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From Combinatorial Chemistry to Cancer-Targeting Peptides

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Abstract: Several monoclonal antibodies that target cell surface receptors have gained approval by the U.S. Food and Drug Administration and are widely used in the treatment of some cancers. These include but are not limited to the anti-CD20 antibody Rituximab, used in lymphoma treatment, as well as anti-HER-2 antibody for breast cancer therapy. The efficacy of this cancer immunotherapy modality is, however, limited by the large size of the antibody (160 kd) and its relatively nonspecific binding to the reticuloendothelial system. This latter property is particularly problematic if the antibody is used as a vehicle to deliver radionuclides, cytotoxic drugs, or toxins to the tumor site. Peptides, peptidomimetic, or small molecules are thus attractive as alternative cell surface targeting agents for cancer imaging and therapy. Cancer cell surface targeting peptides can be derived from known native peptide hormones such as somatostatin and bombesin, or they can be identified through screening combinatorial peptide libraries against unknown cell surface receptor targets. Phage-display peptide library and one-bead one-compound (OBOC) combinatorial library methods have been successfully used to discover peptides that target cancer cells or tumor blood vessel endothelial cells. The phage-display peptide library method, because of its biological nature, can only display L-amino acid peptides. In contrast, the OBOC combinatorial library method allows for bead-surface display of peptides that contain L-amino acids, D-amino acids, unnatural amino acids, or other organic moieties. We have successfully used the OBOC method to discover and optimize ligands against unique cell surface receptors of prostate cancer, T- and B-cell lymphoma, as well as ovarian and lung cancers, and we have used some of these peptides to image xenografts in nude mice with high specificity. Here, we (i) review the literature on the use of phage-display and OBOC combinatorial library methods to discover cancer and tumor blood vessel targeting ligands, and (ii) report on the use of an ovarian cancer targeting ligand, OA02, as an *in vivo* PET imaging probe in a xenograft model in nude mice.

Keywords: Peptide library; cancer cell surface targeting agents; combinatorial chemistry; phage-display; optical imaging; PET imaging; integrin; one-bead one-compound library; on-bead cell binding

Introduction

The hybridoma technology was invented in 1975 by Kohler and Milstein,[1] but it was not until 27 years later that Rituximab (anti-CD20 monoclonal antibody) was approved by the U.S. Food and Drug Administration for the treatment of B-cell lymphoma. Since then, several monoclonal antibodies that target cancer cell surface receptors have been approved for clinical use and many more are currently undergoing clinical trials. The antitumor effects of some of

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these antibodies, e.g., anti-EGFR or anti-HER2/neu, are probably due to the blockage of the promitogenic function of circulating growth factors. Some of these antibodies, e.g., anti-CD20 antibody, inhibit tumor growth through antibody-dependent cellular cytotoxicity (ADCC) effect. Others use a cell-surface-targeting antibody as a vehicle to deliver radionuclides (e.g., Zevalin or Bexxar, anti-CD20 antibody loaded with ^{90}Y or ^{131}I, respectively) or toxin (e.g., myelotarg, anti-CD33 antibody conjugated to calicheamicin) to the cancer cells. Cancer-targeting antibodies utilizing this latter mechanism are problematic because (i) the antibody to the cancer cells is relatively large and has difficulty infiltrating the entire tumor mass, and (ii) the Fc region of the antibody binds to the reticuloendothelial system, resulting in high uptake of radionuclides, cytotoxic drugs, or toxins into bone marrow, liver, and spleen, leading to high toxicity. To overcome these problems, many investigators are exploring the use of antibody fragments or smaller antibody constructs such as minibody and diabody.[2,3] Peptides, peptidomimetics, or small molecules are alternative and possibly more effective targeting agents against cancer. These molecules are chemically stable, small, easy to synthesize and can be readily conjugated to radionuclides, cytotoxic drugs or toxins. Peptides that are *N*- and *C*-terminally blocked, cyclized, and/or contain D-amino acid and unnatural amino acids are generally very stable to proteolysis. These cancer-targeting chemical molecules can be developed through (i) modifying known native ligands against cancer-associated receptors (e.g., octreotide against somatostatin receptor,[4] bombesin against bombesin receptor,[5] and bradykinin analogues against bradykinin receptor[6]), (ii) molecular modeling if the X-ray structure of the receptor or related receptor is known, and/or (iii) screening combinatorial peptide or chemical libraries.[7]

Combinatorial Library Methods

A combinatorial library is usually referred as a collection of 1×10^4 to 1×10^8 different molecules generated by synthetic or biological approaches. The field of combinatorial library chemistry was pioneered by synthesis of the first limited peptide library using multipin technology in 1984,[8] and has since become a powerful tool that not only facilitates the drug discovery process but also provides important information for the fundamental understanding of molecular recognition. To date, there are six general methods of preparing and screening combinatorial libraries: (i) the biological peptide library method (e.g., phage-display peptide library),[9–11] (ii) the spatially addressable parallel library method,[12,13] (iii) combinatorial library methods requiring deconvolution,[14,15] (iv) the affinity selection method,[16] (v) the OBOC combinatorial library method,[17,18] and (vi) self-assembled peptide nucleic acid (PNA) encoded chemical microarrays.[19] Biological peptide libraries can typically accommodate only the 20 natural amino acids. In contrast, synthetic peptide libraries have the potential to incorporate D-amino acids and other unnatural amino acids, as well as specific secondary or scaffolding structures that may enhance biologic activity. In addition to amino acids, other macromolecular components subunits such as monosaccharides,

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nucleotides, lipids, or even small organic moieties can be used. For detailed comparison of these library methods, please refer to our previous review.[20] Of these six methods, only the biological peptide library method and the OBOC method have been applied to the discovery of cancer cell surface targeting ligands.

Phage-Display Peptide Library. Bacteriophage are single-stranded DNA viruses that infect bacteria. The phage genome codes for DNA replication–proteins pII and pX, DNA binding protein pV; major coat protein pVIII, viral assembly proteins pI, pIV, and pXI, and minor coat proteins pIII, pVI, pVII, and pIX.[21] About five copies of pIII protein are displayed on the infectious end of the virus. Smith developed the "fusion phage" in the mid 1980s by inserting foreign DNA fragments into the encoding gene of the pIII protein.[22] This led to the expression of L-amino acid containing peptide on the virion surface, which did not affect virus infectivity. The M13 phage is the most widely used phage-display system because of its high capacity for replication and the ability to receive large DNA inserts into its genome. Trinucleotide sequences that encode specific amino acids can be constructed and inserted into the phage genome either to increase diversity or introduce a bias into the clone population. Linear libraries can be constructed by elimination of trinucleotide coding sequences for cysteines, whereas cyclic libraries can be constructed with even numbered cysteine coding sequences. This extremely powerful method enables the researcher to routinely generate 1×10^8 to 1×10^9 different phages. Furthermore, the size of the grafted peptide or protein is not limited by the constraints of synthetic chemistry, as in the case of the synthetic peptide library. In addition, the library can be easily generated by simply growing the microorganisms. Moreover, the biological library method can take advantage of known protein folds (e.g., immunoglobulin fold, zinc-finger fold, or conotoxin fold) by grafting random oligopeptides on such tertiary folds. However, this biologic approach suffers from major disadvantages, such as (i) only the natural L-amino acid peptide libraries (20 eukaryotic amino acids) can be incorporated into these libraries; (ii) complicated bicyclic, compact scaffolding, branched structures, or molecules with special chemistry of cyclization are impossible; and (iii) screening assays of the biological libraries are generally limited to the binding assays (e.g., panning) and some functional assays such as protease substrate determination.

Spatially Addressable Parallel Library Method. In this method, a collection of compounds is synthesized in a spatially addressable format. The library is screened, depending on the library method used, either by a direct solid phase binding assay or by a solution phase assay. The positive compound is then located and its chemical structure determined. Because the structure of each of these compounds is predetermined, decoding is not needed. The major drawback of this method is that only limited number of compounds can be synthesized and therefore the library is very small. The spatially addressable parallel library method includes multipin technology,[8] SPOT synthesis,[23] NanoKan,[24,25] and chemical microarray.[26,27]

Combinatorial Library Methods Requiring Deconvolution. In this method, mixtures of compounds are first synthesized and then subjected to biological testing. This library approach includes the iterative approach,[14,28] the positional scanning approach,[29] the orthogonal partition approach,[30] the recursive deconvolution approach,[31] and the dual recursive deconvolution approach. The major advantages of this approach are that a large number (1×10^6 to 1×10^8) of peptides can be synthesized and screened and many existing biological assays, including functional assays, can be adapted to this screening process (if it is a solution phase assay). In addition, because the structure of the active compounds can be deduced without need for further structure elucidation, decoding is not needed. However, in general, additional synthesis and testing, which are time-consuming,

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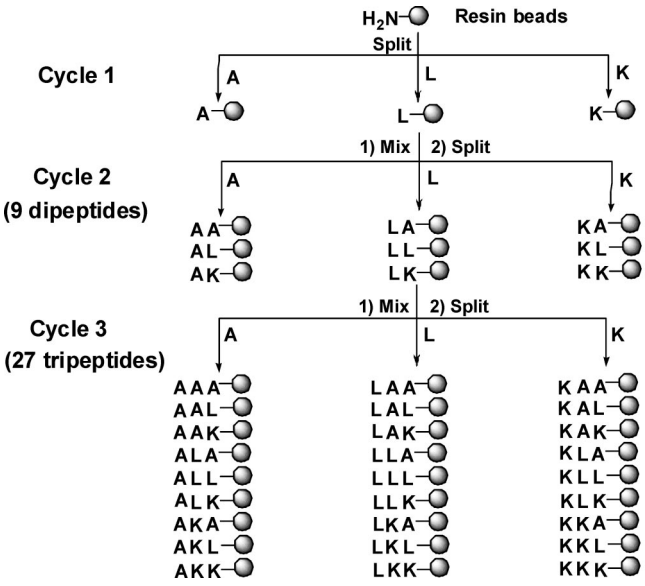

Figure 1. The "split-mix synthesis" method to generate a one-bead one-compound combinatorial library. A, L, and K are building blocks (in this case, amino acids).

are needed to reach a final solution. This method works quite well only if there is one predominant motif for the target protein. Targets with multiple binding motifs likely lead to scrambled and sometimes uninterpretable results.

Affinity Selection Method. In this method, an affinity column with immobilized receptor is used to select the ligand from a solution library (mixture), which is usually synthesized with a "split-mix" synthesis method (Figure 1)[14,17,33] to obtain an equimolar mixture of random compounds. After thorough washing, the bound ligands are eluted and structure determined.[34] This method has been applied successfully for combinatorial oligodeoxynucleotide libraries but with only limited success in peptide libraries larger than 10000 peptides. Major concerns about this method include (i) nonspecific binding, and (ii) if more than one predominant motif is present in the mixture, the result may be uninterpretable. This is because concurrent microsequencing of the retrieved peptide mixture is performed rather than sequencing individual peptides. There is no simple method of isolating a single peptide for sequencing from a large library.

OBOC Combinatorial Library Method. OBOC library is synthesized on solid phase beads using a "split-mix" synthesis method (Figure 1) such that each bead displays only one chemical entity.[17,18] Each 80–100 μm bead contains approximately 100 pmol of the same compound. The OBOC library, consisting of millions of beads, is then subsequently subjected to mass screening. The methods of screening include on-bead binding and functional assays,[35] as well as solution phase biological assays. For the on-bead binding assays, standard enzyme-linked colorimetric assays, fluorescent assays, or radionuclide assays can be used. Another approach is the use of a color probe for library screening, thus taking advantage of the ease with which positive beads may be identified by using color change. The OBOC method has also been successfully utilized in identification of cell surface binding ligands, using whole-cell binding assays (see below). In this assay, OBOC library is incubated with living cells; the resulting beads coated with a monolayer of cells are then handpicked as positive beads and characterized. Another application of the OBOC library method has been in the identification of protease substrates and inhibitors.[36–39] Here, highly porous resins such as PEGA [bis(2-acrylamidoprop-1-yl) poly(ethylene glycol) cross-linked dimethyl acrylamide and mono-2-acrylamidoprop-1-yl [2-aminoprop-1-yl] poly(ethylene glycol)] (Calbiochem-Novabiochem, San Diego) were used for the peptide library construction because it allowed the enzyme to gain access to the bead interior. Besides binding assays, the OBOC method has been adapted to solution phase assays. An elegant and potentially powerful approach to screen the OBOC libraries is the *in situ* solution phase releasable assay,[40–42] in which the compound-bead libraries are immobilized in a thin layer of agar. Compounds from each bead are then released to the immediate vicinity

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surrounding each bead for solution phase screening in the semi-solid matrix.

The main advantages of the OBOC method include (i) a large number (1×10^6 to 1×10^8) of compounds can be synthesized and screened rapidly, using either one or a combination of both on-bead binding and solution phase assays; (ii) it does not require deconvolution where multistep synthesis and screening are generally required; (iii) multiple peptide ligands with completely different motifs can often be identified in a single screen. One major disadvantage of the OBOC method is that each library compound is tethered to the solid support via a linker such as polyethylene glycol, and may result in steric hindrance between the cellular receptor and the library compound. However, in some instances, the linker may be beneficial, e.g., the linker can be used as a convenient handle to link the cancer targeting ligand to the therapeutic payload.

Self-Assembled PNA-Encoded Chemical Microarrays. In this method, the PNA-encoded peptide or small molecule library is prepared by the "split-mix" synthesis method and cleaved from the resin to form an encoded solution-phase library such that each library compound is tethered to a PNA code via a hydrophilic linker.[19] The library is then mixed with the target protein and later exposed to a planar oligonucleotide microarray of predetermined sequences. Alternatively, the encoded soluble library can be hybridized to the oligonucleotide microarray prior to incubation with the target protein. The identity of the positive library compound that interacts with the target protein can be determined by knowing the nucleic acid sequences of the oligonucleotide microarrays. Like the OBOC method, a whole cell binding assay can also be applied to the encoded planar chemical microarrays, although this has never been reported.

Phage-Display Peptide Libraries as Source of Cancer Targeting Ligands

The process of selecting phage clones that bind a specific target (also known as "panning") has been applied (i) *in vitro* to purified proteins that are often immobilized on Petri dish or beads; or to whole cells from various origins, and (ii) *in vivo* in experimental animal models and human. Ligands identified through *in vitro* protein or cell panning need to be validated and evaluated for *in vivo* targeting. A by-no-means exhaustive list of phage peptides identified to different cell types and proteins are shown in Table 1.

***In Vitro* Panning with Cancer-Associated Cell Surface Receptor Protein Targets.** Common technical considerations in screening and selection of phage-display libraries include presentation of the target protein in native form, number of phages used, stringency of selection process, competitive selection, and subtractive panning.[43] Complete step-by-step protocols for *in vitro* screening can be found in the article by Kay *et al.*[44] El-Mousawi et al. screened a linear 16-mer peptide phage-display library with immobilized recombinant Flt-1 and identified N*XX*EIE*XYX*W*XXXXX*Y as a motif that binds with high affinity to this VEGF receptor[45] They showed that this peptide could inhibit the effects of VEGF on human endothelial cells. Peptides that bind to CD-21 B-Cell lymphoma cell surface marker has also been identified by *in vitro* phage panning.[46] One of the five peptides identified (RMWPSSTVNLSAGRR) was evaluated for potential chemotherapeutic targeting by conjugating this peptide to a copolymer drug carrier Hydroxypropylmethacrylamide (HPMA). The connection between FGF, angiogenesis and cancer was made over a decade ago.[47,48] FGF is thought to induce endothelial proliferation and angiogenesis, which in turn can lead to rapid tumor growth. In various studies, FGF antagonists have been shown to decrease angiogenesis and reduce tumor growth.[49–51] Panning of a 26-mer peptide M13-phage library with purified extracellular domain of FGF-R and Sf9 cells led to the identification a novel peptide agonist of FGF-R ("C-19"), which was different from known members of the FGF family.[52]

***In Vitro* Panning with Live Cancer Cells.** Panning of a phage-display library with living cells is advantageous for

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Table 1. Cell Targeting Peptides Identified by Phage-Display Library Methods

	sequence	*in vivo* imaging	therapy
	Ligands to Normal Organs/Cells/Body Fluids		
muscle[118]	ASSLNIA		
lung[119]	QPFMQCLCIYDASC, RNVPPIFNDVYWIAF VFRVRPWYQSTSQS		
sperm[120]	XLWLLXXG		
microglia[121]	SFTYWTN		
synovium[122]	CKSTHDRLC		
urothelium[123]	I/LGSGL		
pancreatic islets[124]	CVSNPRWKC, CHVLWSTRC		
cardiomyocytes[125]	WLSEAGPVVTVRALRGTGSW		
plasma[126]	CGLIIQKNEC, CNAGESSKNC		
	Ligands to Specific Tumor Cells		
breast cancer tissue[59]	PRP, SSSPL, SPW, PHSK, KHST, TLLS, SSTA, TSAH, LSAN		
lung cancer[60]	VSQTMRQTAVPLLWFWTGSL MTVCNASQRQAHAQATAVSL RGDLATLRQLAQEDGVVGVR		61
bladder cancer[57]	CXNXDXR(X)/(R)C		
squamous cell carcinoma[62]	CGKRK, CDTRL		
pancreatic islet cell carcinoma[67]	RSR, KAA, RGR		
medullary thyroid cancer[68]	HTFEPGV, SRESPHP		
neuroblastoma[127]	VPWMEPAYQRFL	128	
medullary thyroid carcinoma[129,68]	HTFEPGV, SRESPHP		
colon carcinoma[58]	VHLGYAT		
invasive colon cancer[130]	CPIEDRPMC	96	
hepatocellular carcinoma[131]	TACHQHVRMVRP		
sup-88 human b-cell lymphoma[132]	KNGPWYAYTGRO,NWAVWXKR, YXXEDLRRR, XXPVDHGL		
LNCaP prostate cancer[133]	DPRATPGS		
wac-2 human neuroblastoma[127]	HLQLQPWYPQIS		
chronic lymphocytic lymphoma (CLL)[134]	LVRSTGQFV, LVSPSGSWT, ALRPSGEWL, AIMASGQWL, QILASGRWL, RRPSHAMAR DNNRPANSM, LQDRLRFAT, PLSGDKSST		
HL 60 human lymphoma & B-16 mouse melanoma[135]	IELLQAR		
head & neck carcinoma[136]	TSPLNIHNGQKL		
	Ligands to Cell Surface Receptors/Proteins		
$\alpha v\beta 3$, $\alpha v\beta 5$[137]	(RGD-4C) CDCRGDCFC	107	
$\beta 3$	GRDSPK-Cypate	86, 95	
$\alpha 5\beta 1$[138]	GACRGDCLGA (cyclic)		
$\alpha 6\beta 1$[139]	FGRIPSPLAYTYSFR, HRWMPHVFAVRQGAS VSWFSRHRYSPFAVS		
VEGF-R1[45]	NXXEIEXYXWXXXXXY, ATWLPPR RRKRRR, ASSSYPLIHWRPWAR		
CD- 21[46]	RMWPSSTVNLSAGRR, PNLDFSPTCSFRFGC, GRVPSMFGGHFFFSR		
FGF-R[52]	AESGDDYCVLVFTDSAWTKICDWSHFRN		
aminopeptidase P[140]	CPGPEGAGC		
aminopeptidase N[141]	CNGRCVSGCAGRC CVCNGRMEC, NGRAHA		
prostate specific antigen (PSA)[142]	CVFXXXYXXC, CXFXXXYXYLMC CVXYCXXXXCYVC, CVXYCXXXXCWXC		
	Ligands to Vasculature		
human vasculature[143]	RGD		144
mice prostate[65]	SMSIARL, VSFLEYR		
vasculature of various tumors[64]	CGSLVRC, CGLSDSC		
	Ligands to Lymphatics		
tumor lymphatics[145]	CGNKRTRGC		

the following reasons. First, the cell surface receptors are presented in the context of a natural biological cell membrane. Second, novel peptide ligands can be identified to unknown cell surface receptors. In addition, one has the option of selecting for phages that bind and internalize inside the cells. Selection of cell surface binding phage as opposed to internalized phage can be accomplished by modifying the washing techniques.[53] Kolonin *et al.* recently reported the screening of phage-display peptide libraries with the NCI panel of 60 cell lines.[54] In this study, tri-peptide ligands that bound across multiple cell types were identified. It was observed that the similarity in ligand binding among these various cell types correlated with gene expression profiles of their cell surface receptors. In other recent studies, investigators have used the phage-display technique to identify peptides against DU-145 and PC-3 human prostate carcinoma,[55] malignant human glial cancers,[56] human bladder cancer,[57] human colon cancer,[58] breast cancer,[59] and human lung cancer.[60] Zitmann *et al.*[55] identified DUP-1 peptide through screening a prostate cancer cell line and demonstrated that the targeting peptide was internalized into the cells after 1hr. The biodistribution of ^{131}I labeled DUP-1 in mouse xenograft models showed accumulation in the tumors within 5 min with a %ID/g of 5 and 7 for DU-145 and PC-3 tumors, respectively. Except for the kidney, the tumor-to-organ ratio in perfused DU-145 tumor-bearing mice was greater than 2. Lee *et al.* screened cyclic CX_7C peptide phage libraries with cells isolated from a human bladder cancer (HT-1376) xenograft.[57] It is important and interesting to note that in this study, the selected peptide "CSNRDARRC" bound not only to bladder cancer cells isolated from a xenograft tumor but also to carcinogen-induced bladder tumors. It is specific for tumors and does not bind normal mouse bladder cells, normal rat kidney cells, or HUVEC cells. Selectivity of this peptide for tumor cells was also validated by testing primary tissue samples from patients with transition cell carcinoma. This peptide also bound to exfoliated cells isolated from the urine of a bladder cancer patient. Oyama *et al.*[60] identified certain 20-mer peptides that seemed relatively specific to certain lung cancer cells. These peptides exhibited nonlineage specific characteristics. Rather, they seemed to bind cell types with similar kinds of cell surface receptors (referred to as landscapes). The H1299 binding peptide "VSQTMRQTAVPLLWFWTGSL" (H1299.1) was found to have the highest selectivity for large cell lung cancer. Synthetic forms of the isolated phage-display peptides presented in a tetrameric form were shown to have an increased affinity for their cognate receptor. Doxorubicin conjugated H1299.1 peptide have been used for specific chemotherapeutic targeting[61] (see section on therapeutic targeting). A similar tetrameric H1299 binding peptide (H1299.2) "SGYAAWPASGAWTGTAPCSAGTASGSAK" was biotinylated and conjugated to streptavidin-coated Qdots for *in vitro* fluorescence imaging of these cells.

***Ex Vivo* Selection of Tumor Binding Phages.** Screening of phage-display peptide libraries with dissociated cells obtained from biopsy lesions has been referred to as *ex vivo* panning.[62] However, phage selection in this study was performed with isolated cells and not with intact tissue sections. For decades, physiologists and pharmacologists have been using isolated perfused organs for their studies. Technically, it should not be difficult to perfuse isolated organs or tumor with phage-display libraries through the big vessels. This system may enable one to screen phage-display libraries in a much more controlled fashion, such as adding high concentrations of blocking agents against certain receptors in the absence of blood cells or plasma.

***In Vivo* Selection of Tumor Targeting Phages Using Xenograft Models.** Work pioneered by Ruoshlahti, Arap, Koivunen, and Pasquilini on selection of phages that bind to tumor vasculature has resulted in numerous publications and therapeutic studies [63–66]. It seems, however, that the focus is now shifting from using xenograft mouse models to transgenic and clinically relevant disease models. In recent

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studies by the Ruoslahti group, *in vivo* screening of cyclic peptide phage-display libraries with transgenic mouse models of squamous cell carcinoma (SCC) led to the identification of specific peptides that bound either dysplastic/premalignant skin lesions ("CSRPRRSEC") or malignant SCC tumors (CGKRK and CDTRL).[62] Using a similar approach, researchers screened transgenic mice with the RIP1-Tag2 pancreatic islet cell carcinoma tumors with phage to identify peptide sequences that bound selectively to hyperplastic lesions ("RSR") in comparison to tumors ("KAA").[67] These peptides (RSR, KAA, and RGR) which colocalized with endothelial and pericyte cell markers, could potentially be used for imaging of preneoplastic lesions. Another interesting point in this study is that although cyclic libraries were used for screening, the peptides identified were linear. This is because the peptides were truncated as a result of stop codons within the random insert and frameshift mutations that changed cysteine to valine. Studies by Bockman *et al.* focused on using the transgenic mouse models of the *RET* proto-oncogene induced medullary thyroid cancer (MTC) to identify phage-display peptides for potential gene therapy and tumor targeting.[68] They found that the initial peptide lead, HTFEPGV, identified as human MTC targeting peptide was different from SRESPHP, which targeted transgenic mouse MTC; and that HTFEPGV actually bound nonspecifically to nontargeting organs in this mouse model. In an effort to optimize clinical phage screening techniques, a new *in vivo* phage screening strategy has recently been described by Kolonin *et al.*[69] Using a "synchronous selection" technique, panning for multiple organs was done simultaneously, and the data were subjected to complex statistical analysis to identify tripeptide motifs that bind selectively to each organ. A different *in vivo* peptide phage panning approach has also been reported by Newton *et al.*, in which a "micropanning assay" was used to identify phage clones that bound preferentially to PC-3 prostate carcinoma cells over human embryonic kidney cells. The resulting phage clone (G-1) with the sequence "IAGLATPGWSHWLAL" was fluorescently labeled with the near-infrared fluorophore AlexaFluor 680 and evaluated for *in vitro* and *in vivo* targeting of PC-3 prostate carcinomas in SCID mice.[70]

***In Vivo* Selection of Phages in Patients with Cancers.** In a recent study by Krag *et al.*,[71] patients with late stage melanoma, breast, and pancreatic cancer were infused with random phage-display peptide libraries or phage-display short chain Fv antibodies in either single or multiple panning experiments. In one of the breast cancer patients, one of the peptides identified, GSPQCPGGFNCPRCDCGAGY, occurred with very high frequency and warrants further characterization and studies on the identity of the target protein. One peptide clone with the sequence MRIRCAAAWRATGTHCSLRA identified from a melanoma patient appeared to be specific to that individual patient's tumor, and did not bind human melanocytes and other melanoma cell lines except SK-MEL-5. This peptide showed a sequence homology to human EGF-like protein. In principle, phage-display selection can be performed on individual patients, leading to "personalized" therapeutics. However, performing pharmacology–toxicology studies on each peptide isolated for each patient will pose tremendous logistic and regulatory problems.

Discovery of Cancer Cell Surface Targeting Ligand through OBOC Combinatorial Library Methods

Phage-display peptide libraries are commercially available; the method is highly efficient, inexpensive, amenable to both short and long peptides, and can be carried in most molecular biology laboratories. However, as mentioned above, one major drawback of phage-display peptide libraries is that only the 20 eukaryotic L-amino acid peptides can be displayed on phages. L-Amino acid peptides are generally highly susceptible to proteolysis, particularly if the *N*- and *C*-termini are not blocked. Converting a phage-display peptide into a proteolytic stable form that still retains a high binding activity and specificity to the cell surface receptor is not trivial. OBOC combinatorial libraries, on the other hand, are based on synthetic chemistry; therefore, one may easily incorporate D-amino acids, unnatural amino acids, many other organic building blocks, and secondary structures into the library design. Furthermore, the OBOC method can be used for rapid optimization of the initial lead compounds. For example, we

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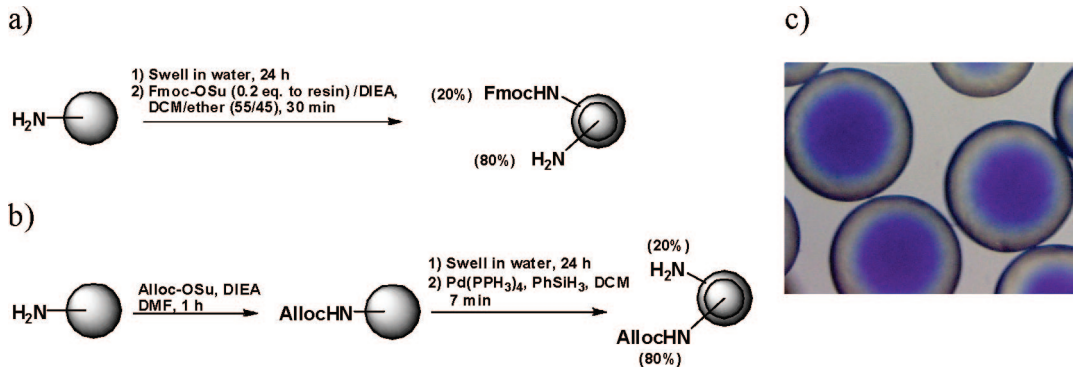

Figure 2. (a) Preparation of bilayer bead using PAP bilayer approach. (b) Preparation of bilayer bead using PAD bilayer approach. (c) Photomicrograph of the topographically segregated bifunctional bead. Free amines at the inner core of each bead stained blue because they reacted with bromophenol blue.

have succeeded in using a highly focused and encoded OBOC peptidomimetic library and a high stringency screening assay to identify a high-affinity ($IC_{50} = 2$ pM) and high-specificity peptidomimetic compound that bind to activated $\alpha4\beta1$ integrin of lymphoid cancers.[72]

OBOC Combinatorial Peptide and Peptidomimetic Library Synthesis and Encoding. As mentioned above, the OBOC combinatorial peptide and peptidomimetic libraries can be generated using a "split-mix" synthesis approach. For OBOC libraries used for on-bead binding assay (e.g., whole cell binding as described below), we prefer to use TentaGel resin (Rapp Polymere, Tubingen, Germany) as solid support because of its uniformity in size and "nonstickiness", as well as its suitability in a wide range of organic solvents and water.

For the OBOC peptide libraries that are comprised of sequenceable α-amino acids (including 20 eukaryotic, many amino acid derivatives, and unnatural amino acids), the synthesis is straightforward by using a standard solid-phase peptide synthesis method. 9-Fluorenylmethoxycarbonyl (Fmoc)/*tert*-butyl (*t*-Bu) chemistry and *N*-hydroxybenzotriazole (HOBt)/*N, N*′-diisopropylcarbodiimide (DIC) coupling are preferable because the reaction is mild and usually gives cleaner results. In the case of difficult coupling, 2-(*1H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), or 2-(*1H*-9-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) in the presence of *N,N*-diisopropylethylamine (DIEA) might be used. Positive compound beads identified during the screening assays are physically isolated for structure determination. An automatic protein sequencer (e.g., Procise 494, Perkin-Elmer/Applied Biosystems) is routinely used via Edman chemistry to determine the amino acid sequence of these α-amino-acid-containing peptides. For peptides without a free *N*-terminus (e.g., cyclic peptides utilizing the *N*-terminal amino group for cyclization), branched peptides, peptides with one or more nonsequenceable building blocks (e.g., β- and γ-amino acids), or peptidomimetics containing a small molecule moiety, the OBOC library synthesis requires a different synthetic strategy and chemical encoding. Chemical coding tags are added to the bead during the synthetic steps so that the synthetic history of each compound bead in the chemical library can be recorded. The coding tag can then be decoded by either Edman microsequencing or mass spectrometry. If both the library compounds and their corresponding coding tags coexist at the bead surface where the interaction between target proteins and library compounds occurs, the coding tags may interfere with the screening. To eliminate such interference, the coding tags should be in the bead interior. We have recently developed two simple, yet highly robust, methods to prepare topologically segregated bilayer beads. The first method is called the partial amine-protection (PAP) bilayer approach.[73] In this method, the TentaGel resin bead is first swollen in water and the outer layer of the bead is exposed to a mixture of dichloromethane (DCM) and diethyl ether containing an amine-derivatizing reagent (e.g., *N*-(9-fluorenylmethoxycarbonyloxy) succinimide (Fmoc-OSu)). Meanwhile, the bead interior remains in water without any derivatizing reagent. As a result, derivatization is confined to the outer layer of the bead (Figure 2a). The thickness of the outer layer (Fmoc-protection percentage) of beads is dependent on the amount of FmocOSu used (e.g., 0.2 equiv.) to resin total loading. Another approach is called the partial Alloc-deprotection (PAD) bilayer method.[74] In this method, the amino groups of the TentaGel beads are reacted with *N*-(allyloxycarbonyloxy) succinimide (Alloc-OSu) to achieve full protection with the Alloc group and then thoroughly swollen in water, followed by deprotection with palladium in DCM for a predefined limited time. This results in beads with a deprotected outer layer (free *N*-termini) and an Alloc-

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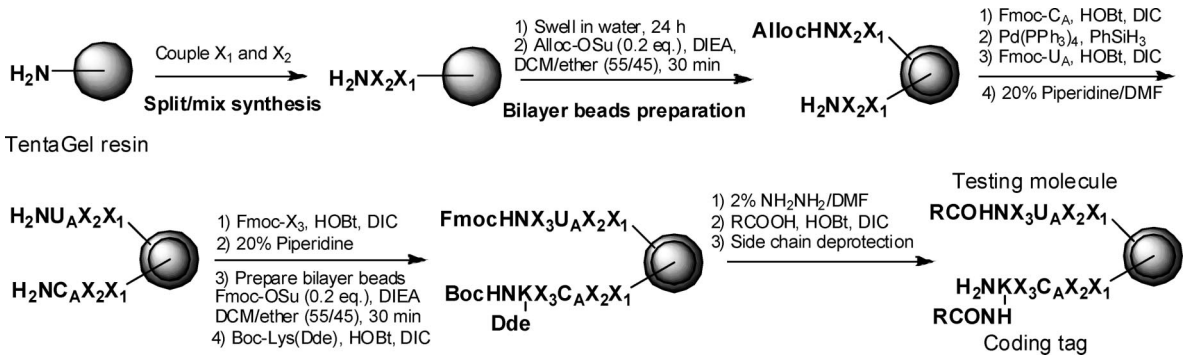

Figure 3. Synthetic and encoding approach for an OBOC peptidomimetic library (an example). X_1, X_2, X_3, and C_A stand for sequenceable amino acids. U_A represents nonsequenceable amino acids, which are encoded by C_A. K stands for L-lysine. R = alkyl or aryl.

protected inner core. Figure 2b illustrates the method for the generation of bilayer beads by the PAD bilayer approach. The thickness of the outer layer (Alloc-deprotection percentage) of beads is dependent on the duration of deprotection. Figure 2c shows photomicrograph of the topographically segregated bifunctional bead, with the inner core free amine stained with bromophenol blue. It is important to point out that for both methods the "bilayer-step" can be employed at any stage of the library synthesis and can be repeated multiple times.

With the above-mentioned bilayer beads on-hand, we have successfully developed several encoding methods to encode non-sequenceable peptide, peptidomimetic, and even small molecule libraries.[73,75,76] In our encoding systems, the library compounds display on the outer layer of the bead and the coding tags reside in the bead interior. Such a configuration not only facilitates the encoding process but also allows us to minimize interference by the coding tags during screening. The coding tag can then be decoded by either Edman microsequencing or mass spectrometry. An example of a peptide-encoded peptidomimetic library is shown in Figure 3. The advantage of microsequencing decoding is that no cleavage and retrieval of coding tags are needed. However, Edman sequencing is time-consuming and expensive compared with mass spectrometry analysis. In addition, microsequencing is not as readily available to most laboratories as mass spectrometry is.

"Ladder-sequencing" [77] and "ladder-synthesis" [78] are the two common MS decoding methods for OBOC peptide libraries. The major disadvantage of the "ladder-synthesis" method is the presence of peptide ladders on the bead surface, which interferes with biological screening. The "ladder-sequencing" method is restricted to libraries with sequenceable peptides or peptoids (*N*-substituted oligoglycine). Recently, we reported on the application of both "ladder-synthesis" and bilayer bead concepts to encode OBOC nonsequenceable peptide and peptidomimetic libraries.[75] Figure 4 shows the general synthetic scheme and MS-based decoding strategy of such peptide libraries. Prior to library synthesis, we assembled a cleavable linker (CL) comprised of methionine (cleavage site by CNBr), arginine (for easily protonation), 3-(4-bromophenyl)-β-alanine (to generate a characteristic isotopic doublet for mass peaks), and 2,2′-ethylenedioxy-bis(ethylamine) monosuccinamide (Ebes, as a spacer, for improved solubility, and to produce a big mass shift for each compound beyond the noise region of matrix ions). During library construction, the PAD bilayer step was employed prior to each Fmoc–amino acid coupling cycle. As a result, we were able to remove the Alloc protecting group, layer by layer, from the bead surface to the bead interior. By the end of library construction, each peptide bead carried a complete library compound ($X_5X_4X_3X_2X_1$) on the outer layer, and four truncated ladder members ($X_5X_4X_3X_2$, $X_5X_4X_3$, X_5X_4, and X_5) in the bead interior. The advantages of the newly developed "ladder-synthesis" approach include that (i) only a single building block is used for coupling during each coupling step, therefore eliminating the problems caused by the differential coupling rates of two different building blocks (as in standard "ladder-synthesis" method); (ii) this method can apply to peptides comprising of both sequenceable and non-sequenceable building blocks; (iii) the undesirable interference of ladder tags with the biological screening can be avoided; and (iv) the library beads generated by this method are amenable to both MALDI-TOF MS and Edman microsequencing if the compound is a sequenceable peptide.

From our experience in using OBOC combinatorial libraries, even weak ligands can be readily identified because of a high local concentration of ligands on the surface of the TentaGel bead (~100 mM). Therefore, to identify high affinity compounds from focused libraries, we increase the

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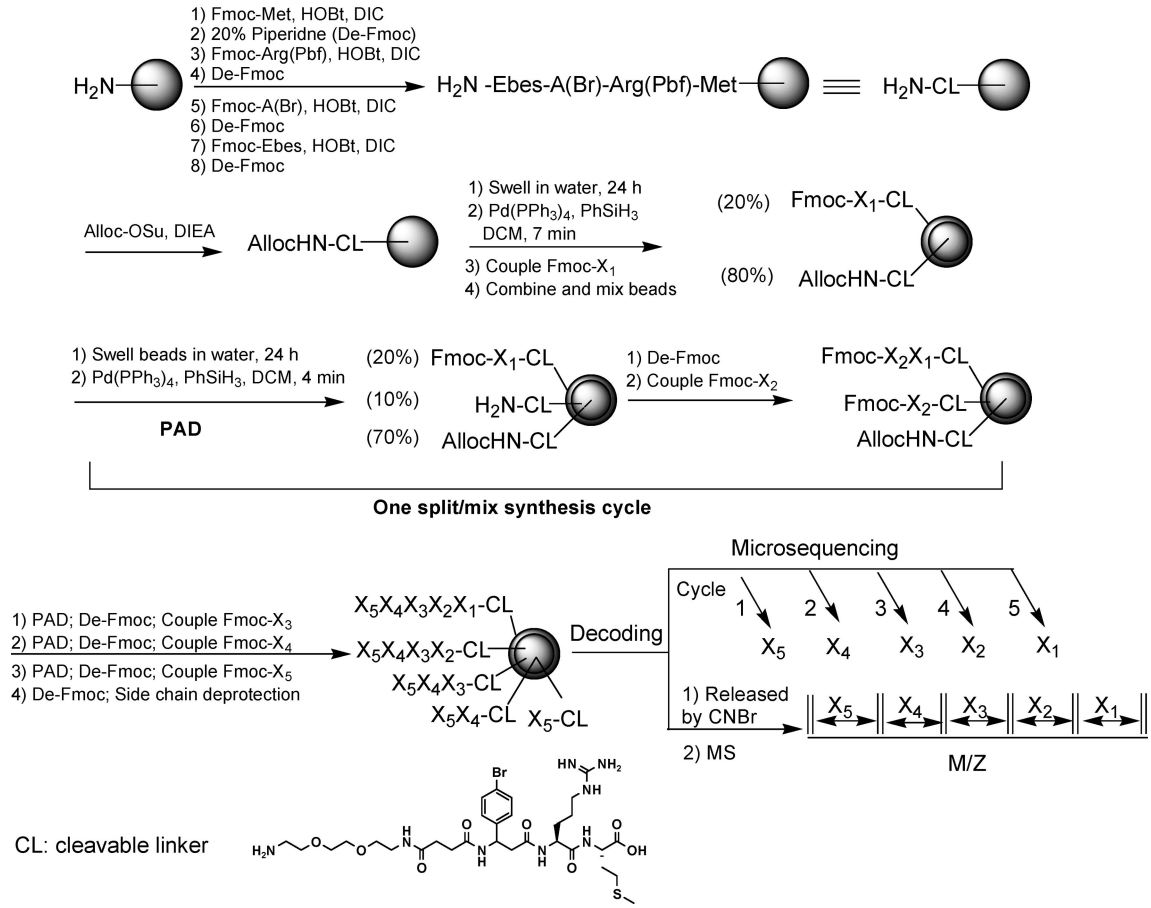

Figure 4. Synthesis of MS-encoded OBOC peptide library using newly developed ladder-synthesis method. Met, L-methionine. Arg, L-arginine. Pbf, 2,2,4,6,7-pentamethyldihydro-benzofuran-5-sulfonyl. A(Br), 3-(4-bromophenyl)-β-alanine. Ebes, 2,2′-ethylenedioxy-bis(ethylamine) monosuccinamide.

stringency of the screening conditions by lowering the concentration of target protein (probe) or incorporating soluble competing ligands in the screening buffer.[72] An alternative approach is to lower the concentration of ligands on the bead surface by blocking a predetermined portion of the amino group within the bead. However, by doing so, there will be an insufficient quantity of peptide or coding tags left in each bead for structure determination. By taking advantage of bilayer beads, we can prepare OBOC combinatorial libraries that are down-substituted on the bead surface but fully substituted in the bead-interior. This configuration enables one to screen the library at a much higher stringency and yet retain full (100%) substitution in the bead interior for structure decoding (see below).[79]

OBOC Library Screening for Cell Surface Ligands. We have developed a screening method in which living cells are incubated with a library of beads to identify compounds or ligands that bind to cell-surface receptors. This technique can be applied to both suspension and adherent cell cultures, as well as fresh cancer cells isolated from patient blood, ascitic fluid, pleural fluid, or biopsy specimens. The screening process begins with extensive washing of the bead library followed by harvesting and washing of the cells. Trypsin can be used to strip the adherent cells off the culture flask. However, it is important to minimize the exposure time of the cells to the enzyme in order to avoid damaging some cell surface receptors that are required for ligand binding. The cells are then incubated with the beads in tissue-culture-treated plates under favorable growth conditions. Gentle agitation is sometimes necessary to increase the "contact time" between beads and cells. Beads with the appropriate ligands that bind to the cell surface receptors will be coated by a monolayer of cells and considered as "hits", as shown in Figure 5. Such beads can be identified and isolated under a microscope with a hand-held micropipette. Because there are hundreds of different macromolecules on the cell surface and more than 100 000 distinct compound beads are screened, it is expected that some of the hits are due to nonspecific binding. We have developed two "subtraction screening" methods to eliminate such false positive beads. In the first method, we first screen the OBOC library with cancer cells, isolate the beads coated with cancer cells, and then recycle the bead by stripping the cells off the bead with 8 M guanidium chloride followed by thorough washing. The recycled beads are then tested for binding with normal cells. Those beads that bind to both cancer and normal cell types

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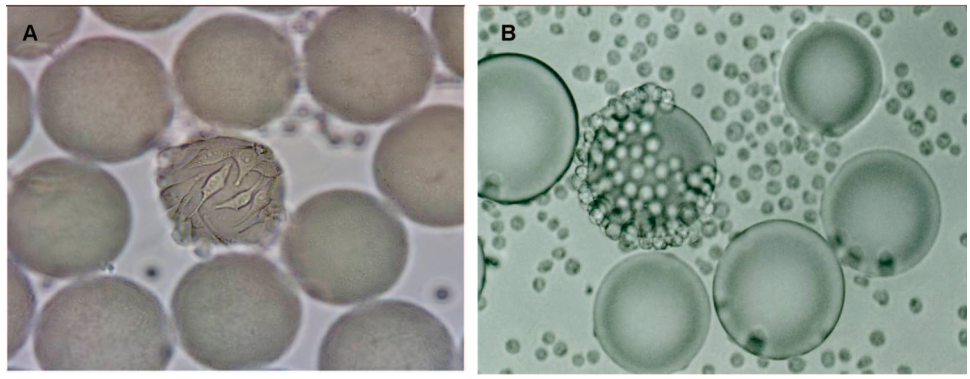

Figure 5. Photomicrographs of a positive bead ("hit") identified from screening random OBOC combinatorial peptide libraries with (A) adherent cancer cells and (B) nonadherent cancer cells.

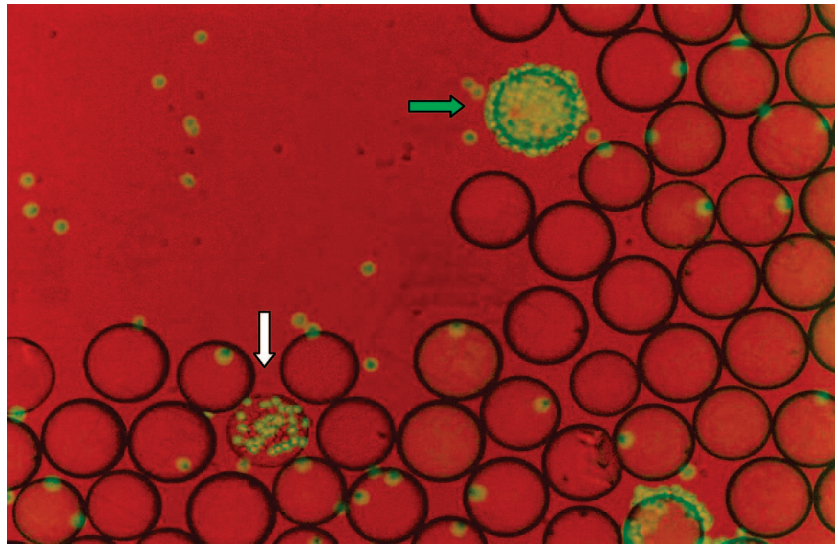

Figure 6. Photomicrograph of "dual-color screening method". Green arrow points to a bead that bound only the fluorescent labeled cells ("true positive"); white arrow points to a bead that bound both unlabeled "control" cells and labeled cells.

are considered false positive. The second method is called a dual-color screening method which involves tagging the cancer cells with a fluorochrome (e.g., calcein AM) and mixing them with unlabeled control cells prior to screening the bead-library. Those beads that only bind to the fluorescent cells are considered "true positive" beads (Figure 6). In addition to assaying for cell binding ligands, one may also screen OBOC libraries for both cell attachment and cell function. In our earlier work, we noticed that in some beads, the bound cells remained round, whereas in other beads, the cell attached and then flattened out on the surface of the beads. Besides screening for specific cell morphological changes, we have also screened for peptides that induce specific cell function such as cell signaling or apoptosis. For example, we may use a GFP transfected cell line in which GFP will be expressed upon activation of a specific cell signaling pathway. For the discovery of pro-apoptotic peptides, we may add caspase-3 fluorescent substrate to the bead library to identify beads that are coated with cells undergoing apoptosis.

Cancer Cell Surface Targeting Ligands Identified by the OBOC Method. In the last few years, we have reported the use of the whole-cell bead binding assay to identify cell surface ligands against a number of different cancer cell lines, including both adherent and nonadherent cells. We have also performed experiments on fresh cancer cells isolated from patients. Some of the peptide libraries consist of all L-amino acids, but the majority of our work was performed on cyclic peptide libraries containing both L- and D-amino acids. We have also screened all D-amino acid peptide libraries. The surface of a living cell is generally negatively charged due to the sialic acids and phospholipid head groups. Therefore, polylysine has been commonly used to immobilize living cells. To avoid nonspecific anionic–cationic interactions between the cells and the peptide-bead surface, we purposely lower the amount of basic amino acids (lysine and arginine) in our library construction. We generally do not eliminate these basic residues totally because a basic residue may be required for binding. This is certainly true for the RGD motif for $\alpha v\beta 3$ integrin. Upon further optimization with focused libraries, we have found that unnatural amino acid substitu-

Table 2. Cancer Targeting Ligands Identified by OBOC Combinatorial Library Methods

ligands to specific tumor cell surface receptors	sequence	*in vivo* imaging
WEHI-231 murine lymphoma cell line[7,146]	XWY(D/T/V), wGeyixvx, lwxxpewi, kwxGpxw	
WEHI-279 murine lymphoma cell line[7,146]	RWID, RWFD, xtxGmxkx, xGrfxswx	
α3-integrin ovarian adenocarcinoma (CaOV-3, ES-2, SKOV-3, OVCAR-3); glioblastoma (A172) and metastatic breast cancer (MDA-MB 231)[83]	c(d/D)GXGXXc	82
α3-integrin NSCLC[147]	cNGXGXXc	
α4β1 integrin Jurkat T-cell lymphoma[7,148]	XXXpLD(I/F/V), xLDFpXXX, cXLD(I/V/F)c, cXXLDIc, cLDIXXc, cWDXXXc, xxxxp-Nle-DIxxxx	
α4β1 integrin Raji B-cell lymphoma	cLDYWDc, cWDLDHHc, sppLDIn, eapLDId, fypLDFf, FSIpLDI, QSYpLDF	
α4β1 integrin Jurkat T-cell lymphoma[72]	"LLP2A" peptidomimetic and a series of analogues	72, 149
α4β1 integrin ES-2 ovarian adenocarcinoma	c-Nle-DWEEc, c-Nle-DVDEc, c-Nle-D-Chg-YMc, cSD-Nle-D-Chg-c, yminp-Nle-DIdnhh, vswap-Nle-DIgspd, vqgp-Nle-DIafvl, vgnvp-Nle-DIgqea, wdinp-Nle-DIgsfn, wsrip-Nle-DIqeps	
α4β1 integrin SKOV-3 ovarian adenocarcinoma	cLDI-Chg-Hyp-Yc, c-Nle-D-Chg-NDFc, c-Nle-D-Nle-PhgDc, cDEL-Nle-EWc	
α6β1 integrin DU145 prostate cancer cell line[150–151]	LNNIVSVNGRHX, DNRIRLQAKXX, kmviywkaG, kGGrhykfG, yiknrkhhG, kikmviswkG	

tion and/or addition of organic moieties often lead to the development of ligands with higher affinity and specificity. The results of OBOC peptide library screening performed in our laboratory are summarized in Table 2. Some of the ligands identified have interesting biological properties other than cell binding. For example, wGeyidk peptide, in tetrameric form, was able to induce specific tyrosine phosphorylation of a few cellular proteins when attached to the WEHI-231 murine lymphoma cell surface. The hybrid peptide kikmviswkG (HYD-1), based on overlapping sequences of some of the positive peptides identified for DU145 prostate cancer cell, was able to block the attachment of DU145 cells to anti-β1 antibody, fibronectin, laminin 1, laminin 5, and collagen IV. In addition, it also inhibited the tumor cell attachment to immobilized dermal fibroblast.[80] Very recently, we have performed a structure–function relationship of this hybrid peptide on PC3N prostate cancer cells, and determined that the most potent element of HYD1 necessary to support cell adhesion was kmvixw, to block cell migration, it was xkmviswxx and to inhibit ERK cell signaling, ikmviswxx was required.[81]

We recently reported on the use of a highly focused peptidomimetic library (based on the preferred building block in each residue) and high stringency screening to identify a high-affinity peptidomimetic ligand ($IC_{50} = 2$ pM) that target activated α4β1 of T- and B-lymphoma xenografts with high sensitivities and specificities.[72] The chemical structure of this novel targeting agent, LLP2A, is shown in Figure 7A. Because there is an organic moiety at the *N*-terminus and the tripeptide entirely consists of unnatural amino acids, the molecule is highly resistant to proteolysis because there was no sign of degradation in human plasma when incubated at 37 °C for 18 days. Using similar methods, we have developed a D-amino acid containing cyclic octa-peptide ligand (OA02) against the α3β1 integrin of ovarian cancer. This ligand (Figure 7B), when conjugated to Cy5.5 or Alexa680 near infrared fluorescent dye, was able to image α3 integrin expressed in ovarian adenocarcinoma xenografts in nude mice.[82]

Use of OBOC Combinatorial Library Approaches To Identify and Optimize Ovarian Cancer Targeting Peptides

Identification of Ovarian Cancer Targeting Ligands through OBOC Random Cyclic Peptide Library. To identify novel peptide ligands against ovarian cancer cell surface receptors, we used SKOV-3 ovarian adenocarcinoma cell line to screen four random OBOC cyclic peptide libraries (cX_6c, cX_7c, $cZ_{4-8}c$, and $c(U/Z)_{5-7}$ c); wherein X = 19 L-amino acids except cysteine, c = D-cysteine, U = 8 unnatural amino acids, 'Z = 17 L-amino acids (no arginine (R), cysteine (C), or lysine (K)). On the basis of the common motif identified from these random libraries, we synthesized and screened four focused cyclic libraries: c(N/D)G (U/X)(U/Z)(U/Z)(U/Z)c, cNGXGXZc, and $cX^*_1GX^*_3GX^*_5X^*_6c$, wherein X* = 42 amino acids (mostly unnatural), N = L-asparagine, D = L-aspartic acid, G = glycine). Peptide bead

(80) DeRoock, I. B.; Pennington, M. E.; Sroka, T. C.; Lam, R. S.; Bowden, G. T.; Bair, E. L.; Cress, A. E. Synthetic peptides inhibit adhesion of human tumor cells to extracellular matrix proteins. *Cancer Res.* **2001**, *61*, 3308–3313.

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(83) Aina, O. H.; Marik, J.; Liu, R.; Lau, D. H.; Lam, K. S. Identification of novel targeting peptides for human ovarian cancer cells using "one-bead one-compound" combinatorial libraries. *Mol. Cancer Ther.* **2005**, *4*, 806–13.

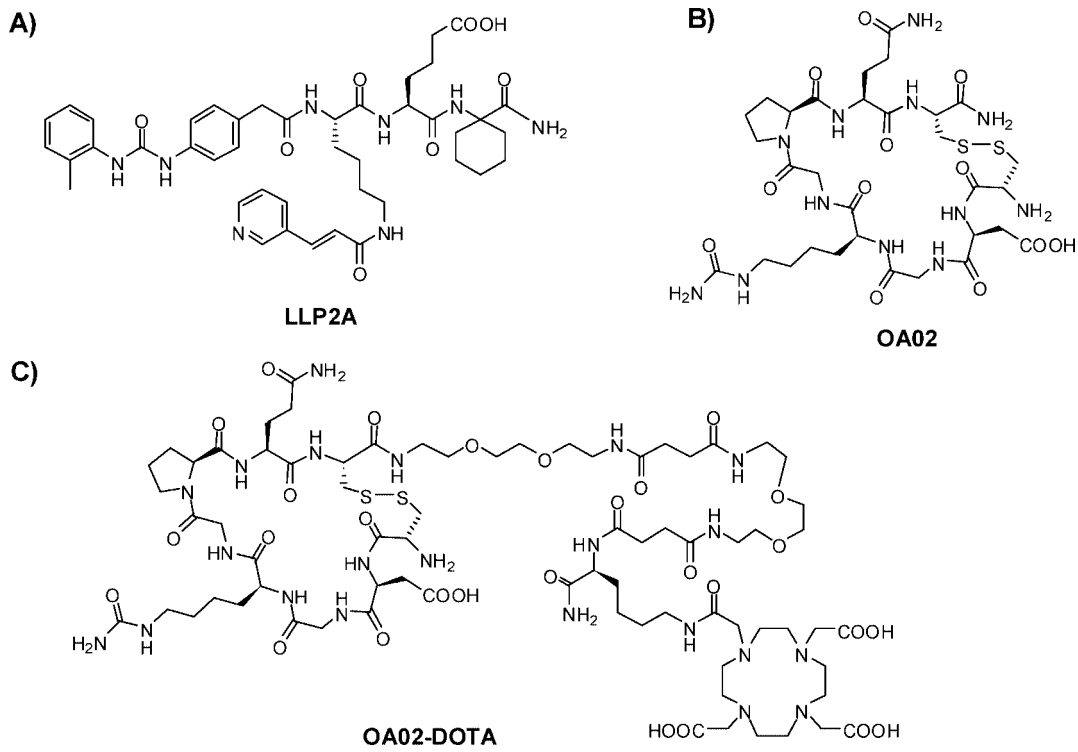

Figure 7. Chemical structures of selected cancer-targeting ligands.

libraries were incubated with ovarian adenocarcinoma cell lines as previously described and beads covered with single or multiple layers of cells were isolated. Once the peptide bead sequences were determined, they were resynthesized and further characterized. Structure–activity relationship studies (e.g. alanine-walk) were performed on three of the isolated peptides to determine which amino acid residues are crucial for receptor–ligand interaction. Molecular modeling was also performed to predict the secondary structure of these peptides. NMR measurements of four representative cXGXGXXc cyclic peptides supported the molecular modelling results. The calculations predicted a β-turn-like structure at the *N*-terminal end of the molecule involving the first four amino acids cXGX, whereas conformation of the *C*-terminal end appeared to be more variable.[83]

Optimization of Ovarian Cancer Targeting Ligands. By using focused OBOC peptide libraries containing unnatural amino acids as well as more stringent library screening methods, we identified several novel cyclic peptide ligands with the following motif: (cdG [Cha/Chg/Phe/Tyr/Nle/Cit/HCit]G[HCit/Hyp/Phe]X-c). Sequences containing unnatural amino acids and D-isomers identified from the "focused" peptide bead library screening are shown in Table 3. In our primary (random, all L-isomer peptide) library screening, cell binding to 100% surface-substituted beads was observed usually after 6–24 h, about 0.01% of the total beads screened were positive with 15–20 cells bound per bead. Using the 100 and 20% surface-substituted "focused" $cX_1GX_3GX_5X_6c$ libraries described here, cell binding was observed within 30 min with 25–35 cells bound per bead in all the ovarian cancer cell lines screened. From a sample of 200 μL (~150 000 beads) of the focused-library beads screened (24 h post incubation), an average of 60 beads total (0.04%) were positive for all cell lines tested. We selected seven representative peptides from the new motifs identified, resynthesized these peptides at 100 and 10% surface substitution on TentaGel beads, and tested binding to a panel of tumor as well as "normal" cell lines (SKOV-3 ovarian cancer, ES-2 ovarian cancer, Raji lymphoma, U118MG glioblastoma, K562 promylecytic leukemia, Jurkat lymphoma, MDA-MB 231 mammary adenocarcinoma, N87 gastric carcinoma, SKMEL-3 melanoma, A2058 melanoma, and IMR-90 normal human lung epithelium). The results are shown in Table 4. The data shows that ovarian adenocarcinomas as well as the U118MG glioblastoma, A2058 melanoma, and MDA-MB231 breast cancer cell lines bind very strongly to all of the peptides. Results shown are after 24 h incubation with the peptide beads. The glioblastoma and A2058 melanoma cells seem to bind more strongly than the ovarian cancer cells to the surface down-substituted beads (10% substitution). Jurkat and Raji lymphoma cells showed no binding to the beads, and N87 gastric showed weak binding. IMR-90 lung epithelial cell binds preferentially to three of the seven peptides.

It is interesting to note that in Table 3, there was a bias towards the dGMG motif by CaOV-3 ovarian cancer cells. This has led us to believe that although these peptides bind to the same integrin receptor, there may be subtle differences in the integrin conformation or activation state among different normal and cancer cell lines. It has been suggested that the differences may be due to glycosylation patterns of the receptor, which is one of the conclusions from the article by Lee *et al.*[57]

Table 3. Seven Distinct Motifs Identified from Screening 100% Substitution[a] and 20% Substitution Focused cXGXGXXc OBOC Combinatorial Peptide Libraries with Ovarian Cancer Cell Lines, CaOV-3, ES-2, OVCAR-3, and SKOV-3; Peptide Cyclization via the –SH Groups of the Flanking D-Cysteines

motif 1	motif 3	motif 5	motif 6
cdG-Cha-G-Chg-Qc	cdG-Phe[1]-GQ-Phe[4]-c	cdG-Tyr[1]-GTMc[1]	cdGLGWGc
cdG-Cha-G-HCit-Qc	cdG-Phe[1]-GTYc	cdG-Tyr[2]-G-Aic-Qc	cdGLGQ-Bta-c
cdG-Cha-G-HCit-Sc[a]	cdG-Phe[2]-G-Aib-Sc	cdG-Tyr[2]-G-Phe[4]-Lc	cdGLG-Phe[4]-Tc
cdG-Cha-GITc[a]	cdG-Phe[2]-GP-Cha-c[a]	cdG-Tyr[2]-GI-Pra-c	cdG-Nle-GIAc
cDG-Cha-G-Hyp-Nc	cdG-Phe[2]-GT-Pra-c[a]	cdG-Tyr[2]-G-Phg-Mc	cdG-Nle-G-Phe[1]-Phe[4]-c
		cdG-Tyr[2]-G-Pra-Gc[a]	cdGIGPQc[a]
		cdG-Tyr[2]-G-Nle-Gc	

motif 2	motif 4 (CaOV-3 only)	motif 7
cdG-Chg-G-Hyp-Nc	cdGMG-HoSer-Nle-c	cdG-Cit-G-Phe[3]-Mc
cdG-Chg-G-Hyp-Yc	cdGMGS-Cha-c	cdG-HCit-GPQc**
	cdGMGSGc	cdG-HCit-GT-Nva-c

[a] Sequences identified from the 100% surface substitution library; all others are from the 20% surface substitution library. Single-letter representation for amino acids is according to standard convention, except for those amino acids without single letter representation: Bta = benzothienylalanine, Cha = cyclohexylalanine, Chg = α-cyclohexylglycine, HCit = homocitrulline, Hyp = hydroxyproline, Phe[1] = 3-chlorophenylalanine, Phe[2] = 4-methylphenylalanine, Phe[3] = 3,4-di chlorophenylalanine, Phe[4] = 4-nitrophenylalanine, Aib = α-aminoisobutyric acid, Pra = propargylglycine, HoSer = homoserine, Tyr[1] = 4-methyltyrosine or 4-methoxyphenylalanine, Tyr[2] = 3-nitrotyrosine, Aic = 2-aminoindane-2-carboxylic acid, Phg = Phenylglycine, Nle = norleucine, Cit = Citrulline, Nva = Norvaline, ** = "OA02".

Table 4. Binding Specificities of Seven Representative Peptides from Each Identified Motif (from Table 3) Tested against a Panel of Cancer Cell Lines and Normal Lung Epithelial Cell[a]

peptide	%	SKOV-3	ES-2	Raji	K562	Jurkat	U118-MG	MDA-MB 231	N87	SKMEL-3	A2058	IMR-90
cdG-Cha-G-HCit-Qc	100	++++	++++	−	−	−	++++	++++	++	−	++++	++++
cdG-Chg-G-Hyp-Nc	100	++++	++++	−	−	−	++++	++++	++	−	++++	++++
cdG-HCit-GPQc	10	++	++	−	−	−	++++	++++	+	−	++++	++
cdGIGPQc	100	++++	++++	−	−	−	++++	++++	+	−	++++	+
cdGLGQ-Bta-c	100	++++	++++	−	−	−	++++	++++	++	+	++++	++++
cdG-Phe[2]-GP-Cha-c	10	++	++	−	+	−	+++	++++	+	+++	++++	−
cdG-Tyr[2]-GI-Pra-c	100	++++	++++	−	−	−	++++	++++	++	−	++++	−

[a] Semiquantitative relative binding activity: ++++ = very strong binding, +++ = strong binding, ++ = moderate binding, + = weak binding, − = no binding; % denotes surface substitution of peptide on the bead surface. (SKOV-3 ovarian, ES-2 ovarian, Raji lymphoma, K562 myeloma, Jurkat lymphoma, U118MG glioblastoma, MDA-MB 231 mammary adenocarcinoma, N87 gastric carcinoma, SKMEL-3 melanoma, A2058 melanoma, and IMR-90 normal human lung epithelium).

In Vivo PET Imaging of Ovarian Cancer in Xenograft Model Using the Optimized Ovarian Cancer Targeting Ligand

***In Vivo* Imaging with Peptides.** ^{111}In-labeled octreotide, a cyclic peptide against somatostatin receptor, has been used for clinical imaging of neuroendocrine tumors for many years. Clinical evaluation of ^{99m}Tc-RGD as an imaging agent to detect malignant melanoma in patients diagnosed with metastatic disease showed high rates of detecting nodal metastasis.[84] In the last decade, many investigators have begun to evaluate the *in vivo* imaging potential of various cancer targeting peptides in xenograft model with a number of different imaging modalities: optical, PET, SPECT, and MRI. Some investigators used known peptide hormone analogues. For example, Becker *et al.* reported the use of octereotide-near-infrared fluorescent dye conjugate and optical imaging to image pancreatic tumors.[85] Many investigators use the well-known cyclic RGD peptides that target $\alpha v\beta 3$ as the imaging probes.[86–89] Conti's group in 2004[90] dem-

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onstrated that RGD-Cy5.5 conjugate could be used to image $\alpha v\beta 3$ integrin expressing glioblastoma xenografts. Many reports have since followed on optical imaging of $\alpha v\beta 3$ integrin and pharmacokinetics studies of RGD multimers and analogues in various xenograft models.[91–95] In a recent article by Liu *et al.*, ^{99m}Tc-labeled RGD peptide was used for SPECT imaging of $\alpha v\beta 3$ expressing MDA-MB-435 breast cancer xenografts in nude mice.[89] The images and biodistribution data from these experiments showed improved uptake of the tetrameric RGD ligand in comparison to the monomer or dimeric analogues. Others have used ligands identified through phage-display peptide libraries. For example, Weissleder et al. reported on the use of an $\alpha 5\beta 1$ binding peptide "RPMC"-Cy5.5 conjugate for the imaging of orthotopically transplanted HT29 colon tumors in a mouse model.[96]

PET Imaging with Ovarian Cancer Targeting Peptide. With the advent of small animal PET imaging technology, major strides have been made in development of PET reporter probes that enable research of biological processes.[97] PET imaging has been used successfully for detection and staging of many cancers in humans.[97,98] Small animal PET imaging technologies enable us to (i) evaluate novel imaging agents, (ii) perform sequential studies in the same animal (monitor therapeutic response), and (iii) use far fewer animals than required for traditional biodistribution studies. Radiolabeled peptides have been used as PET reporter probes. For example, RGD peptides that target the $\alpha v\beta 3$ integrin (in monomeric, dimeric or tetrameric form; pegylated or glycosylated; conjugated to ^{18}F or ^{64}Cu) have been used for PET imaging of nonsmall cell lung cancer, glioblastoma, melanoma, and breast cancer xenografts.[88,99–103] Natural peptides, e.g., somatostatin and bombesin, have also been used in PET imaging of receptor overexpressing tumors.[104–106] The use of radiolabeled multimeric RGD ligands was recently reviewed by Liu.[107] We have developed the "OA02" peptide into a PET imaging probe and evaluated its potential use in *in vivo* PET imaging of ES-2 ovarian carcinoma xenografts. This work will be briefly discussed below.

Synthesis and ^{64}Cu Loading of Cyclic cdG-HCit-GPQc-Ebes-Ebes-K(DOTA)-NH$_2$ (OA02-DOTA). The chemical structure of OA02-DOTA is shown in Figure 7C. Standard Fmoc chemistry was used for solid phase synthesis of cdG-HCit-GPQc-Ebes-Ebes-K (Alloc) on Rink amide

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(102) Chen, X.; Park, R.; Hou, Y.; Khankaldyyan, V.; Gonzales-Gomez, I.; Tohme, M.; Bading, J. R.; Laug, W. E.; Conti, P. S. MicroPET imaging of brain tumor angiogenesis with 18F-labeled PEGylated RGD peptide. *Eur. J. Nucl. Med. Mol. Imaging* **2004**, *31*, 1081–9.

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(107) Liu, S. Radiolabeled multimeric cyclic RGD peptides as integrin alphavbeta3 targeted radiotracers for tumor imaging. *Mol. Pharmaceutics* **2006**, *3*, 472–87.

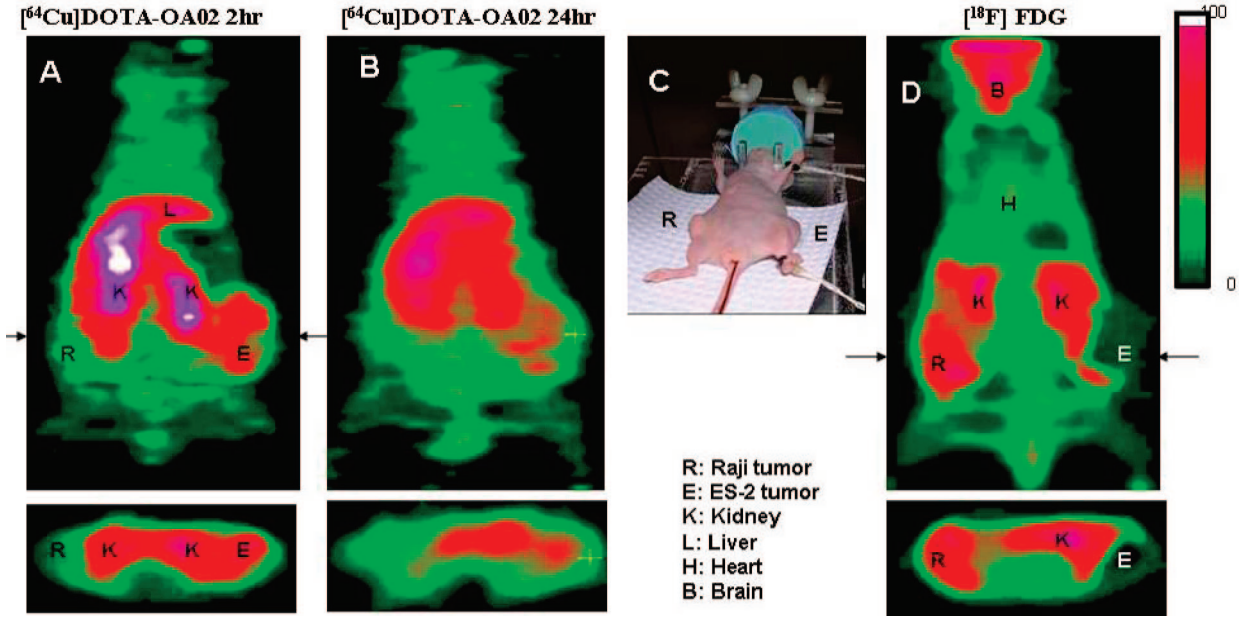

Figure 8. Coronal and sagittal sections of MAP reconstructed MicroPET images of ovarian cancer (ES-2) and lymphoma (Raji) bearing mouse. (A) An image of the mouse two hours postinjection with [^{64}Cu] DOTA-OA02 showing high signal in the ES-2 tumor; (B) An image taken 24 h after injection showing some signal still in the ES-2 tumor; (C) Picture of an anesthetized nude mouse on the imaging bed showing both tumors and (D) Image of the same animal injected with [^{18}F] FDG 24hours after the initial [^{64}Cu] DOTA-OA02 injection showing high signal in the Raji tumor and low signal in the ES-2 tumor.

resin. The hydrophilic linker Fmoc-Ebes-OH was synthesized as previously described.[108] The Alloc protecting group was removed from the lysine side chain with tetrakistriphenylphosphine palladium, and DOTA-NHS ester was coupled to the lysine. Side-chain protecting groups of amino acids were removed with a trifluoroacetic acid:triisopropylsilane: 1, 2-ethanedithiol:water mixture. The peptide was then cyclized via the flanking D-cysteines to form a disulfide bond. The compound was lyophilized, purified, and analyzed with HPLC, and verified with MALDI-TOF mass spectrometer. ^{64}Cu (5.0–10 mCi; specific activity 20–200 mCi/μg [109]) was incubated with OA02-DOTA (0.5–1 mg) in ammonium acetate, pH 7, at 40 °C for 60 min. Ethylenediamine tetraacetic acid (EDTA) was added to scavenge nonspecifically bound ^{64}Cu. The product was purified and evaluated by TLC.

MicroPET Imaging of Ovarian Cancer Xenografts with OA02-DOTA-[^{64}Cu]. MicroPET images acquired at 15 min showed radioactivity in the kidneys and bladder. Figure 8 shows a representative maximum a posteriori (MAP) reconstructed image of an ovarian cancer bearing mouse two hours post injection with [^{64}Cu] DOTA-OA02 (Figure 8A). It shows high radioactivity in the ES-2 tumor, kidney, and liver. No radioactivity was observed in the negative control Raji tumor in all the mice scanned. The 24 h post injection scans showed residual radioactivity at liver, kidney, and ES-2 tumor (Figure 8B). A picture of the mouse on the scanner bed shows both Raji lymphoma and ES-2 ovarian tumor xenografts (Figure 8C). [^{18}F] FDG scans of all the animals showed high activity in the Raji tumor, kidney, bladder, brain and heart; with moderate activity in the bone, muscle and brown fat (Figure 8D). Very little radioactivity was seen in the ES-2 tumors. In one experiment, a mouse developed a small metastatic lesion at the right brachial lymph node after i.p. injection with SKOV3 ovarian cancer cells (Figure 9A). Interestingly, this lesion was easily detected with [^{64}Cu] DOTA-OA02 as the imaging probe (Figure 9B), but not with the [^{18}F] FDG scans (Figure 9C). Upon histological examination, there were signs of nodal invasion with tumor cells.

Cell Surface Ligand as Capturing Agent for Circulating Cancer Cells

We have previously reported the use of polystyrene microbeads coated with ovarian cancer targeting ligand to isolate OVCAR-3 ovarian cancer cells from whole blood that had been spiked with these cells.[83] More recently, we demonstrated that LLP2A, the high affinity lymphoma targeting ligand,[72] when coated on beads, can be used as a tool to isolate lymphoma cells from whole blood. In this experiment, Jurkat T-lymphoma cells were first labeled with 10 μM calcein AM (Becton Dickinson, Bedford, MA) for one hour at 37 °C. The labeled Jurkat cells were then washed and resuspended in culture medium. Peripheral blood mono-

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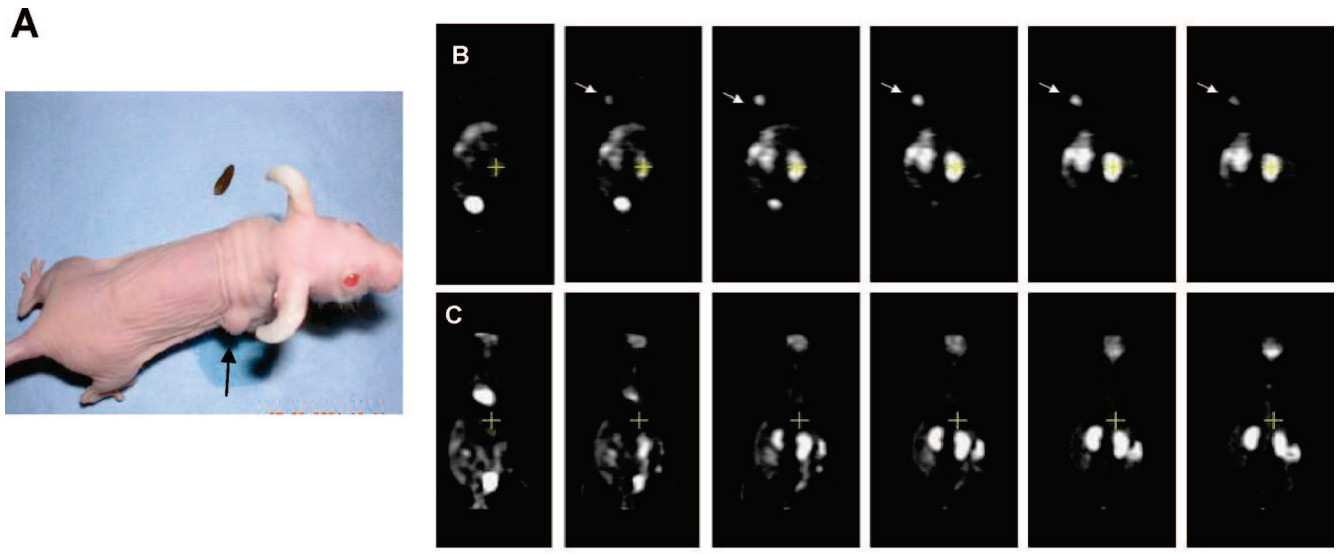

Figure 9. (A) Ovarian cancer xenograft bearing mouse (i.p. injection with SKOV-3) with metastastatic lesion to the right brachial lymph node (arrow). (B) Coronal sections of MAP reconstructed MicroPET images of the mouse after i.v. injection with [^{64}Cu] DOTA-OA02 cyclic peptide, with good radio-uptake to the right brachial lymph node. (C) Same mouse was imaged with [^{18}F] FDG 24 h after the [^{64}Cu] scan, with obvious lack of radio-uptake in the brachial lymph node, but high uptake in brain and heart.

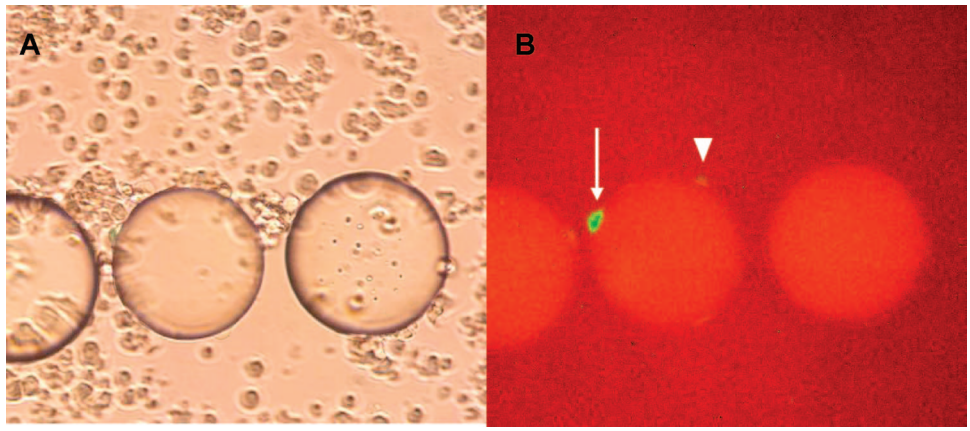

Figure 10. An LLP2A-coated bead captured Jurkat cells among a mixture of Jurkat cells and PBMC cells at 1:100 000 dilution. Jurkat cells were first labeled with calcein AM to make them appear green under fluorescence microscope. Calcein-labeled Jurkat cells were mixed with PBMC at 1:100 000 dilutions. LLP2A beads were added, incubated with the cell suspension for one hour with gentle shaking, and examined under a fluorescent microscope, with (A) full background white light and (B) little background light. Two Jurkat cells (white arrow and arrowhead) bound to the middle bead appeared green while the background PBMC at the bottom of the plate did not have any fluorescence. One fluorescent cell (white arrowhead) is out of focus and behind the bead.

nuclear cells (PBMC) were obtained from peripheral blood of healthy volunteers using Ficoll-hypaque density centrifugation method. Calcein AM-labeled Jurkat cells were mixed with unlabeled PBMC at varying ratios (1:10 to 1:1 $\times$ 10^6). The cell mixture (1×10^7 cells) was then incubated in a 60 mm culture dish with approximately 1500 LLP2A-coated beads (LLP2A synthesized on 90 μm TentaGel beads), shaken gently for 1 h at room temperature and examined under a fluorescence microscope. We found that calcein AM-labeled and unlabeled Jurkat cells bound to LLP2A beads equally well, but calcein-labeled and unlabeled PBMC did not. Furthermore, we were able to demonstrate that LLP2A-coated beads could be used to retrieve Jurkat cells from a PBMC preparation that had been spiked with a small number of Jurkat cells (1:1 $\times$ 10^5 Jurkat:PBMC ratio) (Figure 10).

Therapeutic Targeting

As indicated in the Introduction, peptides, peptidomimetics, or small molecules represent excellent alternative cancer-targeting agents, besides monoclonal antibodies, to deliver radioactive or cytotoxic payloads to the tumor. The final ligand-drug could be formulated at a small monomeric form, an intermediate size oligomeric form, or a large polymeric

form. The payload could be small, such as a radionuclide (e.g., ^{131}I), a radiometal chelate (e.g., [^{90}Y]-labeled DOTA), or a doxorubicin molecule, or it could be larger, such as a drug-loaded liposome or drug-loaded nanoparticle. Most investigators use one single kind of ligand as a delivery vehicle, but the incorporation of multiple different ligands into the same drug conjugate may increase tumor targeting specificity and affinity.

The $\alpha v\beta 3$ integrin (overexpressed in neovascular endothelial cells) is a popular target for cancer-targeting studies. EMD 121974 (Cilengitide), a cyclic RGD peptide have been used in clinical trials for glioblastoma and other cancers.[110,111] In recent preclinical studies on vascular targeting of peptides, NGR and RGD conjugated to doxorubicin have been shown to decrease tumor burden, reduce toxicity, as well as enhance the efficacy of Doxil in human breast cancer xenografts.[63,112,113] More recently, dimeric RGD peptide conjugated to Paclitaxel has also been used to evaluate therapeutic activity in a metastatic model of breast cancer.[114]

As mentioned earlier, peptides identified from screening lung cancer cells H1299 and H2009 have been conjugated to doxorubicin for therapeutic targeting.[61] The results from this study show good potential of specific therapeutic targeting of specific tumor cells. In doxorubicin-H1299.1-treated cells, H1299 cells showed the largest decrease in viability. The same was the case for the H2009.1 peptide, which targeted H1648 cells. The most encouraging result from this study is that normal lung fibroblasts, which were very sensitive to treatment with free doxorubicin, were unaffected by the doxorubicin-peptide conjugates. Another novel peptide identified from phage library screening of nasopharyngeal carcinoma, "RLLDTNRPLLPY", has been used in experimental targeting of doxorubicin in SCID mouse xenograft models of this tumor.[115] In this study, a significant decrease in tumor burden was observed in mice treated with the peptide-Lipo-dox when compared to treated control mice.

Perspective

Cancer-specific cell surface ligands identified from combinatorial libraries can be used for drug delivery, imaging, or diagnostics. These ligands are also useful for cell adhesion and cell signaling studies and as substrates for tissue engineering. A good portion of the peptides identified through screening random combinatorial libraries (phage-display or OBOC methods) are integrin-binding ligands. This is not surprising as integrins tend to bind to short sequence motifs and they are abundant on the cell surface. Through screening random OBOC peptide libraries, we have also identified

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many ligands that do not bind to integrins; however, the identity of many of their targets remains undetermined. One way to identify the cell surface receptor to which these ligands bind is to evaluate the ability of a series of antibodies against various cell surface proteins to inhibit cell binding to the peptide-bead. An alternative method is to use the ligands to prepare photo- or chemical-affinity probes so that the receptors can be tagged and subsequently identified with mass spectrometry.

In addition to facilitating lead compound identification, combinatorial chemistry can be used to rapidly optimize the initial lead molecules (identified from library screening) or compounds that are known to bind to the receptor. On the basis of the structural information of these lead compounds, highly focused chemical libraries can be designed and screened under high stringency conditions. These approaches often lead to the discovery of ligands with higher affinity. Binding specificity can also be built into the screening steps, such as dual-color screening (Figure 6), in order to eliminate binding of ligands to normal cells. Both the phage-display peptide and OBOC combinatorial library methods are distinct, and they each have unique advantages and disadvantages. The phage-display library method affords the screening a larger number of peptides and also longer peptides, whereas the OBOC method allows one to use unnatural amino acids and organic moieties. Although not yet reported in the literature, it is certainly advantageous to combine these two powerful methods in cell surface ligand identification. For example, 15-mer all L-amino acid cancer cell surface ligands identified with the phage-display method can be used as a template to develop focused OBOC libraries that include D-amino acids, unnatural amino acids, and organic moieties for subsequent lead optimization. The screening of phage-display peptide libraries in cancer patients[71] is a bold move and its utility in the identification of useful cancer-targeting peptides against human cancers remains to be proven.

In the past decade, there have been great advances in the molecular imaging field. Cancer cell surface targeting ligands developed through modification of known peptide hormones or identified through screening combinatorial libraries will undoubtedly play an important role as molecular imaging probes in clinical cancer detection and staging in the future. The use of these ligands as vehicles to deliver therapeutic agents or radionuclides to tumors, however, remains problematic. For example, many *in vivo* imaging studies with RGD containing peptides have demonstrated good tumor uptake, but undesirable uptake in liver, kidney, and sometimes GI tract is a concern. For the targeting ligands to be effective as vehicles to deliver cytotoxic drugs, radionulcides, or toxins to the tumor, the ligands' nonspecific uptake into normal organs must be eliminated. There is a need to discover RGD-containing ligands that target only tumor blood vessels and not liver cells. Our recently reported

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LLP2A peptidomimetic ligand for malignant lymphoma targets lymphoma xenografts with high specificity.[72] Even though liver uptake of LLP2A was very low, kidney uptake remains rather high. Some of the solutions to lower renal uptake are to increase the hydration of the animal and to give drugs that interfere with renal uptake of the peptides that have filtered through the glomeruli. Alternative approaches are to develop cleavable linkers (between the peptide and the therapeutic payload) that are labile in the kidney[116] or cleavable on demand, using an exogenous agent.[117] Identification of such cleavable linkers can also be accomplished through combinatorial chemistry.

Abbreviations Used

Alloc = allyloxycarbonyl; Alloc-OSu = *N*-(allyloxycarbonyloxy) succinimide; *t*-Bu = *tert*-butyl; CNBr = cyanogen bromide; ^{64}Cu = copper-64; DCM = dichloromethane; DIC = diisopropylcarbodiimide; DIEA = *N,N*-diisopropylethylamine; DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; Ebes = *N*-(9-fluroenylmethoxycarbonyl)-4-{2-[2-(2-amino-ethoxyl) ethoxy] ethylamino}−4-oxobutanoic acid (*N*-Fmoc-2,2′-(ethylenedioxy)bis(ethylamine) monosuccinamide; EDTA = ethyleneditetraacetic acid; ^{18}F = fluorine-18; FDG = fluorodeoxyglucose; Fmoc = 9-fluorenylmethoxycarbonyl; Fmoc–OSu = *N*-(9-fluorenylmethoxycarbonyloxy) succinimide; HATU = 2-(*1H*-9-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU = 2-(*1H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt = *N*-hydroxybenzotriazole; MALDI-TOF = matrix assisted laser desorption–ionization-time of flight; MRI = magnetic resonance imaging; MS = mass spectroscopy; NIR = near infrared; OBOC = one-bead one-compound; PAD = partial alloc deprotection; PAP = partial alloc protection; PEGA = bis(2-acrylamidoprop-1-yl) poly(ethylene glycol) cross-linked dimethyl acrylamide and mono-2-acrylamidoprop-1-yl [2-aminoprop-1-yl] poly(ethylene glycol); PET = positron emission tomography; PNA = peptide nucleic acid; PyBOP = benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; RGD = arginine–glycine–aspartic acid; SCID = severe combined immuno deficient; SPECT = single photon emission computed tomography.

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