Identification of novel targeting peptides for human ovarian cancer cells using ''one-bead one-compound'' combinatorial libraries

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Abstract

Using ''one-bead one-compound'' combinatorial chemistry technology, we generated random peptide libraries containing millions of 90 μm TentaGel beads, each with its own unique amino acid sequence. A cyclic random 8-mer library was screened with CAOV-3 (a human ovarian adenocarcinoma cell line) and beads with a unique ligand that bind to the cell surface receptors were coated by one or more layers of cells. These positive beads were isolated, stripped, and microsequenced. Several peptide motifs were identified from these screenings, some of which were novel and unique, e.g., cDGX$_4$GX$_6$X$_7$c. Structure-activity relationship studies of this peptide revealed that the L-aspartate residue at position 2, the two glycines at positions 3 and 5, and the two D-cysteines at the amino and COOH terminus are critical for activity. In addition, a hydrophobic residue was preferred at position X$_4$, whereas amino acids at positions X$_6$ and X$_7$ were more variable. Binding of this peptide to a number of different cancer cell lines and normal cells was also determined and we observed that peptides with this motif bound preferentially to three other human ovarian cancer cell lines (ES-2, SKOV-3, and OVCAR-3) as well as a human glioblastoma cancer cell line (A172). Structural analysis of the peptides using high-resolution nuclear magnetic resonance spectroscopy revealed strong conformational similarity among all peptides with cX$_1$GX$_4$GX$_6$X$_7$c motif. Blocking study with a panel of anti-integrin antibodies strongly suggests α3 integrin present on these ovarian adenocarcinoma cells is the target receptor for this peptide. [Mol Cancer Ther 2005;4(5):806–13]

Introduction

Ovarian cancer is diagnosed in >22,220 American women yearly, and an estimated 16,000 die each year, making ovarian cancer the leading cause of death from gynecologic malignancy. Despite treatment with intensive chemotherapy, radiation therapy, and surgery, the survival rate of patients with stage III ovarian cancer is only 15%; prognosis for patients with stage IV disease is even worse. Even patients with low-stage, high-grade tumors have a long-term survival rate of only 40% to 50%. Because many patients, even those with advanced disease, have ovarian cancer confined to the peritoneal cavity, i.p. therapy is an attractive option to pursue, especially if an effective targeting agent is available.

The clinical success and Food and Drug Administration approval of antibodies such as Rituxan and Zevalin [anti-CD20 monoclonal antibodies (mAb) against B cell non–Hodgkin's lymphoma], Herceptin (anti-Her2/neu mAb against breast cancer), Mylotarg (anti-CD33 mAb against acute myeloid leukemia), Campath (anti-CD52 mAb against B cell chronic lymphocytic leukemia), and Erbitux (anti-epidermal growth factor receptor mAbs for metastatic colorectal cancer) in the treatment of human cancers has validated the cell surface targeting approach for cancer therapy. One limitation of using mAbs to target cancer is that the antibody molecule is relatively large with a molecular weight of 160,000, making it difficult to reach into the interior of large tumor masses where the blood supply is inadequate (1–3). To overcome this problem, some researchers are developing antitumor single chain antibodies that are smaller in size (M_r = 25,000; refs. 4–8). Another major problem with mAb therapy is the nonspecific binding of the antibody molecules by the reticuloendothelial system such as the liver, spleen, and bone marrow. For example, imaging with [^{111}In]-labeled anti-CD20 antibody (same antibody as Zevalin) in humans showed high and prolonged uptake in liver and spleen (9). We believe cell surface–binding peptides are useful alternative agents for targeting cancer. Peptides are considerably smaller than mAbs and generally do not bind to the reticuloendothelial system. They are chemically stable and relatively easy to derivatize. Peptides are, however, susceptible to proteolytic degradation *in vivo* unless their NH_2 and COOH termini are blocked, they are cyclized, or they contain D-amino acids. Octreotide

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(a cyclic octapeptide containing two D-amino acids that binds to somatostatin receptor) has in fact been used as a therapeutic and radioimaging agent for patients with carcinoid tumors (10). The discovery and application of therapeutic cancer targeting peptides have been reviewed (11).

Most investigators use phage-display combinatorial peptide library approaches to discover cell surface binding ligands. In this method, phage-display peptide libraries could be panned by purified cell surface proteins or by intact cells. Alternatively, phage-display peptides can be selected *in vivo* by injecting phage-display libraries i.v. into tumor-bearing mice followed by retrieval of bound phages from the excised tumor. This latter approach targets murine neovasculature within the human xenograft rather than the tumor cells directly. One major limitation of phage-display combinatorial peptide library is that only peptides with L-amino acids can be displayed. In 1991, we first reported the ''one-bead one-compound'' (OBOC) combinatorial library method (12). In essence, when a ''split-mix'' synthesis method is used to generate a combinatorial library, each bead expresses only one chemical entity (12, 13). Random libraries of millions of beads can be screened in parallel for a specific acceptor molecule (receptor, antibody, enzyme, virus, whole cell, etc.). We have developed several screening methods for the OBOC libraries and have successfully employed these methods to identify ligands for many biological targets. Because of the synthetic nature of the method, OBOC combinatorial libraries may contain D-amino acids, unnatural amino acids, and even nonpeptidic moieties. Furthermore, cyclic, turned, or branched OBOC combinatorial libraries can be easily constructed and screened. Peptides containing these unnatural amino acids, and those with more constrained structures, are much more resistant to proteolysis. In the last few years, we have applied the OBOC combinatorial library method to successfully discover peptide ligands for a number of different human cancer cell lines (14–18).

Once lead compounds are identified in the primary library screen, we perform standard structure-activity relationship studies such as alanine walk, in which each residue is replaced with an alanine, one at a time. Based on such studies, critical residues can be determined and can be used as part of the template for the design of focused or secondary libraries for further optimization of the lead compounds.

Here, we report on the use of ovarian adenocarcinoma cell lines (SKOV-3, CaOV-3, OVCAR-3, and ES-2) to screen OBOC combinatorial cyclic octapeptide libraries and the identification of novel peptide ligands for these cells. We believe that some of these ligands have great targeting potential for ovarian cancer.

Materials and Methods

Synthesis of Random Peptide Libraries

Using the OBOC combinatorial library methodology, we synthesized random peptide libraries on 90 μm polyethylene glycol–grafted polystyrene beads (TentaGel, Rapp Polymere, Tubingen, Germany) which acts as the solid support. Peptide libraries (linear or cyclic) were synthesized by a split-mix synthesis approach as previously described (12, 19–21). These libraries contained millions of peptide beads with each bead carrying a peptide with distinct amino acid sequence. Standard solid phase peptide synthesis method with 9-fluorenylmethyoxycarbonyl chemistry was used to synthesize the peptide libraries (22). For down-substituted libraries, the resin was reacted with 0.2 equivalent of Fmoc Osu dissolved in ether/DCM with 0.8 equivalent DIEA. The resin was then separated into multiple aliquots and each aliquot was reacted with 3-fold molar excess of a single N^{α}-(9-fluorenylmethyoxycarbonyl-amino acid). Coupling was initiated by the addition of a 3-fold molar excess of 1-hydroxybenzotriazole and diisopropylcarbodiimide. The coupling reactions were driven to completion and monitored by the ninhydrin test. Then the aliquots were thoroughly washed, mixed, and deprotected with 20% piperidine, thoroughly washed again, and redivided into multiple aliquots for the next cycle of coupling. After the desired cycles of ''split synthesis'' were completed, the side chain–protecting groups were removed with mixture containing trifluoroacetic acid/triisopropylsilane/1,2-ethanedithiol/water (94:1:2.5:2.5, v/v/v/v) for disulfide cycling via the flanking D-cysteines, the library was gently mixed in a large volume (1,000 mL) of 5% DMSO in water for 24 hours. The peptide beads were subsequently washed and stored in PBS with 0.05% sodium azide.

Cell Lines

SKOV-3 (ovarian adenocarcinoma), OVCAR-3 (ovarian adenocarcinoma), ES-2 (ovarian clear cell carcinoma), and CaOV-3 (ovarian adenocarcinoma) cell lines were purchased from American Type Culture Collection (Rockville, MD). SKOV-3, CaOV-3, and ES-2 were maintained in 90% McCoy's 5a medium with 1.5 mmol/L L-glutamine, 10% fetal bovine serum, 50 units/mL penicillin, and 50 mg/mL streptomycin. OVCAR-3 was maintained in 80% RPMI 1640 with 2 mmol/L L-glutamine, 10 mmol/L HEPES, 1.0 mmol/L sodium pyruvate and supplemented with 0.01 mg/mL bovine insulin; 20% fetal bovine serum.

Peptide Library Screening

The previously reported cell-growth-on-bead assay (16) was used in our library screening. About 200 μL of peptide bead library (equivalent to approximately 150,000 beads) previously stored in PBS / 0.05% sodium azide was placed in a 2 mL fritted disposable polyethylene column and washed five times with sterile PBS, five times with sterile water, once with 70% ethanol, and twice with tissue culture medium. OVCAR-3, SKOV-3 and CaOV-3 cells were dissociated with 0.05% trypsin (w/v)/0.53 mmol/L EDTA. ES-2 cells were dissociated with 0.3% (w/v) EDTA in double-distilled water according to dissociating condition specified by American Type Culture Collection. The beads were then transferred to 100 × 20 mm nonpyrogenic tissue culture dishes and then incubated with 1×10^6 cells/mL of cells suspension in complete media. Plates were incubated at 37°C in a rotating incubator for 1 to 24 hours and

inspected under the light microscope. Beads with a unique ligand that interacted with cell surface receptors were coated by one or more layers of the ovarian cancer cells. These positive beads were then isolated with a micropipette under an inverted microscope. The bound cells were then stripped off the beads with 8 molar guanidine hydrochloride; beads were washed several times in water and 70% ethanol and then rescreened with fresh cells to ensure true positive binding. Beads that were positive after two or three rounds of screening were physically isolated under a microscope, treated with 8 mol/L guanidine hydrochloride, washed, and processed for sequence analysis.

Solid Phase Synthesis of Identified Peptide Ligands

Standard solid phase peptide synthesis method with 9-fluorenylmethyoxycarbonyl chemistry was used to synthesize the individual peptide ligands on 90 μm TentaGel beads (22). The same procedure was used to synthesize peptide beads for structure-activity relationship studies.

Nuclear Magnetic Resonance Spectroscopy

The nuclear magnetic resonance (NMR) data were collected on a Bruker Avance 500 spectrometer. The samples were dissolved in DMSO-d_6 at 5 mmol/L concentration and data were collected at 298 K. Assignments were based on a combination of one-dimensional-NMR, two-dimensional-TOCSY and, two-dimensional-NOESY. The NOESY mixing time was 300 ms TOCSY spectra were collected with 80 ms mixing time. The data for chemical shift temperature coefficients were collected in range 298 to 313 K with 5 K step.

Identification of the Receptor Binding DGLG Peptide Bead

In order to identify possible receptors for these novel DGLG peptides, we carried out a BLAST search on the University of Minnesota protein sequence data base web site.[1] We searched for naturally occurring extracellular matrix proteins with the -DGLG- motif: among the numerous proteins that contained this motif was collagen Iα, gelsolin precursor, and thrombospondin precursor.

Inhibition of Cell Binding to Peptide Beads

Collagen-specific integrin investigator kit (ECM 425) was purchased from Chemicon International, Temecula, CA. Function-blocking anti-α1 (FB12), anti-α2 (P1E6), anti-α3 (ASC-1), anti-αvβ3 (LM609), anti-αv (P3G8), anti-β1 (P5D2), and anti-β3 (25E11) antibodies were used. Serial dilutions of each antibody (5, 2.5, 1, 0.5, 0.25, and 0 μg) were added to 500 μL of cell suspension containing approximately 15,000 cells and incubated on ice for 30 minutes. Peptide beads were then added to the cells and incubated in a rotator-incubator at 37°C, 5% CO_2 for 1 hour. The total number of cells bound to beads was then counted. Separate experiments were done with two different cell lines (ES-2 and SKOV-3).

Isolation of Ovarian Cancer Cells from Mixture with Whole Blood

A 1:7 dilution of whole blood in complete McCoy's media was first prepared. Five microliters of this suspension was added into sterile flow cytometry tubes containing 1,000 OVCAR-3 cells in a 1 mL volume. Ten microliters of either cDGWGPNc or ''blank'' peptide beads (approximately 7,500) was added to the tubes and incubated in a rotator-incubator at 37°C, 5% CO_2 overnight. The suspension was decanted into 12-well plates and observed. Subsequently, the beads were taken through serial washes with PBS (until there was little or no trace of whole blood left) and then observed again under the microscope.

Results

Identification of Ovarian Cancer Targeting Peptides

Several cyclic peptide libraries were screened with live human ovarian cancer cell lines (CaOV-3, SKOV-3, OVCAR-3, and ES-2). Each of these peptide library compounds contained two D-Cys residues (at the amino and carboxyl termini), and each was cyclized by a disulfide bond. Figure 1A shows a photomicrograph of a typical screening experiment. Several peptide ligands were identified, many of which could be grouped into five distinct motifs (Table 1). Motifs 1 and 2 are similar but also different: cDGXGXXc versus cNGXGXXc. Motif 3 -NGR- has been previously described by us (16) and others (23). Motif 4 is RGD, which is a known ligand for many integrins including αvβ3. Four of the strongest binding ligands were selected and characterized with respect to their binding specificities (Table 2). Three of these four peptides (cLDWDLIc, cDGLGDDc, and cDGWGPNc) show strong preferential binding to ovarian cancer cell lines (CaOV-3, SKOV-3, and ES-2) and do not bind to T lymphoma, prostate cell lines nor B lymphoma (except for cLDWDLIc, which binds weakly to Raji cells). In addition, they do not bind or bind only weakly to breast and a non–small cell lung cancer cell line. Interestingly, two of these peptides also bind strongly to A172, a human glioblastoma cell line. Peptide cNGRFEHc, on the other hand, is relatively nonspecific and binds to many different malignant cell lines as well as benign cells. We have also successfully used one of these peptides, cDGWGPNc, to retrieve ovarian cancer cells that have been mixed into whole blood (Fig. 1B). Even though the peptide beads were in a suspension containing millions of RBC, lymphocytes, and mononuclear cells they bound OVCAR-3 cells specifically. Each bead shown bound >30 cells and there were hardly any OVCAR-3 cells left free in the suspension. This indicates that the peptide bead does not bind to normal peripheral blood cells. In addition, these peptides either bind very weakly or not at all to normal lung fibroblast (IMR-90) or normal breast epithelium, making them excellent candidates for both imaging and therapeutic targeting agents for ovarian cancer.

Structure-Activity Relationship Studies

In order to determine the key residues necessary for cell binding, we did an ''alanine walk'' on three of the ovarian cancer–specific cyclic peptides shown in Table 2, by replacing each amino acid with alanine, one at a time. The results of this study are shown in Table 3. It is clear

[1] http://alces.med.umn.edu.

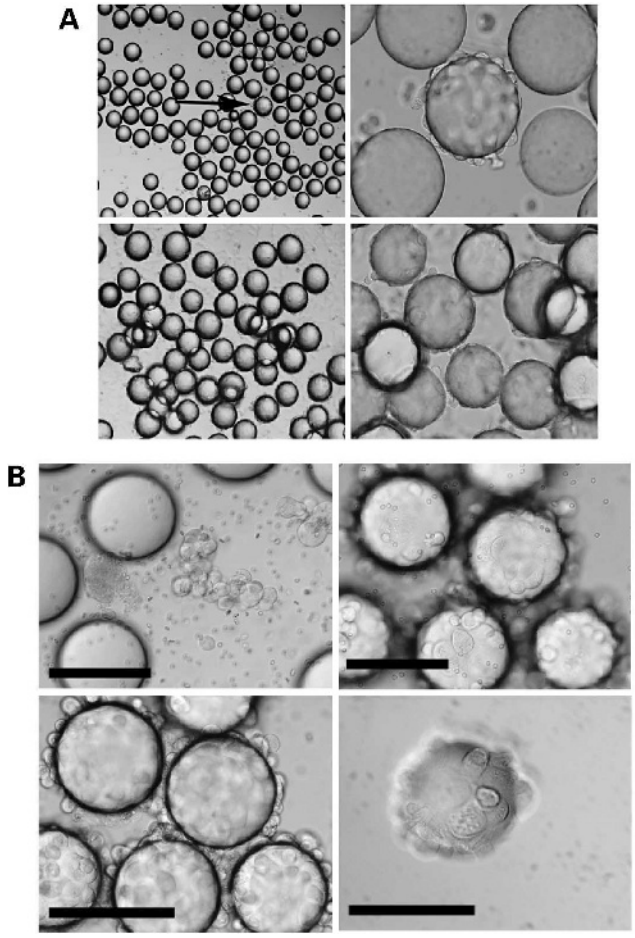

Figure 1. A, micrograph of library screening with live ovarian adenocarcinoma cells (*top*). *Arrow,* ''positive'' bead. Picture was taken after 2 h under Olympus IX 70 microscope using a digital camera. *Left,* 4× magnification; *right,* 40× magnification. Micrograph of retesting of a single ''positive'' peptide synthesized on TentaGel beads (*bottom*). *Left,* 10× magnification; *right,* 20× magnification. **B,** micrograph showing isolation of OVCAR-3 tumor cells from a mixture of these tumor cells with whole blood. *Top,* negative control ''blank'' beads were unable to isolate these tumor cells (*left*); positive cDGWGPNc beads bound to the OVCAR-3 cells in the mixture (*right*). *Bottom,* after several washing steps, only the OVCAR-3 cells remained tightly bound to the peptide beads (*right* and *left*). *Bars*, 100 μm.

from this study that some of the residues are irreplaceable and are crucial for specific cell binding, and peptide cyclization with disulfide bond seems to be very important as well. For cDGLGDDc and cDGWGPNc peptides, replacing Cys-1, Gly-3, Gly-5, and Cys-8 with Ala completely eliminate the binding activity. The minimal motif for ovarian cancer cell binding for these two peptides is cDGXGXXc.

NMR Studies of the cXGXGXXc Peptides

Through library screening, we have identified several cyclic peptides with a cXGXGXXc motif for CaOV-3, ES-2, OVCAR-3, and SKOV-3 ovarian cancer cell lines and A549 non–small cell lung cancer cell line [peptide no. (1) cNGRGEQc, (2) cNGQGEQc, (3) cNG-Nle-GWGc, (4) cDGWGPNc, (5) cDGLGDDc, (6) cDG-Nle-G-Hyp-Tc, (7) cNG-Nle-GPNc, and (8) cNGLGM-Nle-c]. To obtain information about the secondary structure of these peptides, we carried out the Monte Carlo–based conformation search (24). The lowest energy conformers were further optimized using semiempirical methods (AM1; ref. 25). The predicted conformation of five cXGXGXXc peptides is shown in Fig. 2A. The calculation predicts a β-turn like structure at the NH_2-terminal part of the molecule involving the first four amino acids D-Cys-Aaa-Gly-Aaa, whereas conformation of the COOH-terminal part of the molecule seemed to be more variable. Four peptides 1, 4, 6, and 7 were synthesized and appropriate NMR measurements were done to support the abovementioned molecular modeling results. The peptides were synthesized on Rink MBHA resin (GL Biochem, Shanghai, China) and resulting peptide amides were purified on HPLC and characterized by HRMS. The [^{1}H]NMR spectra were recorded in DMSO-d_6 at 500 MHz and the standard algorithm based on two-dimensional-TOCSY and two-dimensional-NOESY was used to assign all proton signals. The long-distance NOE contacts involving backbone protons αCH and NH are shown in Fig. 2B for all measured peptides. The strong NOE contact between NH of Gly^3 and NH of Aaa^4 in peptides 1, 4, 6, and 7, together with weak NOE contacts between NH of Aaa^4 and αCH of Aaa^2 in peptides 4 and 7, weak NOE between NH of Aaa^4 and αCH of D-Cys^1 in peptide 1, and weak NOE contact between NH of Aaa^4 and βCH of Aaa^2 in peptide 6 indicates structural similarity in the NH_2-terminal part of the molecule between peptides 1, 4, 6, and 7. Some of these NOE contacts are characteristic for β-turns type I and II found in proteins (26), which confirms our previous prediction (by molecular modeling) about β-turn-like structures in NH_2-terminal part of the macrocycle involving Gly^3 in i+2 position and Asp^2/Asn^2 in i+1 position of the turn.

The NOE contacts in the COOH-terminal part of the molecule revealed the role of Pro^6 (peptides 4 and 7) or Hyp^6 (peptide 6) as a conformation-stabilizing amino acid. The NOE contacts are depicted in Fig. 2B. The strong NOE contacts between amide protons of Cys^8 and Aaa^7 in peptide 4, 6, and 7 together with the distinctive *trans*-Pro NOE contact between αCH of Gly^5 and δCH of Pro^6/Hyp^6, NOEs between βCH of Pro^6/Hyp^6 and NH of Aaa^7 confirm the conformational similarity among peptides 4, 6, and 7 in the COOH-terminal part of the molecule. The distinctive strong NOE contact between NH of Glu^6 and NH of Gln^7 in peptide 1 (data not shown) indicates the presence of turn with Glu^6 in i+2 positions. Thus, the COOH-terminal part of the macrocycle of 1 shows conformational similarity with peptides 4, 6, and 7 with Pro^6 or Hyp^6 in i+2 position of the turn. The proposed conformation of peptide 4 is shown in Fig. 2C.

Integrin-Blocking Antibody Studies

A collagen-specific integrin investigating kit (ECM 425, Chemicon) containing function-blocking anti-α1 (FB12), anti-α2 (P1E6), anti-α3 (ASC-1), anti-αvβ3 (LM609), anti-αv (P3G8), anti-β1 (P5D2), and anti-β3 (25E11) antibodies was used in a cell bead–binding inhibition assay to evaluate the cell surface receptor for the cyclic DGLG

Table 1. Five distinct cyclic peptide motifs for four ovarian cancer cell lines (CaOV-3, SKOV-3, OVCAR-3, and ES-2) identified from screening several OBOC combinatorial peptide libraries

Motif 1	Motif 2	Motif 3	Motif 4	Motif 5
cDGLGDDc	cNGQGTAc	cNGRH-Hyp-Vc	cIGRGDVFc	cLDWDLIc
cDGWGPNc	cNGQGTDc	cNGRHHFc	cSFRGDFIc	c-Nle-D-Chg-NDFc
	cNGQGENc	cNGRNVRGc	cLRGDLTFc	c-Nle-D-Nle-Phg-Dc
	cNGQGESc	cNGRFEHc	cRGDKLQYc	c-Nle-DWEEc
	cNGQG-Nle-Sc	cSNGRFNMc		c-Nle-D-Chg-YMc
	cNG-Nle-GEAc			c-Nle-DVDEc
	cNG-Nle-G-Nle-Lc			
	cNG-Nle-GTMc			

NOTE: Cyclization via the -SH groups of the flanking D-cysteines. Single-letter representation for amino acid according to standard convention, except for those amino acids without single letter representation: Nle, norleucine; Chg, cyclohexylglycine; Phg, phenylglycine; Hyp, hydroxyproline.

peptide identified in our library screen. Of all the antibodies tested, only anti-α3 (ASC-1) was able to inhibit the binding of both ES-2 and SKOV-3 cells to the peptide beads. Binding of ES-2 cells to the cyclic peptide bead was completely abolished even at concentrations as low as 0.5 μg/mL (the concentration suggested by the manufacturer for integrin inhibition assays is 1-10 μg/mL; Fig. 3). This strongly suggests that α3 integrin is the target receptor for the cyclic DGLG peptide.

Discussion and Conclusion

Targeted therapy has become the goal of cancer treatment. In the post-genomic era, clinical diagnosis and treatment regimen is more and more dependent upon the molecular characteristics of tumors. Molecular markers of biopsy specimens will be tested and categorized according to the probability of response to treatment based on the presence or absence of a molecular target. The importance of cancer cell surface receptors as therapeutic targets is exemplified by the successful use of monoclonal antibodies such as Rituxan, Zevalin, Herceptin, and Mylotarg in the clinics. We believe peptides are better cell surface–targeting agents than monoclonal antibodies, particularly when the antibodies are used as carriers for cytotoxic payloads such as chemotherapy or radionuclides. It is because peptides are: (*a*) smaller and therefore can penetrate large tumors better, (*b*) less likely to bind to the reticuloendothelial system such as liver, spleen, and bone marrow, and (*c*) easy to derivatize chemically. Peptides are probably not very immunogenic if they are short, not covalently attached to a carrier protein, and administered without any immunoadjuvants.

Although many researchers have used phage-display peptide library methods to identify cancer cell surface–binding ligands [see review by Aina et al. (11)], we have been using the OBOC approach to screen cancer cell lines or fresh cancer cells derived from cancer patients. The

Table 2. Binding specificities of the three cyclic peptides against a large number of cancer cell lines and normal cells

Cancer cell type	cLDWDLIc	cDGLGDDc	cDGWGPNc	cNGRFEHc
OVCAR-3 (ovarian)	−	++	+	++++
CaOV-3 (ovarian)	+++	++	+++	+++
SKOV-3 (ovarian)	+	+++	++++	++++
ES-2 (ovarian)	+++	++++	++++	++++
Raji (B lymphoma)	++	−	−	−
Ramos (B lymphoma)	−	−	−	−
Jurkat (T lymphoma)	−	−	−	−
LnCaP (prostate)	−	−	−	+
PC-3 (prostate)	−	−	−	+++
DU-145 (prostate)	−	−	−	−
MDA-MB 435 (breast)	−	−	−	++
MDA-MB 231 (breast)	−	+	+	++
T47D (breast)	−	−	−	−
MCF-7 (breast)	−	−	−	++
A549 (lung)	−	+	+	++
A172 (brain)	−	+++	+++	+++
IMR-90 (normal lung fibroblast)	−	−	−	+++
NBE (normal breast epithelium)	+	−	−	+++

NOTE: Semiquantitative relative binding activity: ''++++'' very strong binding (>30 cells per bead), ''+++'' strong binding (20–30 cells per bead), ''++'' moderate binding (10–20 cells per bead), ''+'' weak binding (5–10 cells per bead), ''−'' no binding.

Table 3. Relative binding activity of peptide analogues (alanine walk) to ES-2 and SKOV-3 ovarian cancer cells

Peptide	Activity		Peptide	Activity		Peptide	Activity	
	ES-2	SKOV3		ES-2	SKOV-3		ES-2	SKOV-3
cDGLGDDc	+++	++++	cDGWGPNc	+++	++++	cLDWDLIc	++	+
ADGLGDDc	–	–	ADGWGPNc	–	–	ALDWDLIc	–	–
cAGLGDDc	–	–	cAGWGPNc	–	–	cADWDLIc	–	–
cDALGDDc	–	–	cDAWGPNc	–	–	cLAWDLIc	–	–
cDGAGDDc	–	–	cDGAGPNc	+++	+++	cLDADLIc	+	–
cDGLADDc	–	–	cDGWAPNc	–	–	cLDWALIc	–	–
cDGLGADc	+++	++++	cDGWGANc	–	+	cLDWDAIc	++++	+++
cDGLGDAc	+++	+++	cDGWGPAc	+++	++++	cLDWDLAc	+++	+
cDGLGDDA	–	–	cDGWGPNA	–	–	cLDWDLIA	–	–

NOTE: ''c'', D-cysteine, the uppercase single letters represent L-amino acids (standard convention), peptides with two flanking D-cysteines were cyclized by disulfide bonds. Semiquantitative relative binding activity: ''++++'' very strong binding (>30 cells per bead), ''+++'' strong binding (20–30 cells per bead), ''++'' moderate binding (10–20 cells per bead), ''+'' weak binding (5–10 cells per bead), ''–'' no binding.

main advantage of the OBOC combinatorial library method is that unnatural amino acids, D-amino acids, organic moieties, cyclic structures, or branched structures can easily be incorporated into the library, making it much more versatile and diverse. In addition, peptides containing D-amino acids and organic moieties are generally much more resistant to proteolysis, which is critical for effective cancer therapeutics. Using this approach, we have identified several unique ligands for lymphoma (17), prostate cancer (14, 15), and lung cancers (16, 18). Here, we report the identification of short cyclic peptide ligands that bind to cell surface receptors of four ovarian adenocarcinoma cell lines (CaOV-3, ES-2, SKOV-3, and OVCAR-3). These ligands were identified from screening various combinatorial peptide libraries with these cell lines followed by the testing of the ''positive'' beads to eliminate nonspecific binders prior to micro-sequencing. Several novel motifs were identified through these screening efforts (Table 1). Some of these motifs, such as NGR (motif 3), RGD (motif 4), and Nle-D-W (motif 5) have been previously reported. For example NGR was identified (23) with a phage-display technique as a binding motif for aminopeptidase N. RGD is a known motif for several different integrins such as $\alpha v\beta 3$ and $\alpha v\beta 5$. Nle-D-W was identified as a ligand for $\alpha 4\beta 1$ integrin (18). The NGR peptide is nonspecific and binds to many different cell types including normal cells. We chose to focus our effort on the cXGXGXXc motif because of its apparent affinity and specificity to ovarian cancer cell lines. Structure-activity studies of cDGLGDDc and cDGWGPNc (motif 1) revealed that cDGXGXXc was the minimum required sequence for cell binding, and cyclization is crucial for activity. A series of related peptides cNGXGXXc (motif 2) was also identified in our library screen. This is similar to the cNGRGEQc peptide that was previously reported by us as ligands for the A549 non–small cell lung cancer cell line (16). Computer modeling and NMR studies of cDGXGXXc and cNGXGXXc peptides strongly suggest that both of these peptide motifs display a β-turn conformation, which is stabilized by disulfide cyclization (Fig. 2). We evaluated the binding of cDGLGDDc and cDGWGPNc peptides to

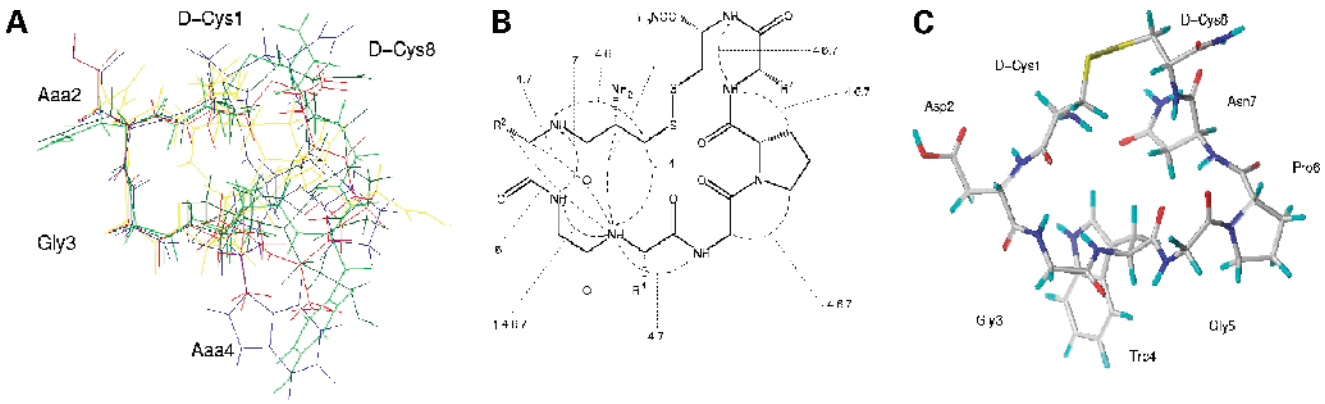

Figure 2. A, an overlay of proposed conformation of peptides with cXGXGXXc motif. **B,** NOE contacts found in peptides: (1) cNGRGEQc, (4) cDGWGPNc, (6) cDG-Nle-G-Hyp-Tc, (7) cNG-Nle-GPNc. **C,** proposed conformation of peptide (4).

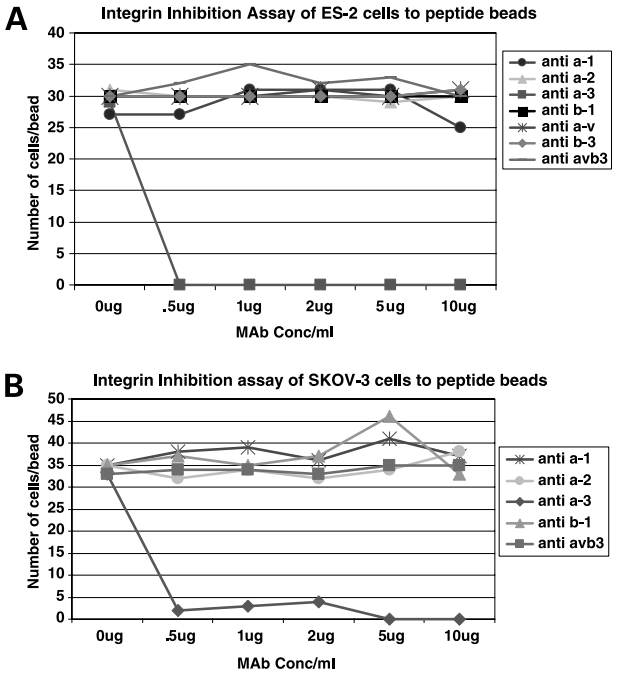

Figure 3. Dose-response curve of inhibition of ovarian tumor cells (ES-2 and SKOV-3) attachment to peptide beads using serial dilutions of α1, α2, α3, αv, αvβ3, and β1 function blocking integrin antibodies.

a panel of cancer cell lines and observed that this peptide motif preferentially binds to ovarian cancer cells (Table 2). Interestingly, it also binds strongly to a glioblastoma cell line (A172).

We have previously reported the use of plain and magnetic peptide beads to retrieve A549 non–small cell lung cancer cells from blood (27). In this study, we were able to show that we could also use cDGWGPNc peptide beads to retrieve OVCAR-3 ovarian cancer cells from whole blood that was previously spiked with these cells (Fig. 1B). No binding of mononuclear cells, red cells, or platelets to these peptide beads was detected. This observation, together with the fact that these peptides did not bind to ''normal'' lung fibroblasts or ''normal'' breast epithelial cells (Table 2), strongly suggest that these peptides are cancer-specific, making them prime candidates for the development of ovarian cancer targeting agents. Because normal ovarian epithelial cells are not readily available, we do not know if cDGWGPNc peptide will bind to these cells. However, when we tested normal Chinese hamster ovary-K1 cells (American Type Culture Collection) with this peptide, there was no binding. In any case, we believe that there may be differential expression and up-regulation of some integrins between normal and tumor ovarian epithelium. Nevertheless, this peptide can still be useful as a therapeutic or imaging agent even if it binds normal ovarian epithelium as long as there is no nonspecific targeting of other vital organs.

Through function-blocking antibody studies (Fig. 3), we were able to determine that cDGXGXXc binds specifically to α3 integrin receptor. Previously, we reported that binding of the cNGRGEQc peptide to A549 non–small cell lung cancer could be blocked by both anti-α3 and anti-β1 integrin antibodies (16). However, in this current study, anti-β1 integrin antibody had little or no blocking effect on the binding of ovarian cancer cells to cDGLGDDc or cDGWGPNc peptides. Furthermore, as shown in Table 2, cDGWGPNc and cDGLGDDc bind strongly to the ovarian cancer cell lines but only weakly to A549 cells. Together, this data suggests that the α3 integrin receptors on the ovarian cancer cell lines and the non–small cell cancer line may be somewhat different, perhaps on their partner β chains. We can speculate that the glioblastoma cell line (A172) expresses high levels of α3 integrin receptor and thus binds strongly to these peptides. α3β1 is known to be expressed in the developing nervous system and is crucial in the organization of cortical neurons in the brain as well as in neuronal migration (28).

The fact that the -DGLG- motif identified in our library screening is contained in collagen Iα is of interest. First, this form of collagen is mostly present in the interstitium, which suggests that cells with metastatic potential may have up-regulated their cell surface receptors to bind to this extracellular matrix protein. Second, ovarian tumor cells have been known to synthesize as well as bind to extracellular matrix proteins in order to migrate. It is conceivable that the -DGLG- motif on collagen, displayed in a β-turn conformation, is the native binding motif for α3-integrin.

Ovarian cancer cells have been known to invade both the peritoneal mesothelium and submesothelial extracellular matrix. Integrins are important in this process because their ligands are components of the extracellular matrix. Studies have shown that the level of integrin expression by some cancers can be directly linked to the ability of these cells to metastasize and invade the extracellular matrix (29, 30). Antibodies to both β1-integrin subunit and CD44 (ovarian cell surface molecule) have been used to inhibit the migration of ovarian carcinoma toward extracellular matrix proteins (31). Several integrins, such as α5β1, αvβ3 and αvβ5, play critical roles in promoting tumor metastasis and angiogenesis (for review, see refs. 32, 33). Antagonists of these integrins are now in clinical trials for cancer therapeutics. Integrin β1, α1, α2, α3, α4, α5, α6, as well as αvβ3 are expressed at varying levels (mostly high) in CAOV-3, SKOV-3, and OVCAR-3 ovarian cancer cell lines, whereas similar patterns of β1 and αvβ3 expression have been found in primary ovarian cancer tissue samples (34). RGD peptides (a known sequence in fibronectin), which is a ligand for αvβ3 and αvβ5, has been used experimentally to inhibit peritoneal seeding of human ovarian cancer cells in nude mice (35).

We have determined that the ovarian cancer targeting peptides (cDGLGDDc and cDGWGPNc) identified through OBOC combinatorial library method bind to α3 integrin. It has been reported that naturally occurring tumors such as astrocytomas, melanomas, gliomas, medulloblastoma, and gastric tumors have up-regulated expression of α3β1

integrin (36). We believe that the integrin-binding peptides reported here are excellent lead compounds for further development of therapeutic and imaging agents not only for human ovarian cancer but for these tumors as well.

To optimize these peptide leads, we have recently designed and synthesized a focused or secondary combinatorial library based on the cXGXGXXc motif. When this library was screened under high stringency, we identified several ligands that bind strongly to ovarian cancer cell lines. One of these ligands was able to effectively image ovarian cancer in a xenograft model both optically with peptide-Cy5.5 conjugates and with a micropositron emission tomography scanner using [^{64}Cu]-1,4,7,10-tetra-azacylododecane-peptide conjugate as the imaging agent (37).[2]

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