also applied in the construction of library **2**, **3** and **4** to minimize the number of residues in the peptide coding chain that resided on the bead interior. The full length cyclic octapeptides were displayed on the bead surface, whereas the corresponding coding tag, a truncated linear tri-peptide (x₃x₅x₆), was presented on the bead interior of Library **2**, a five-member peptide (x₁x₂x₃x₅x₆) was presented on the bead interior of Library **3**, and a four-member peptide (Lys(R₄)x₃x₅x₆) was presented on the bead interior of Library **4** for Edman microsequencing. These libraries were screened against MDA-MB-231 breast cancer cells, however, no significant cell binding was observed in these three focused OBOC combinatorial cyclic peptide libraries. This suggested an L-amino acid was required in some or all of the x₃, x₅ and x₆ positions, and a free *N*-terminus was necessary for MDA-MB-231 breast cancer cell binding.

To confirm the screening results from the whole cell binding assay, cdGLGBNc (**1**, wherein B = hydroxyproline), one of the representative cyclic peptide ligands, was synthesized on TentaGel resin. MDA-MB-231 cells binding assay showed that all peptide ligand **1** beads were fully covered by MDA-MB-231 cells within 15 minutes (Supplementary Figure 1). Interestingly, this same peptide was also identified from OBOC library screening experiments with U-87MG glioblastoma cell lines.²⁴

Specificity of peptide ligand **1** to α 3 integrin

Integrins are a family of cell surface proteins composed of α and β subunits, which mediate cell-matrix and cell-cell adhesion.²⁵ Lundstrom *et al*²⁶ reported that the aggressive MDA-MB-231 human breast carcinoma cell line expressed high levels of α 2 β 1 and α 3 β 1 integrins at the cell surface. We have previously identified c(D/N)GXGXXc as the peptide binding motifs for α 3 β 1 integrin in lung and ovarian cancers.^{27, 28} Since **1** contains the same cXGXGXXc motif, α 3 β 1 integrin is likely to be the receptor for peptide ligand **1**. To confirm this, MDA-MB-231 breast cancer cells were treated separately with a series of anti-integrin monoclonal antibodies (anti α 1, α 2, α 3, α 4, α 5, α 6, α V, β 1, β 2, β 3, β 4, β 5) prior to incubation with peptide ligand **1** beads. We found that only anti α 3 antibody could completely block MDA-MB-231 breast cancer cell binding to peptide ligand **1** beads (Supplementary Figure 2) indicating that the molecular target for **1** was indeed α 3 integrin. The binding specificity of **1** to α 3 integrin was further confirmed with flow cytometry. **1** was first biotinylated at the C-terminus via a hydrophilic spacer²⁹ to minimize the interference by streptavidin (Scheme 1

and Supplementary Scheme 2). K562 cells expressing $\alpha 5$ integrin and K562 cells transfected with $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 6$, $\alpha 9$ and $\alpha v\beta 3$ integrins were then incubated with biotinylated **1**, followed by streptavidin-PE staining. As shown in Figure 1, only $\alpha 3$ -transfected K562 cells exhibited strong binding, whereas both K562 parent cells and those transfected with other integrins did not. This further demonstrates the binding specificity of **1** to $\alpha 3$ integrin on MDA-MB-231 cells.

Binding affinity of peptide ligand **1** to MDA-MB-231 Cells

The $\alpha 3\beta 1$ integrin has been reported to recognize several extracellular matrix proteins, including some laminins, type IV collagen, fibronectin, thrombospondin-1, and entactin/nidogen.^{30–33} Binding interactions between $\alpha 3\beta 1$ integrin and peptides derived from these native proteins have been demonstrated via adhesion inhibition assays,^{34–39} but the measured affinities were low. In our initial experiments, we used flow cytometry⁴⁰ and biotinylated **1** to directly determine the binding affinity of **1** to $\alpha 3\beta 1$ integrin expressed on MDA-MB-231 cells. MDA-MB-231 cells were treated with or without anti- $\alpha 3$ integrin antibody (10 μ g) prior to sequential incubation with various concentrations of biotinylated **1** and Streptavidin-Phycoerythrin (PE). The fluorescence signal obtained in the presence of anti- $\alpha 3$ integrin blocking antibody represented nonspecific binding of **1**. The fluorescence signal obtained in the absence of blocking antibody represented total binding of **1** to MDA-MB-231 cells. Specific binding to $\alpha 3\beta 1$ integrin was calculated by subtracting nonspecific binding from total binding. Using this method, the apparent dissociation constant (K_d) for the specific binding of peptide ligand **1** to MDA-MB-231 cells was determined to be 0.4 $\pm$ 0.07 μ M (Figure 2).

The molecular interaction between peptide ligand **1** and $\alpha 3$ integrin

Molecular interactions between the peptide ligand **1** and $\alpha 3$ integrins were elucidated using K562 myeloid leukemia cells transfected with various mutant $\alpha 3$ integrins. Laminin-5, a native ligand for integrin $\alpha 3\beta 1$,⁴¹ is a unique laminin variant which promotes cell adhesion and migration. Kruonis *et al*⁴² reported that mutations of Tyr-186 and Trp-188 to Ala blocked $\alpha 3\beta 1$ /laminin-5 interaction, while mutation of Asp-122 to Ala increased the affinity of $\alpha 3\beta 1$ adhesion to laminin-5. Zhang *et al*⁴³ suggested that both Thr-162 and Gly-163 are critical for $\alpha 3\beta 1$ adhesion to laminin-5 and its regulation. Since laminin-5 is the predominant natural ligand for $\alpha 3\beta 1$ integrin, we selected 26 mutations in proximity to these critical amino acids for binding assays with **1**. Biotinylated **1** was first incubated with K562 and a series of $\alpha 3$ mutant-transfected K562 cells, followed by streptavidin-PE, and analyzed binding by flow cytometry. Anti- $\alpha 3$ -Alexa488 was used to normalize cell surface expression of $\alpha 3$ integrin in K562 cells. The binding of **1** to each mutant was determined after normalization for expression levels of $\alpha 3$. Majority of the K562 cells which expressed mutant $\alpha 3$ bound to **1** at levels comparable with cells expressing wild-type $\alpha 3$. However, the D158A, T162A, G163A, M164A and Y186A mutations resulted in a significant drop in binding affinity to **1** (Figure 3). This binding profile was consistent with the fact that Thr-162, Gly-163, and Tyr-186 are crucial for binding of laminin-5 to $\alpha 3\beta 1$. Interestingly, while Asp-158 and Met-164 have not been implicated in laminin-5 binding, we found that D158A and M164A mutations led to a decrease in peptide ligand **1** binding. Together, these data suggest that peptide ligand **1** and laminin-5 may recognize an overlapping, but not totally identical, set of residues on the $\alpha 3$ integrin receptor.

Design, synthesis and screening of two highly focused OBOC peptide libraries

To identify high binding affinity ligands to MDA-MB-231 cells, two highly focused OBOC peptidomimetic libraries with the general sequence cdGX₃GX₅X₆c were designed and synthesized based on the above structure - activity relationship (SAR) results. The simplified synthetic route is presented in Supplementary Scheme 3. In synthesizing both focused libraries,

the X₆ position was diversified with 36 natural and unnatural L-amino acids (Supplementary Table 4). Since the X₅ position had a preference for hydroxyproline, proline and threonine in library **1**, we included three amino acids plus 8 β -turn-promoting amino acids in the X₅ position of this library (Supplementary Table 5). Sequence determination of peptides containing these unsequenceable amino acids was simplified via use of our topographically segregated bilayer bead method which involved incorporation of these amino acids on the outer layer of the library beads, and corresponding sequenceable α -amino acids (the peptide coding tag) on the bead interior.^{23, 44} Since the X₃ position showed a preference for phenylalanine and leucine analogues (described above), we introduced 8 L-phenylalanine analogues and 29 L-phenylalanine derivatives (29 carboxylic acids were coupled to a potential amino group via the reduction of a nitro group of 4-Nitro-L-Phenylalanine) to the X₃ position of library **A** (Supplementary Table 6), and 10 leucine analogues and 26 L-lysine derivatives were introduced to the X₃ position of library **B** (Supplementary Table 7). To increase screening assay stringency such that peptide ligands with higher binding affinity than peptide ligand **1** could be discovered, 10 μ M peptide ligand **1** was added to the screening solution to compete with the interaction between MDA-MB-231 cells and the immobilized compounds on the library beads. Under these conditions, the two highly focused libraries (libraries **A** and **B**) were screened against MDA-MB-231 breast cancer cells. A total of six positive beads were selected from both libraries A and B for microsequencing. Their amino acid sequences are presented in Table 1. Strong consensus was observed among the peptide sequences, with Tyr(3-NO₂) and Cha in the X₃ position, Hyp in the X₅ position, and polar amino acids such as Asn, HoSer, Thr and 4-Pal in the X₆ position. Further modification at the X₃ position using L-phenylalanine and L-lysine derivatives did not increase the binding affinity to MDA-MB-231 cells.

Identification and characterization of the peptidomimetic ligand **2**

To compare binding affinities of peptide ligands identified from the highly focused libraries **A** and **B** (Table 1) to that of peptide **1**, various concentrations of biotinylated **1** and peptide ligands (Table 1) were incubated with MDA-MB-231, followed by streptavidin-PE staining and analysis using flow cytometry. The IC₅₀ of all the identified peptide ligands, presented in Table 1, indicated higher binding affinities to MDA-MB-231 cells than peptide ligand **1**.

Given its high binding affinity for MDA-MB-231 cells, peptide ligand **2** (cdGTyr(3-NO₂)GBNc, wherein B = hydroxyproline, IC₅₀=57nM), was resynthesized and its structure was characterized by ESIMS, 1D- and 2D-NMR. ESIMS data showed a pseudomolecular ion [M + H]⁺ at *m/z* 886.6 which is consistent with C₃₂H₄₃N₁₁O₁₅S₂. The proton and carbon chemical shift of **2**, as presented in Supplementary Table 8, was assigned on the basis of the analysis of both 1D-NMR (¹H NMR and ¹³C NMR) and 2D-NMR data (HMQC, HMBC, TOCSY and NOESY). NOESY was used to establish the conformation of **2**. The proton correlations of **2** were showed in Scheme 2. In NOESY, proton correlations between the amide protons of Tyr(3-NO₂) and Gly4, and the amide protons of D-Asp and Gly2, suggested that both proton groups must be in *cis*- conformation. NOE cross peaks were observed between the amide protons of D-Asp and Gly4, indicating that the amide protons of D-Asp, Gly2, Tyr(3-NO₂) and Gly4 should be on the same side of the ring. In addition, the α CH of Gly4 and the N-CH of Hyp, the α CH of Asn and the β CH of Hyp, and the NH of both Asn and D-Cys (C-terminal) showed correlation in the NOESY, which suggested that Gly4, Hyp and Asn are located on the same side of the ring. Moreover, the presence of NOEs between the β CH of D-Cys (C-terminal), the α and β CH of D-Cys (N-terminal), and the absence of NOEs between the two parts of the ring (for example, the N-terminal part (cdG-Tyr(3-NO₂)) and the C-terminal part(G-Hyp-Nc)) defined the cyclic **3** as having a β turn-like structure.

To further demonstrate that **2** can act as a specific ligand to α 3 integrin on MDA-MB-231 cells, biotinylated **2** was incubated with MDA-MB-231 cells, followed by secondary staining with

streptavidin-Alexa488. Figure 4 clearly demonstrates positive MDA-MB-231 staining by the biotinylated **2**. Binding of the biotinylated **2** to MDA-MB-231 cells was fully blocked by co-incubation with either excess free peptide **2** or anti $\alpha 3$ integrin antibody, confirming that the peptide **2**, like **1**, also binds specifically to $\alpha 3$ integrin on MDA-MB-231 cells.

Near-infra red (NIR) optical imaging of breast cancer xenografts

Modern optical imaging techniques offer real-time and high-resolution imaging of fluorophores embedded in tissues.⁴⁵ Near-infrared (NIR) fluorescence-based imaging is useful for non-invasive *in vivo* imaging in small animals because of high tissue penetration and minimal auto-fluorescence with NIR light.⁴⁶ Cy5.5 has been used previously by several groups as a convenient NIR fluorescent dye for *in vivo* detection of tumors.^{47, 48} Here, we used *in vivo* and *ex vivo* NIR optical imaging to investigate the targeting efficiency of peptide **2** in nude mice bearing breast cancer xenografts. Biotinylated **2** was first incubated with streptavidin-Cy5.5 to form peptide ligand **2** -SA-Cy5.5 complex. Various doses of peptide ligand **2** -SA-Cy5.5 were injected into the tail vein of nude mice bearing MDA-MB-231 xenografts to establish an optimal dose that had the highest ratio of tumor to normal tissue fluorescence intensity. This was found to be approximately 7.2 nmol peptide ligand **2** -SA-Cy5.5 (data not shown). The mice were scanned with the fluorescence imager at various intervals for up to 72 hours (Supplementary Figure 3). Tumor uptake of the NIR signal was found to peak at about 6 hours post-injection and slowly washed out, although significant signal remained at the tumor site even after 72 hours.

To validate the specificity of the targeting compound **2**, nude mice bearing orthotopic MDA-MB-231 mammary fat pad xenografts on one side and K562 chronic myeloid leukemia xenografts (expressing $\alpha 5 \beta 1$ integrin) on the contralateral side as a negative control, were treated with peptide ligand **2** -SA-Cy5.5 by tail vein injection. Fluorescence uptake by the MDA-MB-231 xenograft was found to be much higher than that of the K562 xenograft. Additionally, when an anti- $\alpha 3$ integrin blocking antibody was administered prior to the administration of peptide ligand **2** -SA-Cy5.5, *ex vivo* imaging of the MDA-MB-231 tumor (Figure 5) showed a significant drop in fluorescence intensity. There was moderate and high fluorescence uptake in the liver (Figure 5) and kidneys (data not shown), respectively. The high renal uptake could be explained in part by the known affinity of streptavidin for kidney.⁴⁹ Together, these results indicate that peptide ligand **2** is relatively specific against $\alpha 3$ integrin in MDA-MB-231 xenografts.

Conclusions

The OBOC combinatorial library method, using the “split-mix” synthesis approach, enables one to rapidly synthesize hundreds of thousands to millions of compounds. With the development of the bilayer bead methods we described, OBOC peptide libraries as well as encoded peptidomimetic, small molecule, and macrocyclic OBOC libraries can be readily synthesized and decoded. These methods substantially increase flexibility in library design and potential diversity of OBOC combinatorial libraries. Through screening four OBOC peptide libraries against live MDA-MB-231 cells, a series of peptide ligands were identified, and important structure and activity relationships were elucidated. These include the findings that: (i) cyclic octapeptides with residues D-Cys-1, D-Asp-2, Gly-3, Gly-5, L-Hyp-6, and D-Cys-8 are crucial for MDA-MB-231 cell binding; (ii) L-amino acids in position 4, and 7 and a free *N*-terminal are necessary for MDA-MB-231 cell binding; and (iii) small polar amino acids, such as Asn, Thr and homoserine in position 7 are preferred for MDA-MB-231 cell binding. Based on this information, we constructed two highly focused OBOC peptidomimetic libraries incorporating these features. Using a highly stringent competitive cell-based screening method, peptide ligand **2** was discovered and determined to exhibit both high affinity binding to MDA-

MB-231 breast cancer cells ($IC_{50}=57\text{nM}$), and high specificity for $\alpha 3$ integrin. NMR studies of **2** strongly suggest that **2** displays a β -turn conformation. The binding profile of **1** and laminin-5 to $\alpha 3$ integrin mutants has provided valuable molecular recognition information. For example, the binding site of **1** on $\alpha 3$ integrin was found to overlap with but not entirely identical to that of laminin-5. *In vivo* and *ex vivo* near infra-red fluorescence optical imaging studies have confirmed the targeting efficiency and specificity of **2** for the MDA-MB-231 breast tumor xenograft model. Also, **2** has been proven capable of delivering optical probes to mouse-implanted breast tumors derived from MDA-MB-435, MDA-MB-436, and MDA-MB-468 cell lines (data not shown). Work is currently underway in our laboratory to evaluate the binding profile of **2** to cancer cells obtained directly from primary breast cancer tumors. Should **2** prove to bind strongly to a significant fraction of clinical breast cancer specimen, it will be an excellent candidate for use in efficient vehicle delivery of radionuclides (such as ^{64}Cu , ^{67}Cu or ^{90}Y), cytotoxic agents, or drug loaded-nanoparticles for breast cancer imaging and therapy.

Experimental Section

Materials

TentaGel S NH_2 resin (90 μm , 0.26 mmol/g) was purchased from Rapp Polymere GmbH (Tübingen, Germany). Rink amide MBHA resin (0.5 mmol/g), amino acid derivatives, HOBt, and DIC were purchased from GL Biochem (Shanghai, China). All solvents and other chemical reagents were purchased from Aldrich (Milwaukee, WI) and were analytical grade. ESIMS was performed with Finnigan LCQ. Analytical HPLC was performed on a Waters 2996 HPLC system equipped with a $4.6 \times 150\text{ mm}$ Waters Xterra® MS C18 5.0 μm column, and employed a 20 min gradient from 100% aqueous H_2O (0.1% TFA) to 100% CH_3CN (0.1% TFA) at a flow rate of 1.0 mL/min. Preparative HPLC was performed on a System Gold 126NMP Solvent Module (Beckman) with a C18 column (Vydac, 5 μm , 2.5 cm i.d. $\times$ 25 cm). A gradient elution of 0–60% B over 25 min followed by 60–100% B over 25 min followed by 100% B for 5 min was used at a flow rate of 7 mL/min (solvent A: H_2O /0.1% TFA; B: Acetonitrile/0.1% TFA). Anti $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, αV , $\beta 1$, $\beta 2$, $\beta 3$, $\beta 4$, $\beta 5$ integrin antibodies were purchased from Chemicon (CHEMICON International, Inc). MDA-MB-231 cells, MDA-MB-435 cells, MDA-MB-436 cells and MDA-MB-468 cells were obtained from American Type Culture Collection (Manassas, VA). K562 cell line and K562 cells transfected with $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 6$, $\alpha 9$ and $\alpha \text{V}\beta 3$ integrins were generous gifts from Dr. Yoshikazu Takada.

Synthesis of initial and focused “one-bead one compound” peptide libraries

The OBOC libraries were synthesized on TentaGel S NH_2 resin using the bi-layer bead encoding strategy²¹ and “split-and-pool” method.¹⁷ A bi-phasic solvent and topologic segregation approach was used to create bilayer beads, in which the library compounds are displayed on the outer layer and the coding peptide tags on the bead interior. The initial OBOC peptide libraries **2**, **3**, and **4** and the focused OBOC peptide libraries **A** and **B** were constructed on such bilayer beads using Fmoc chemistry.⁵⁰ Library **1** was directly assembled on the TentaGel beads using the “mix and split” method. The detailed synthesis routes are presented in the Supplementary section.

On bead cell screening assay

MDA-MB-231 cells adherent to the bottom of a T75 flask were trypsinized with 0.05% trypsin-EDTA and neutralized with culture medium. Floating cells were collected, spun down, and resuspended in 10mL culture medium in a 10cm Petri dish. The library beads were washed sequentially with ethanol, water, and PBS. The beads were then incubated with suspended MDA-MB-231 cells, and the entire dish was agitated at a speed of 40rpm inside an incubator at 37°C under 5% CO_2 . The plate was then examined under an inverted microscope every 15 minutes.

Peptide synthesis

Fmoc chemistry⁵⁰ and HOBt/DIC coupling were used to synthesize the cyclic peptides on Rink amide resin (loading 0.5mmol/g). A three-fold molar excess of Fmoc-protected amino acids to resin were used for coupling. The reaction was monitored with the ninhydrin test. The Fmoc group was deprotected with 20% piperidine in DMF. The peptides were finally cleaved from Rink resin using a TFA cocktail containing 82.5% TFA: 5% phenol: 5% thioanisole: 5% H₂O: 2.5% triisopropylsilane (TIS), followed by precipitation in cold diethyl ether. The crude peptides were cyclized in 50mM NH₄HCO₃ buffer with charcoal⁵¹ and then purified by preparative RP-HPLC. The desired cyclic peptides were confirmed by ESI MS.

Flow cytometry

80–90% confluent MDA-MB-231 cells were dissociated with 0.05% trypsin-EDTA and neutralized with culture medium. K562 cells and a series of alpha3 mutant-transfected k562 cells in suspension culture were directly used without digestion. 3×10^5 cells in each sample were incubated with biotinylated **1** at different concentrations in 50 μ L PBS containing 10% FBS and 1 mM MnCl₂ for 30 minutes on ice. To determine the level of signal from non-specific binding, the samples were incubated with 10 μ g anti- α 3 integrin blocking antibody for 30 min prior to incubation with biotinylated **1**. After peptide incubation, each sample was washed three times with 1 mL PBS containing 1% FBS. Samples were incubated with a 1:500 dilution of streptavidin-PE (1mg/mL) for 30 minutes on ice followed by a single wash with 1 mL PBS containing 1% FBS. Finally, the samples were analyzed via flow cytometry (Coulter XL-MCL); histogram analysis with determination of mean fluorescence intensity (MFI) was conducted. The apparent K_d was calculated using Graph Prism software.

In vitro microscopy

Sample preparation was the same as flow cytometry studies (described above). Instead of running the cells through a flow cytometer, the cells were examined under a fluorescence microscope. To conduct the blocking experiment, unlabeled cyclic peptide **2** and anti- α 3 integrin antibody were incubated with the cells together with the biotinylated **2**. After the last wash, the cell pellet was resuspended in 100 μ L PBS and loaded into a cytospin centrifuge vial (Cytospin3, SHANDON). The cells were spun down onto slides at 2000 rpm for 2 min, mounted using DAPI-containing fluorescence mount solution (Invitrogen) and examined under a fluorescence microscope (IX81, Olympus). (DAPI exposed with 1ms, Alexa488 exposed with 100ms. Image Software: slidebook)

Tumor xenografts

Animal studies were performed according to a protocol approved 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 2–3 nipple fat pads with 5×10^6 MDA-MB-231 cells suspended in 200 μ L PBS. In some mice, 5×10^6 K562 chronic myeloid leukemia cells were implanted in the contralaterally as a negative control. When the subcutaneous tumors reached 0.5 to 1.0cm in diameter or 40–50 days after implantation, the tumor-bearing mice were subjected to *in vivo* and *ex vivo* imaging studies.

In vivo and ex vivo optical imaging

Peptide ligand 2-SA-Cy5.5 (1.8 nmol), prepared by mixing 7.2 nmol of biotinylated **2** with 1.8 nmol of streptavidin-Cy5.5 in PBS overnight at 4°C, was injected via the tail vein in an anesthetized mouse before imaging. To conduct blocking experiments, an anti- α 3 integrin antibody was injected via tail vein 1 hour prior to injection of the optical probe. Animals were placed on a transparent sheet in the supine position. Images were acquired with a Kodak IS2000MM Image station (Rochester, NY) with a 625/20 band pass excitation filter, 700WA/

35 band pass emission filter, and 150 W quartz halogen lamp light source set to maximum. Images were captured with a CCD camera set at F stop=0, FOV=150, and FP=0. Data were collected at different time points and analyzed using the Kodak ID 3.6 software by drawing the region of interest (ROI) on the imaged mouse. For *ex vivo* imaging, the mice were euthanized and their organs were excised for imaging.

Data processing and statistics

For determination of tumor contrast, we calculated mean fluorescence intensities of the tumor area and of the normal tissue area by means of the region-of-interest function using Kodak ID Image Analysis Software (Kodak). All the data are shown as mean $\pm$ s.d. of n independent measurements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Binding specificity of **1 to $\alpha 3$ integrin**

K562 cells (expressing $\alpha 5$ integrin) and K562 cells transfected with $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 6$, $\alpha 9$ and $\alpha V \beta 3$ integrins were treated with 1mM Mn^{2+} for 30min, followed by incubation with biotinylated **1** at $1\mu M$ for 30min. After washing 3 times with PBS, the cells were incubated with streptavidin-PE at $2\mu g/mL$ and analyzed with flow cytometry. It is evident that **1** binds specifically to $\alpha 3$ integrin, but not to $\alpha 1$, $\alpha 2$, $\alpha 4$, $\alpha 6$, $\alpha 9$ and $\alpha V \beta 3$ integrins.

Figure 2. Binding affinity of 1 against MDA-MB-231 Cells

MDA-MB-231 cells (3×10^5 cells/sample) were incubated sequentially with biotinylated **1** and streptavidin-PE prior to analytic flow cytometry, and mean fluorescent signal was determined as a function of **1** concentration. (■): total binding; (●): nonspecific binding (after blocking by $10 \mu\text{g}$ anti- $\alpha 3$ antibody); (▲): specific binding was obtained by subtracting nonspecific binding from total binding. The solid line through the specific binding data represents nonlinear regression fits of data to the equation $Y = B_{\text{max}} * X / (K_d + X)$, where K_d is the apparent dissociation constant of the ligand **1**.

Figure 3. The molecular interaction between **1 and $\alpha 3$ integrin**

A series of transfected K562 cells displaying various mutant $\alpha 3$ integrins were incubated with biotinylated **1**, followed by streptavidin-PE, and then analyzed by flow cytometry. Mutations at D158A, T162A, T162F, G163A, M164A and Y186A resulted in a significant loss of binding to **1**. To normalize the data, $\alpha 3$ expression level on these cell lines were also determined with anti $\alpha 3$ antibody, and the ratio of mean fluorescence intensity of **1** binding to mean fluorescence of $\alpha 3$ expression was determined. Error bars denote $\pm$ SD of triplicate experiments.

Figure 4. Fluorescent microscopy of MDA-MB-231 cells stained with biotinylated 2
MDA-MB-231 breast cancer cells were significantly stained by (A) peptide ligand 2 -Bio-SA-Alexa488 compared with (D) control, but this staining was blocked by (B) free peptide ligand 2 and (C) anti- $\alpha 3$ integrin antibody.

Figure 5. *In Vivo* and fluorescence imaging of orthotopic MDA-MB-231 xenograft

In vivo near-infrared fluorescence imaging 6 hrs after tail vein injection of peptide ligand 2 - Bio-SA-Cy5.5. The imaging agent significantly accumulated in the MDA-MB-231 xenografts, but not in K562 tumors. Organs were excised 6hrs after administration of imaging agent; *Ex vivo* near infrared fluorescence imaging was conducted. Peptide ligand 2 -Bio-SA-Cy5.5 accumulated in the MDA-MB-231 xenograft (#3 mouse) and signal was significantly higher than K562 tumor (#4 mouse). The accumulation in breast cancer xenografts could be blocked by pre-treatment with anti- $\alpha 3$ antibody in the #2 mouse. Tumor uptake of SA-Cy5.5 alone without any ligand was shown to be very weak (#1 mouse).

Scheme 1.
Structure of biotinylated 1

Scheme 2.

NOEs of peptide ligand 2 determined by NMR spectroscopy

Table 1

Peptide ligands and their binding affinity

Cmpd	Sequence	IC ₅₀ (nM)
1	c-d-G-L-G-B-N-c	550±0.17
2	c-d-G-Tyr(3-NO ₂)-G-B-N-c	57±0.004
3	c-d-G-Tyr(3-NO ₂)-G-B-HoSer-c	190±0.04
4	c-d-G-Tyr(3-NO ₂)-G-B-T-c	68±0.008
5	c-d-G-Phe(3-Cl)-G-B-T-c	220±0.05
6	c-d-G-Cha-G-B-HoPhe-c	340±0.06
7	c-d-G-Cha-G-P-A-c	290±0.05