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Structure −Activity Relationship Studies of Targeting Ligands against Breast Cancer Cells

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A series of LXY3 (**1**) analogues were designed and synthesized. Their binding affinity was demonstrated using MDA-MB-231 breast cancer cells adherence inhibition assay. Further structure−activity relationship was obtained. Analogue **29** was discovered to have 3.5-fold increase of the binding affinity. Fluorescent microscopy and in vivo and ex vivo imaging studies demonstrated that **29** is an efficient in vivo targeting agent against α3 integrin of MDA-MB-231 breast tumor xenograft implant.

Introduction

Integrins are heterodimeric cell surface proteins consisting of α and β subunits that mediate extracellular matrix and cell−cell interactions and regulate cellular activities such as adhesion, migration, proliferation, and differentiation.[1−3] Many integrins have been found to play an important role in cancer invasion and metastasis, tumor angiogenesis, immune functions, and tissue repair.[4,5] Integrin α3β1 is a promiscuous receptor reported to bind laminin, collagen, fibronectin, and entactin.[6−8] It participates in the phagocytosis of extracellular matrix molecules by human breast cancer cells.[9] Integrin α3β1 is also involved in the initial anchoring of MDA-MB-231 human breast cancer cells to cortical bone[10] and the adhesion and spreading of metastatic breast carcinoma cells to the lymph node stroma.[11] Increasing evidence that integrin α3β1 is significantly up-regulated on MDA-MB-231 cells[9,12,13] suggests that α3β1 would be of great interest as a promising receptor to develop targeting agents for delivering radionuclides, toxins, or cytotoxic agents to the breast tumor site.

Immunotargeting using monoclonal antibodies is a promising approach for early detection and treatment of cancer.[14−16] Solid tumors, however, have proven less responsive in part because of difficulties in the tumor selective delivery of antibodies and potential cytolytic effectors.[17] To circumvent these problems, peptides and peptidomimetics have been intensively studied for tumor imaging and targeting therapy because of their rapid clearance and high tumor penetration. The best-studied is RGD containing ligands developed for radiolabeling and drug targeting.[18−20] "The one-bead−one-compound" (OBOC) combinatorial library method, developed in our lab,[21,22] was widely used to identify peptide and peptidomimetic ligands against various interesting receptors.[23−25] Using this approach, we recently identified a novel cyclic octapeptide **1** (cdGTyr(3-NO_2)GHypNc,[a] LXY3) (Figure 1) to specifically bind to α3 integrin on MDA-MB-231 breast cancer cells. Further in vivo and ex vivo imaging studies revealed that **1** effectively targeted α3 integrin on MDA-MB-231 breast tumor xenograft model.[26]

Here, with the aim to understand structure−activity relationship (SAR) and develop novel **1** analogues with higher binding affinity, we performed a series of structural modifications on the peptide portion of **1**. Their binding affinities to MDA-MB-231 cell lines were evaluated using high efficiency adherence inhibition assay. Modifications of Tyr(3-NO_2) on position 4 successfully yielded several derivatives with higher affinities than **1**.

Results and Discussions

In our search of novel ligands for breast cancer cells through screening various random OBOC libraries, cyclic octapeptides were found to bind to MDA-MB-231 cell lines. Further "alanine scanning" on the positive-cyclic octapeptide clearly revealed that residues D-Cys-1, Gly-3, Gly-5, and D-Cys-8 are necessary for MDA-MB-231 cell binding. Subsequently, D-Asp-2, L-Hyp-6, and small polar amino acids in position 7 were found to be essential for binding against MDA-MB-231 cells in screenings of highly focused OBOC cyclic octapeptide libraries.[26]

On the basis of above preliminary structure−activity relationship (SAR) information, we determined whether changes to certain structural aspects of the peptide segment of **1** influenced binding affinity by replacing the disulfide bridge and systematically modifying amino acid residues from the C to N termini of the sequence. Derivatives are grouped on amino acid substitutions or modifications, including five separate segments of **1**: (i) replacement of the disulfide bridge; (ii) substitution of Asn; (iii) substitution or modifications of Hyp; (iv) substitution of Tyr(3-NO_2); (v) derivatization of the N-terminus (Figure 1).

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[a]Abbreviations: Dab, α,γ-diaminobutyric acid; Tyr(3-NO_2), 3-nitrotyrosine; HoSer, homoserine; Hyp, hydroxyproline; Thz, L-thiaproline; Homotyr, homotyrosine; Fmoc, fluorenylmethoxycarbonyl; ESI, electrospray ionization; NMR, nuclear magnetic resonance; HOBt, *N*-hydroxybenzotriazole; DIC, *N*,*N*′-diisopropylcarbodiimide; PyBrop, bromotripyrrolidinophosphonium hexafluorophosphate; DIEPA, *N*,*N*-diisopropylethylamine; NMM, *N*-methylmorpholine; RP-HPLC, reverse-phase high performance liquid chromatography; TFA, trifluoroacetic acid; DMF, *N*,*N*-dimethylformamide; DCM, dichloromethane. 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.

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LXY1 was previously characterized as a specific ligand against α3 integrin of MDA-MB-231 breast cancer cells with binding affinity $K_d = 0.4 \pm 0.07$ μM via flow cytometry.[26] This method is complicated, tedious, and time-consuming and consequently not suitable for high throughput screening. To circumvent these problems, a simple adherence inhibition assay[23] was developed to determine the half-maximal inhibitory concentration (IC_{50}) of the analogues for their ability to inhibit binding of MDA-MB-231 cells to immobilized LXY1. The relative binding affinity of these novel derivatives are shown in Table 1.

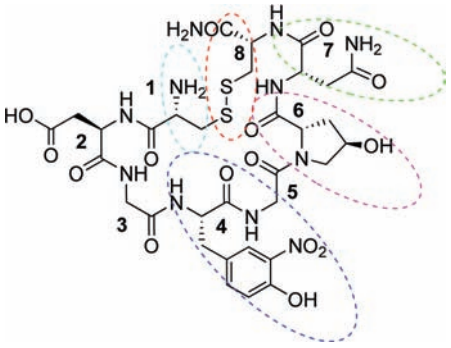


Figure 1. Structure of **1**.

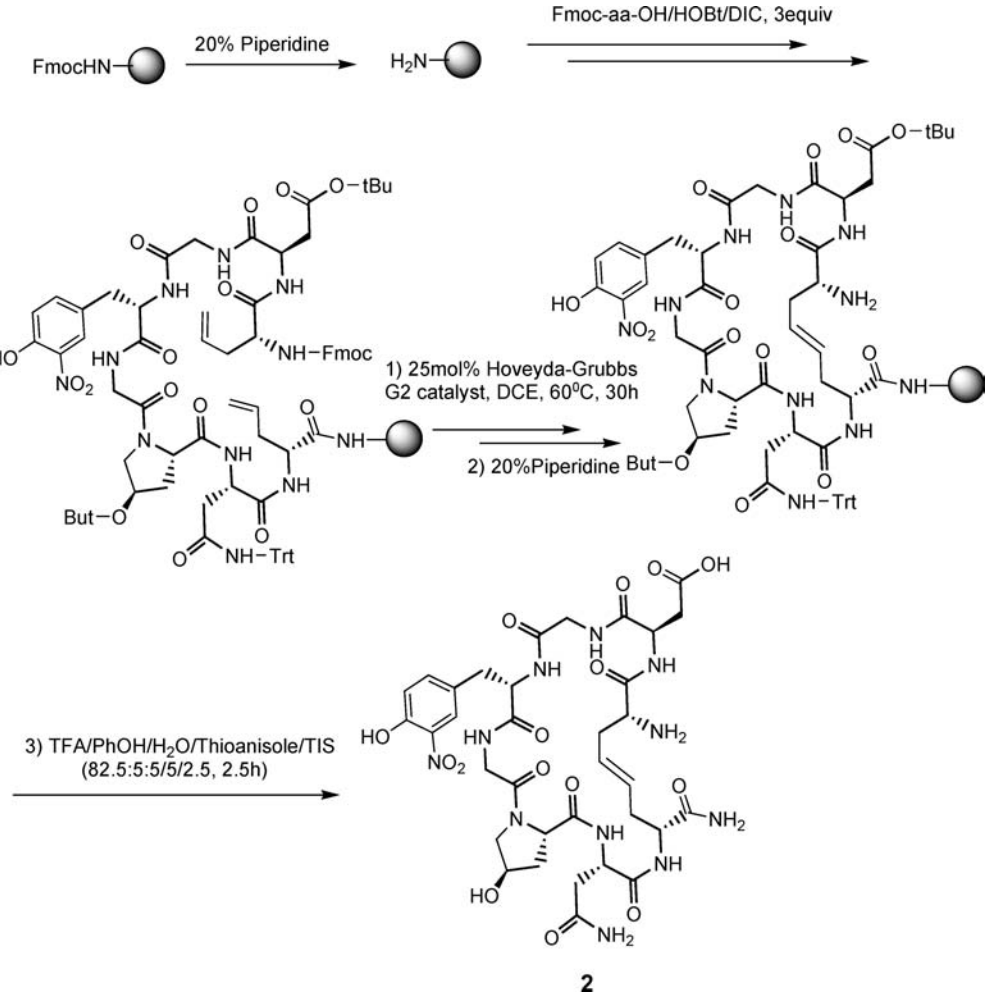
Replacement of the Cystine Bridge. Disulfide bonds are metabolically and chemically unstable structural features in many naturally occurring and synthetic peptides.[27] Replacement of the S−S motif of a cystine bridge with more stable alternatives, such as carbon−carbon bond, carbon−carbon double bond, and amide bond,[28−33] has long been regarded as an attractive strategy to alleviate these problems. Synthesis of the dicarba analogues of **1** (**2**) was achieved by ring-close metathesis[31−35] (Scheme 1). The linear precursor peptide was prepared on Rink amide resin by Fmoc chemistry.[36] D-Allylglycine was incorporated in place of D-Cys to facilitate cyclization and retain original ring size as well. Ring-closing metathesis of resin bound linear peptide was carried out in 1,2-dichloroethane at 60 °C for 30 h by using 25% Hoveyda−Grubbs second-generation ruthenium catalyst. The resulting cyclic olefinic peptides were cleaved from the resin by TFA treatment in the usual manner, followed by Fmoc deprotection. The configuration of the double bond was established by measurement of the *J* coupling constants between the olefinic protons. The high *J* values observed (22.5 Hz) is indicative of trans stereochemistry for the newly formed double bond.

Cyclization with an amide bond is the general strategy to synthesize a cyclic peptide. To maintain the same ring size as **1**, Fmoc-D-Asp(OAll) and Fmoc-D-Dap(Alloc) were selected to couple to the C and N termini, respectively,

Table 1. Analogues of **1** and Their IC_{50}

compd	N-terminus	D-Cys 1	Tyr(3-NO_2) **4**	Hyp **6**	Asn **7**	D-Cys **8**	IC_{50} (μM)
1							7.4
2		D-allylglycine				D-allylglycine	>25
3		D-Dap				D-Asp	>25
4		D-Lys			Asp	no	>25
5		D-Pen				D-pen	>25
6					Asp		17.1
7					Dab		13.6
8				Pro			16.9
9				3, 4-dehydro-Pro			15.3
10				*trans*-4-fluoro-Pro			18.5
11				Thz			10.5
12				(2*s*,4*s*)-4-phenyl pyrrolidine-2-carboxylic acid			>25
13				azetidine-2-carboxylic acid			21.4
14				pipecolic acid			24.1
15			HomoTyr				>25
16			Tyr(3-Cl)				19.8
17			Tyr(3-I)				>25
18			Tyr(3-NH_2)				>25
19			Tyr(3-OCH_3)				>25
20			Tyr(3,5-Br)				>25
21			Tyr(3,5-I)				>25
22			Tyr(3,5-2NO_2)				11.9
23			Phe(2-F)				>25
24			Phe(3-F)				11.3
25			Phe(4-F)				23.7
26			Phe(3-CF_3)				11.9
27			Phe(4-CF_3)				>25
28			Phe(3,4-2F)				6.3
29			Phe(3,5-2F)				2.1
30			Phe(3,4,5-3F)				5.9
31			Phe(2,3,4,5,6-5F)				11.7
32			Phe(2-NO_2)				19.6
33			Phe(3-NO_2)				3.1
34			Phe(2-Cl)				19
35			Phe(3-CN)				>25
36−48	aldehyde						>25
49−58	isocyanate						>25

Scheme 1. Synthesis Route of **2**



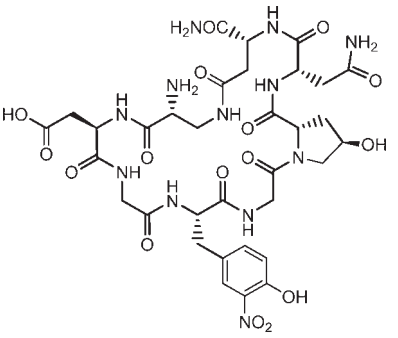
instead of D-Cys. Following assembly of linear peptide on the Rink resin, OAll and Alloc group were removed simultaneously with tetrakis(triphenylphosphine)palladium (0.24 equiv) and phenylsilane (20 equiv) in DCM. The subsequent cyclization was carried out in solid phase with PyBOP/DIEPA as coupling reagents instead of DIC to afford **3** (Figure 2). In the synthesis of cyclic peptide **4**, Fmoc-D-Lys(Alloc), which possesses a long space side chain, was used to simulate the approximate length of the disulfide bridge. Due to the truncation of the C-terminus (D-Cys), Fmoc-L-Asp-OAll was first coupled to the Rink resin instead of Fmoc-L-Asn. L-Asp residue was automatically transformed to L-Asn, followed by cyclic peptide **4** cleavage from the resin. Introduction of bulky penicillamine can often induce resistance to enzymatic cleavage. Cyclic peptide **5** was obtained by substituting D-Cys with D-Pen.

In comparison with **1**, cystine bridge replacement with a carbon−carbon double bond, amide bond, or penicillamine bridge resulted in drastic loss of binding affinity. These results indicate that cysteine bridge disulfide bonds are necessary for the MDA-MB-231 cells binding.

Substitution of Asn with Asp and Dab. In our previous OBOC libraries,[26] various natural and unnatural amino acids, including polar, hydrophobic, and charged amino acids, were introduced to position 7. Small polar amino acids, such as Asn, Thr, and homoserine, were preferred at position 7. To further investigate the importance of small polar amino acids, small charged amino acids (Asp and Dab) instead of Asn were introduced to position 7 to give **6** and **7**, respectively. When substituting with negatively charged amino acid (Asp), binding affinity to MDA-MB-231 breast cancer cells was reduced 2.3-fold. Additionally, when substituting with positive-charged amino acid (Dab), binding affinity to MDA-MB-231 breast cancer cells was reduced 1.8-fold. These observations suggest that a hydrophilic residue is required at position 7, and an electrostatic interaction between ligand **1** and $\alpha 3$ intergrin may not exist around residue Asn.

Substitution of Hyp with Analogues of Pro and Modifications to Hyp. Our initial OBOC library screenings established that hydroxyproline at position 6 is very important for binding affinity.[26] This amino acid limits the rotation about the Nα−Cα bond and can induce β-turn conformation.[37,38] To further probe this structure effects, cyclic amino acids were substituted for Hyp (Figure 3). Proline (**8**), 3, 4-dehydroproline (**9**), and *trans*-4-fluoro-L-proline (**10**) reduced the binding affinity 2- to 3-fold. Thioproline (**11**) resulted in a slight decrease in binding affinity. (2*s*,4*s*)-4-Phenylpyrrolidine-2-carboxylic acid (**12**) and the homologous cyclic amino acids, four-membered azetidine-2-carboxylic acid (**13**) and six-membered pipecolic acid (**14**), resulted in a significant loss of binding affinity. Taken together, these findings indicate that polar hydroxyproline

may be important for cell binding. Encouraged by these results, we therefore hypothesized that further modification of hydroxyproline may increase binding affinity. To test this assumption, we replaced hydroxyproline with (2*S*,4*R*)-4-amino-1-pyrrolidine-2-carboxylic acid, which can provide the extra amino group for easily reacting with different carboxylic acids. As shown in Supporting Information (Scheme S1), commercially available (2*S*,4*R*)-Fmoc-4-amino-1-Boc-pyrrolidine-2-carboxylic acid (Fmoc(2*S*,4*R*)-Abpc) was first converted to (2*S*,4*R*)-Alloc-4-amino-1-Fmoc-pyrrolidine-2-carboxylic acid. Linear peptide was assembled on 20% down-substituted TentaGel S NH_2 (2 g, loading 0.27 mmol/g). After Alloc deprotection, 38 various carboxylic acids (Supporting Information Table S1) were coupled to amino group of (2*S*,4*R*)-Abpc. On-bead cyclization was carried out in the mixture solvent including water, acetic acid, and DMSO (75:5:20) for 48 h. The synthetic route is shown in Supporting Information (Scheme S2). To rapidly evaluate and compare binding affinity to **1**, on-bead cell binding assay was utilized. No cell binding was observed with these analogues within 5 h compared to **1** (Figure S1), indicating that modifications of Hyp led to a decrease in binding affinity.



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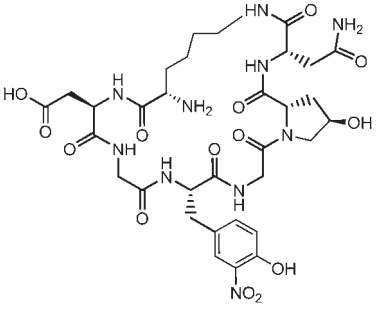

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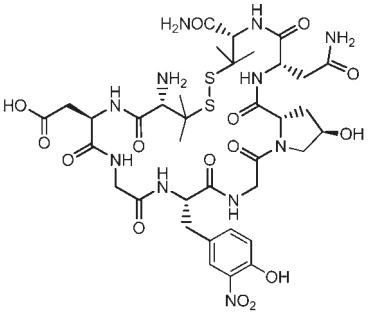

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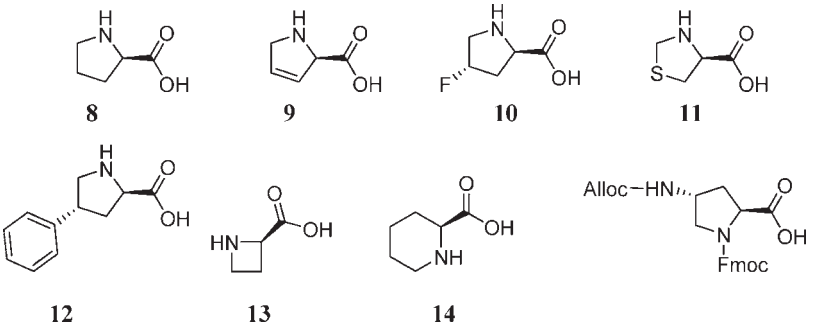
Figure 2. Strutures of **1** analogues with various bridges.

Substitution of Tyr(3-NO_2) with Analogues of Phe and Tyr. Various natural amino acids, unnatural amino acids, lysine derivatives, and phenylalanine derivatives were introduced to position 4 during the initial synthesis of OBOC libraries.[26] Tyr(3-NO_2) at position 4 was determined to show strong binding affinity. To further investigate the importance of Tyr(3-NO_2), a series of analogues of tyrosine and phenylalanine instead of Tyr(3-NO_2) were introduced to position 4 to afford compounds **15**−**35** (Figure 4). Replacement of the nitro group of Tyr(3-NO_2) with different halogens (chloro(**16**) and iodo(**17**)), amino group (**18**), and methoxyl (**19**) resulted in a significant decrease in binding affinity. Introduction of extra halogens (bromo(**20**) and iodo(**21**)) to the aromatic ring dramatically reduced the binding affinity, while introduction of an extra nitro group (**22**) to the aromatic ring slightly reduced binding affinity, which indicated that a polar group (NO_2) that can generate hydrogen bonds is favorable to cell binding.

When Tyr(3-NO_2) was substituted with phenylalanine analogues, compounds with a nitro group at the 3 position (**33**) or fluorine at the 3 and 5 (**29**) positions of the aromatic ring had 2- to 4-fold higher binding affinity than **1**. **28** [Phe(3,4-2F)] and **30** [Phe(3,4,5-3F)] were as active as **1**. In contrast, compounds with fluorine, trifluoromethyl, and nitro group at the 2 or 4 position of the aromatic ring (**23**, **25**, **27**, **32**, **34**) or cyano group at the 3 position of aromatic ring (**35**) showed lower affinity. Compounds with single fluorine and trifluoromethyl at position 3 of the aromatic ring (**24**, **26**) and the analogue with five fluorines (**31**) had slightly less affinity. Taking all of these findings into account, the position of a nitro group and fluorine appeared to be relevant to binding affinity; moreover, the hydroxyl group of Tyr(3-NO_2) was not required for cell binding.



8 **9** **10** **11**

12 **13** **14**

Figure 3. Building blocks used for the substitution of hydroxyproline.

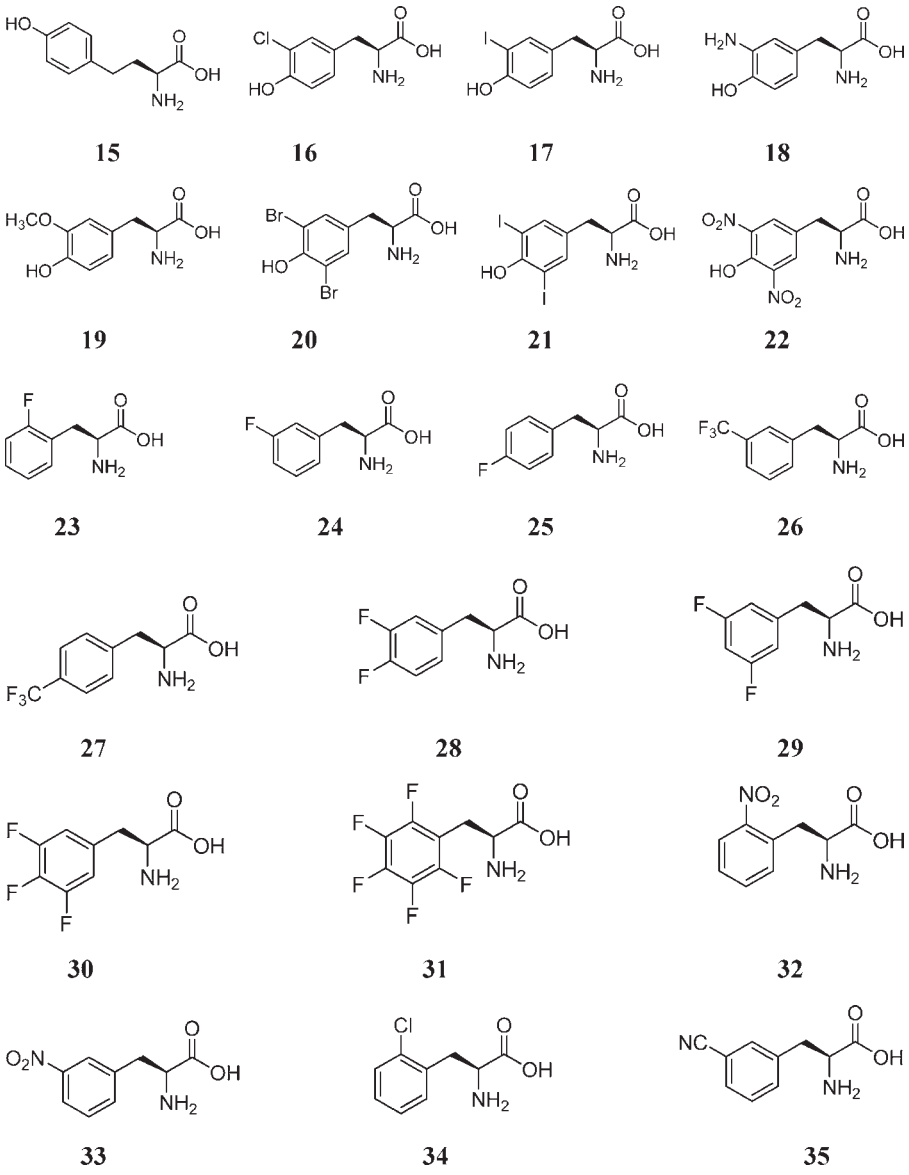


Figure 4. Unnatural amino acids used for the substitution of Tyr(3-NO_2).

Modifications to the N-Terminus. The previous molecular interaction between peptide ligand **1** and α3 integrin demonstrated that the D158A mutation resulted in a remarkable decrease in binding affinity to **1**,[26] indicating that electrostatic interaction between Asp (α3 integrin) and N-terminus of **1** may facilitate cell binding. The reductive alkylation of the N-terminal amino group could render the N-terminus more basic and introduce diverse groups.[39] A series of analogues of **1** were therefore designed and synthesized with the N-terminal amino group alkylated with various aliphatic and aromatic substituents. Linear peptide **1** was first assembled on Rink resin. Unhindered amino group of the N-terminus then underwent reductive alkylation with excess aldehydes (Figure 6) and sodium cyanoborohydride ($NaBH_3CN$) in DMF to give products **36**−**48** (Figure 5). It is important to note that aromatic aldehydes commonly afforded monoalkylated products. Aliphatic aldehydes with less steric hindrance generated monoalkylated and dialkylated products (**38**, **40**). Urea, an attractive pharmacophore, can generate hydrogen bonds and potentially increase binding affinity. The N-terminus

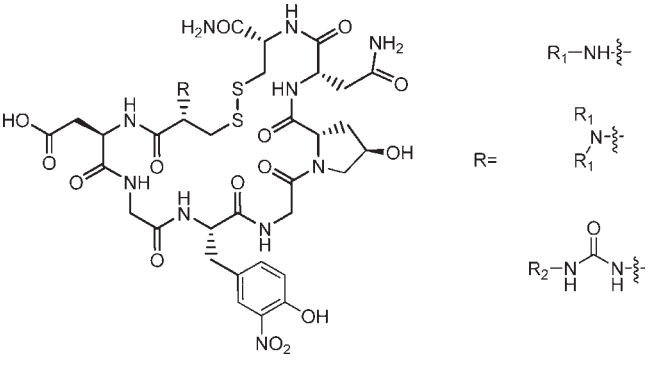


Figure 5. Structure of **1** analogues with N terminal modifications.

primary amino group was treated with various isocyanates (Figure 6) and NMM in DMF to provide products **49**−**58** with a urea group.[39] Compared to **1**, all N-terminal derivatives showed a remarkable decrease in binding affinity, suggesting that the free N-terminal amino group is crucial for cell binding.

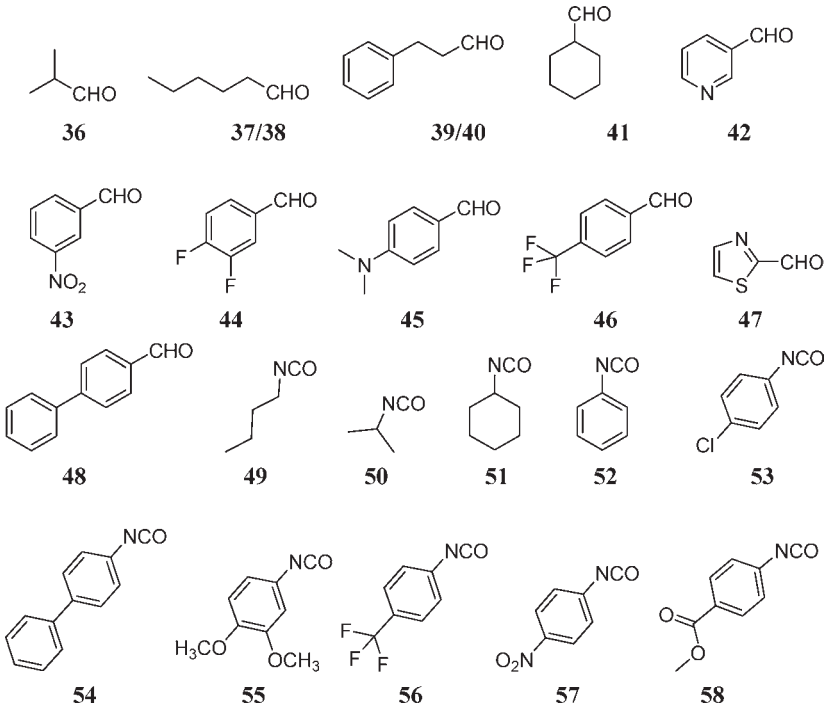


Figure 6. Building blocks used for modifications to N terminus.

Conclusions

Cyclic peptide **1** has been systematically modified from C- to N-terminus to provide 58 analogues. Their binding affinities to $\alpha 3\beta 1$ integrin in MDA-MB-231 breast cancer cells were evaluated by using an adherence inhibition assay. One of the ligands (**29**) demonstrated a 3.5-fold increase of binding affinity compared to **1**. Fluorescence microscopy(Figure S2) and in vivo and ex vivo imaging studies (Figure S3) further demonstrated that **29** efficiently targeted $\alpha 3$ integrin on MDA-MB-231 breast tumor implant in a xenograft model. We previously discovered that D-Asp-2, Gly-3, and Gly-5 were critical for MDA-MB-231 cell binding. The absence of a side chain in Gly possibly provided the conformational flexibility of the cyclic peptide backbone and facilitated the appropriate spatial orientation of pharmacophores, resulting in binding affinity enhancement. For the optimal binding affinity, this report further demonstrates that the peptide portion of the targeting ligands of MDA-MB-231 breast cancer cells must contain (1) the disulfide linkage of D-cystine, (2) short polar amino acid (Asn) at position 7, (3) β-turn inducible hydroxyproline at position 6, (4) phenylalanine analogues with very specific arrangement of polar group (F of NO_2) on the aromatic ring, and (5) a free N-terminal amino group. These features are required for MDA-MB-231 breast cancer cell binding.

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 mm $\times$ 150 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 is H_2O/0.1% TFA; solvent B is acetonitrile/0.1% TFA). Anti $\alpha 3$ integrin antibodies were purchased from Chemicon (CHEMICON International, Inc.). MDA-MB-231 cells were obtained from American Type Culture Collection (Manassas,VA).

Synthesis of 1 Analogue with Various Bridges. Rink amide resin (0.25 g, loading 0.6 mmol/g) was swollen in DMF for 2 h. After Fmoc deprotection with 20% piperidine in DMF twice (5 min, 15 min), the beads were washed sequentially with DMF, MeOH, DCM, MeOH, and DMF, three times each. The beads were then subjected to stepwise assembly of Fmoc-D-allylglycine, Fmoc-Asn(Trt)-OH, Fmoc-Hyp(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Tyr(3-NO_2)-OH, Fmoc-Gly-OH, Fmoc-D-Asp-(OtBu)-OH, and Fmoc-D-allylglycine using Fmoc solid-phase chemistry.[36] Finally, the beads were sequentially washed with DMF, MeOH, DCM and dried under vacuum. The beads were swelled in 3 mL of anhydrous 1,2-dichloroethane (DCE) in a dried flask under a nitrogen atmosphere for 30 min. The Hoveryda−Grubbs catalyst (23 mg, 0.15 mmol) in 2 mL of DCE was added to the flask, and the mixture was stirred at 60 °C for 40 h. The beads were washed with DCM, MeOH, and DMF. Following Fmoc deprotection, the beads were dried under vacuum and treated with a TFA cocktail containing 82.5% TFA/5% phenol/5% thioanisole/5% H_2O/2.5% triisopropylsilane (TIS) at room temperature for 3 h. The cleavage solution was collected, concentrated, and precipitated in cold diethyl ether. The crude product was purified by preparative reverse-phase high performance liquid chromatography (RP-HPLC) to give **2**. ^{1}H NMR (DMSO-d_6, 500 MHz): δ ppm 12.40 (1H, brs), 10.80 (1H, brs), 8.74 (1H, d, J = 10 Hz), 8.69 (1H, d, J = 5 Hz), 8.31 (1H, d, J = 5 Hz), 8.18 (1H, d, J = 5 Hz), 8.05 (1H, brs), 7.95 (1H, d, J = 5 Hz), 7.79 (1H, S), 7.48 (1H, brs), 7.41 (1H, d, J = 10 Hz), 7.39 (1H, d, J = 10 Hz), 7.04 (1H, d, J = 5 Hz), 7.01 (1H, d, J = 10 Hz), 6.91 (1H, brs), 6.86 (1H, brs), 5.52 (1H, m), 5.45 (1H, m), 5.22 (1H, brs), 4.53 (2H, m), 4.38 (2H, m),

4.27 (2H, m), 4.00 (2H, m), 3.96 (2H, m), 3.65 (2H, m), 3.46 (2H, m), 3.05 (2H, m), 2.1−2.8 (overlap, m), 2.02 (1H, m), 1.87 (1H, m). ESI-MS *m*/*z*: calcd for $C_{34}H_{45}N_{11}O_{15}$ $[M + 1]^+$, 848.3; found 848.6. Purity 99%

As described as above, Fmoc-D-Asp (OAll), Fmoc-Asn-(Trt)-OH, Fmoc-Hyp(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Tyr-(3-NO_2)-OH, Fmoc-Gly-OH, Fmoc-D-Asp(OtBu)-OH, and Fmoc-D-Dap(Alloc) were sequentially assembled on Rink amide resin (0.25 g, loading 0.6 mmol/g) using Fmoc solid-phase chemistry.[36] The beads were treated with $Pd(Ph_3)_4$ (0.24 equiv) and $PhSiH_3$ (20 equiv) in DCM for 30 min, twice. After washing, PyBrop (5equiv) and DIEPA (10 equiv) in DMF were added to the beads. The reaction proceeded at room temperature until a Kaiser test was negative. Following Fmoc deprotection, the beads were subjected to TFA cocktail cleavage to afford **3**. ESI-MS *m*/*z*: calcd for $C_{33}H_{44}N_{12}O_{16}$ $[M + 1]^+$, 865.3; found 865.6. Purity 98%.

As described for the synthesis of **3**, Fmoc-L-Asp-OAll, Fmoc-Hyp(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Tyr(3-NO_2)-OH, Fmoc-Gly-OH, Fmoc-D-Asp(OtBu)-OH, and Fmoc-D-Lys(Alloc) were sequentially assembled to Rink amide resin to give **4**. ESI-MS *m*/*z*: calcd for $C_{32}H_{44}N_{10}O_{14}$ $[M + 1]^+$, 793.3; found 793.6. Purity 99%.

After the assembly of Fmoc-D-Pen(Trt), Fmoc-Asn(Trt)-OH, Fmoc-Hyp(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Tyr(3-NO_2)-OH, Fmoc-Gly-OH, Fmoc-D-Asp(OtBu)-OH, and Fmoc-D-Pen(Trt) to Rink amide resin (0.25 g, loading 0.6 mmol/g), the beads were dried and treated with a TFA cocktail. Following precipitation in cold diethyl ether, the crude peptides were dissolved in 100 mL of 50 mM NH_4HCO_3 buffer. Activated charcoal (100 mg) was added to the buffer. The solution was stirred at room temperature until the Ellman test was negative. The reaction solution was filtered, collected, and lyophilized. The crude product was purified by preparative reverse-phase high performance liquid chromatography (RP-HPLC) to give **5**. ESI-MS *m*/*z*: calcd for $C_{36}H_{51}N_{11}O_{15}S_2$ $[M + 1]^+$, 942.3; found 942.6. Purity 98%.

Synthesis of Cyclic Peptide 1 Derivatives with Substitution of Asp, Hyp, and Tyr(3-NO_2). Compounds **6**−**35** were synthesized as **5** using Fmoc chemistry.[36] Peptide cyclization was carried out in the 50 mM NH_4HCO_3 buffer with activated charcoal. The crude product was purified by preparative reverse-phase high performance liquid chromatography (RP-HPLC). The final products were characterized by ESI-MS (Supporting Information). The purity of the final products was determined by analytical HPLC (Supporting Information).

Synthesis of Cyclic Peptide 1 Derivatives with N-Terminal Modification. Linear peptide cdGTyr(3-NO_2)GHypNc was first assembled to Rink amide resin (6 g) using Fmoc chemistry.[36] After Fmoc deprotection, the beads were split into 21 portions. Eleven different aldehydes (5 equiv) (Figure 6) and $NaCNBH_3$ (16 equiv) in DMF were added to each portion of beads. Additionally, 10 various isocyonates (16 equiv) (Figure 6) and NMM (16 equiv) were added to each portion of beads. Each mixture was shaken overnight at room temperature. The beads were then completely washed and dried under vacuum. After cleavage from the beads, the crude products were cyclized and purified to give **36**−**58** (Supporting Information).

Cell Adhesion Assay. Neutravidin (1 mg/mL) was coated on 96-well plates, followed by incubation with biotin-conjugated LXY1 peptides (0.2 μM) (synthesis described in Scheme S3). After washing, the wells were blocked with 1% bovine serum albumin in PBS. MDA-MB-231 cells and serial diluted concentrations of peptides were premixed in 2 mL cluster tubes on ice for 30 min. The mixture in each tube was transferred to the individual well and incubated for 30 min at 37 °C. The unbound cells were gently removed. The wells were washed with PBS. The bound cells in each well were fixed with 3.7% formaldehyde and stained with 0.1% crystal violet overnight at 4 °C. Then 50 μL of 1% SDS was added to each well to dissolve the dye. Each well was recorded with a 96-well microscope reader at 570 nm. IC_{50} was calculated on the basis of the inhibition curves resulting from the concentration-dependent inhibition.

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Supporting Information Available: Synthetic procedures, MS data, and biological data. This material is available free of charge via the Internet at http://pubs.acs.org.

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