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Design, Synthesis, and Application of OB2C Combinatorial Peptide and Peptidomimetic Libraries

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Abstract

The “one-bead two-compound” (OB2C) combinatorial library is constructed on topologically segregated trifunctional bilayer beads such that each bead has a fixed cell-capturing ligand and a random library compound co-displayed on its surface and a chemical coding tag (bar code) inside the bead. An OB2C library containing thousands to millions of compounds can be synthesized and screened concurrently within a short period of time. When live cells are incubated with such OB2C libraries, every bead will be coated with a monolayer of cells. The cell membranes of the captured cells facing the bead surface are exposed to the library compounds tethered to each bead. A specific biochemical or cellular response can be detected with an appropriate reporter system. The OB2C method enables investigators to rapidly discover synthetic molecules that not only interact with cell-surface receptors but can also stimulate or inhibit downstream cell signaling. To demonstrate this powerful method, one OB2C peptide library and two OB2C peptidomimetic libraries were synthesized and screened against Molt-4 lymphoma cells to discover “death ligands.” Apoptosis of the bead-bound cells was detected with immunocytochemistry using horseradish peroxidase (HRP)-conjugated anti-cleaved caspase-3 antibody and 3,3′-diaminobenzidine as a substrate. Two novel synthetic “death ligands” against Molt-4 cells were discovered using this OB2C library approach.

Keywords

OB2C combinatorial library; Apoptosis; Immunocytochemistry; Cell-capturing ligand; Synthetic “death ligand”; Bilayer bead; Proapoptotic agent; Lymphoma

1 Introduction

In 1991, Dr. Lam first introduced the “one-bead one-compound” (OBOC) combinatorial library method and used it to identify short linear peptides that bind to the anti-β-endorphin antibody and streptavidin [1]. The OBOC library is prepared via “split-mix” synthesis [1–3] on resin beads such that each bead displays only one chemical entity. Thousands to millions of compounds can be readily generated and screened concurrently within a short period of time (1–3 weeks). Since its invention, the OBOC library approach has been successfully applied to discover ligands against a variety of biological targets [4–11]. In the last 10 years, we have successfully applied this approach to discover ligands against many cell-surface receptors, such as LLP2A for activated α4β1 integrin [12], LXY1 for α3β1 integrin [13], and LXW7 for αvβ3 integrin [14]. In order to extend its application to discover cell-surface acting functional molecules, we have recently modified the OBOC method by adding a

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known cell-capturing ligand to the surface of every bead in the OBOC library to generate "one-bead two-compound" (OB2C) combinatorial libraries [15]. Thus, each bead will display on its surface a cell-capturing molecule and a random library compound. The chemical coding tag resides in the interior of each bead; therefore, it will not interfere with the screening. When live cells are incubated with such an OB2C library, the cell-capturing ligand will direct the cells to attach to the bead surface, resulting in the display of random library molecules in proximity to the cell surface. Some of these library compounds will interact with a cell-surface receptor, resulting in specific biochemical or cellular response which can be rapidly detected with an appropriate reporter system. For example, propidium iodide (PI) can be used to identify dead cells, caspase-3 fluorescent substrates can be used to identify cells undergoing apoptosis, and antibodies against specific cell-signaling proteins can be used to detect activation or suppression of a specific cell-signaling pathway. Figure 1 depicts the concept of the OB2C library for the discovery of functional ligands that target cell-surface receptors. Cell-surface receptors play important roles in cellular communications between adjacent cells, between cells and their extracellular microenvironment, and in intracellular signal transduction as well. Synthetic molecules that target these receptors are useful reagents in the study of biochemical pathways involving them. Some may even be used as lead compounds for the development of new drugs. The OB2C library method provides a highly efficient and economical way to discover such molecules.

The synthetic molecules on OB2C libraries can be linear, branched, or cyclic peptides (comprised of L- and D-amino acids and unnatural amino acids), as well as peptidomimetics, small molecules, glycopeptides, lipopeptides, or macrocyclic molecules. In this chapter, we shall focus on the design and synthesis of OB2C peptide and peptidomimetic libraries. We will briefly describe the application of this enabling technology to discover novel synthetic "death ligands" against lymphoid cancer Molt-4 cells.

2 Materials

2.1 Materials for Synthesis of OB2C Combinatorial Libraries

1. TentaGel S NH_2 resin.
2. Disposable polypropylene columns with frit (1 mL, 5 mL, 10 mL, and 50 mL).
3. *N*-(9-Fluorenylmethyloxycarbonyloxy) succinimide (Fmoc-OSu).
4. Allyloxycarbonyl-*N*-hydroxysuccinimide (Alloc-OSu).
5. Dichloromethane (DCM).
6. Diethyl ether.
7. *N,N*-Diisopropylethylamine (DIEA).
8. *N,N*-Dimethylformamide (DMF).
9. Methanol (MeOH).
10. Boc anhydride [$(Boc)_2O$].

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11. 4-Methylpiperidine.
12. Tetrakis(triphenylphosphine)palladium(0) [$Pd(PPh_3)_4$].
13. Phenylsilane ($PhSiH_3$).
14. Biotin.
15. Fluorescein isothiocyanate (FITC).
16. *N*-Methylpyrrolidone (NMP).
17. *O*-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU).
18. 6-Chloro-1-hydroxy-benzotriazole (6-Cl-HOBt).
19. 1,3-Diisopropylcarbodiimide (DIC).
20. Trifluoroacetic acid (TFA).
21. Triisopropylsilane (TIS).
22. Phenol.
23. Thioanisole.
24. Fmoc-protected amino acids (*see* Figs. 2 and 3): Fmoc-Acpc-OH, Fmoc-Orn(Boc)-OH, Fmoc-HoCit-OH, Fmoc-Hyp(tBu)-OH, Fmoc-Aib-OH, Fmoc-Nva-OH, Fmoc-Aad(tBu)-OH, Fmoc-Dpr(Boc)-OH, Fmoc-D-3-Pal-OH, Fmoc-D-Tyr(Me)-OH, Fmoc-Phg-OH, Fmoc-Nle-OH, Fmoc-4-Apc(Boc)-OH, Fmoc-Phe(4-Me)-OH, Fmoc-Aic-OH, Fmoc-D-Phe(3-Cl)-OH, Fmoc-HoPhe-OH, Fmoc-D-Chg-OH, Fmoc-Bpa-OH, Fmoc-Cha-OH Fmoc-D-2-Nal-OH, Fmoc-1-Nal-OH, Fmoc-Phe(diCl), Fmoc-Ala-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(tBu)-OH, Fmoc-Glu(tBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Val-OH, Fmoc-Trp(Boc)-OH. Natural amino acids and their D-isomers are designated by the standard three-letter code. Abbreviations of unnatural amino acids: Acpc, 1-aminocyclopropane-1-carboxylic acid; Orn, ornithine; HoCit, Homocitrulline; Hyp, hydroxy proline; Aib, 2-aminoisobutyric acid; Nva, norvaline; Aad, 2-aminohexanedioic acid; Dpr, 2,3-diaminopropionic acid; D-3-PAL, D-3-(3-pyridyl)alanine; D-Tyr (Me), D-*O*-methyltyrosine; Phg, phenylglycine; Nle, norleucine; 4-Apc, 1-amino-1-(4-piperidinyl) carboxylic acid; Phe(4-Me), 4-methylphenylalanine; Aic, 2-aminoindane-2-carboxylic acid; D-Phe(3-Cl), D-3-chlorophenylalanine; HoPhe, homophenylalanine; D-Chg, D-α-cyclohexylglycine; Bpa, 4-benzoylphenylalanine; Cha, cyclohexylalanine; D-2-Nal, D-3-(2-Naphthyl)alanine; 1-Nal, 3-(1-Naphthyl)alanine; Phe(di Cl), 3,4-di-chlorophenylalanine.
25. Twenty-three carboxylic acids (*see* Table 1, #1 to #23).
26. Sixteen isocyanates (*see* Table 1, #24 to #39).

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27. Three sulfonyl chlorides (*see* Table 1, #40 to #42): benzenesulfonyl chloride, 2,5-dichlorothiophene-3-sulfonyl chloride, cyclopentane sulfonyl chloride.
28. Fmoc-Dpr(Alloc)-OH.
29. Fmoc-4-Apc-OH hydrochloride.
30. Sodium diethyldithiocarbamate.
31. 70 % Ethanol in water.

2.2 Materials for Screening of OB2C Libraries

1. Neutravidin.
2. Phosphate-buffered saline (PBS) buffer.
3. Molt-4 lymphoma cells.
4. 4 % Paraformaldehyde.
5. 5 % Bovine serum albumin (BSA).
6. 0.5 % Triton X-100.
7. Rabbit antihuman cleaved caspase-3 antibody.
8. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG.
9. Diaminobenzidine(DAB) tetrahydrochloride.
10. Propidium iodide (PI).
11. 12-Well plate.

2.3 Instruments

1. Perkin-Elmer/Applied Biosystems protein sequencer (ABI Procise 494).
2. An inverted Olympus fluorescent microscope (model IX81).
3. An Olympus dissecting stereomicroscope (SZX12).

3 Methods

Synthesis of an OB2C combinatorial library involves three steps (1) bi-layer bead preparation [16] and introduction of two orthogonal amino-protecting groups to the bead surface; (2) library assembly via "split-mix" approach [1–3] and addition of coding tag; and (3) direct synthesis of ligand (route **A** in Fig. 4), or chemoselective ligation of ligand on bead surface (e.g., via Click chemistry, route **B** in Fig. 4), or through ligand-biotin-neutravidin complexation if a biotin is on the surface (route **C** in Fig. 4, *see* below).

3.1 Preparation of Topologically Segregated Trifunctional Bilayer Beads

We choose TentaGel S NH_2 resin (Rapp Polymere, Tubingen, Germany) for OB2C combinatorial libraries due to its relative uniformity in size and non-stickiness, as well as its compatibility with both water and many organic solvents. The topologically segregated

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bilayer TentaGel beads are prepared by a simple biphasic solvent system [16]. The surface of the bead is exposed to organic solvent(s) that contains a small amount of derivatizing reagent [e.g., Fmoc-OSu, 20 % of whole bead loading], while the interior of the bead remains in water without any derivatizing reagents. The end result is that only the outer layer of the bead is derivatized (**2** in Fig. 5a). In order to visualize this configuration microscopically, the Fmoc on the outer layer is removed, and the exposed amino group is derivatized with FITC (**3** in Fig. 5a). The confocal photomicrographs in Fig. 5b clearly demonstrate the bilayer configuration.

The trifunctional bilayer beads (**4** in Fig. 5c) were prepared with similar approach, but the derivatizing reagents were a mixture of Fmoc-OSu and Alloc-OSu (1:1, total 20 % of bead loading). Therefore, Fmoc and Alloc were co-displayed on the bead surface (Fig. 5c). In brief, TentaGel S-NH_2 resin beads (6.0 g, 1.44 mmol, loading 0.24 mmol/g) were swollen in water for 24 h. Water was removed by filtration, and the solution of Fmoc-OSu (48.6 mg, 0.144 mmol) and Alloc-OSu (28.7 mg, 0.144 mmol) in DCM/diethyl ether (300 mL, 55/45) mixture was added to the wet beads, followed by the addition of DIEA (201 μL, 1.152 mmol). The mixture was shaken vigorously at room temperature for 45 min. After removal of the liquid by filtration, the beads were washed with DMF (5×300 mL) to remove water from inside the beads, followed by MeOH (3×300 mL) and DMF (3×300 mL). A solution of $(Boc)_2O$ (1.006 g, 4.61 mmol) and DIEA (1.606 mL, 9.22 mmol) in DMF (45 mL) was added to the beads. The slurry beads were shaken at room temperature for 1 h. Kaiser test [17] indicated the completion of coupling.

3.2 Design and Synthesis of OB2C Peptide and Peptidomimetic Libraries

3.2.1 Selection of Fmoc-Amino Acids for OB2C Peptide and Peptidomimetic Libraries—Fmoc chemistry is chosen for the synthesis of OB2C peptide and peptidomimetic libraries because it is easy to handle. In addition, a large number of Fmoc-protected natural and unnatural amino acids are commercially available. In order to maximize the structure-activity relationship information from the library screening, we include both L-/D-amino acids and natural/unnatural amino acids in the library. The 42 Fmoc-amino acids for OB2C peptide and peptidomimetic libraries are shown in Figs. 2 and 3. Those amino acids can be unambiguously distinguished with Edman microsequencing using our modified method (*see* Table 2), which uses a slightly modified gradient program that extends elution time from a total of 22 min to 28 min [18, 19].

3.2.2 Design of OB2C Peptide and Peptidomimetic Libraries—The three OB2C combinatorial libraries (L1, L2, and L3) are shown in Fig. 6. In these libraries, a peptide coding tag (80 % of the total loading of single bead) comprised of 42 sequenceable α-amino acids (shown in Figs. 2 and 3) resides inside the beads. Instead of using lysine to make branched peptide, we choose a dipeptide consisting of 4-Apc and Dpr (Fig. 6), which presumably has more rigid conformation and thus less steric hindrance for coupling. In order to allow the OB2C libraries useful for multiple projects, we introduce the cell-capturing ligand on the bead surface of OB2C libraries through the biotin-neutravidin complexation approach (Fig. 4c). The biotin molecule can be used to link any type of cell-capturing ligands through (ligand-biotin)-neutravidin-(biotin-bead) linkages. Neutravidin is a

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tetrameric protein with four unique biotin-binding pockets. It can be used as a convenient protein scaffold to construct heterodimeric, trimeric, or tetrameric ligands by simply mixing the appropriate ratio of biotinylated ligand(s) and biotin(s) with one-fourth molar ratio of neutravidin. For example, a library can be screened for "death ligands" of cancer cell lines with activated α4β1 integrin (e.g., Molt-4 in this chapter) if linked with LLP2A-biotin. The same OB2C library can also be used for discovery of "death ligands" for cancer cell lines with high level of α3β1 integrin (e.g., MDA-MB-231 breast cancer cells and U-87 MG glioblastoma cells) if complexed with LXY1-biotin. For those cell lines without known binding ligands, one may use polylysine (e.g., octa-lysine) as a cell-capturing ligand. Library L1 is an OB2C 8-mer peptide library with the *N*-terminus of 4-Apc acetylated. Library L2 is a tetrapeptide-based peptidomimetic library with the *N*-terminus of 4-Apc derivatized with 23 carboxylic acids, 16 isocyanates, and 3 sulfonyl chlorides (encoded with X_R which are 42 amino acids in Figs. 2 and 3). L3 is similar to L2 but with one additional amino acid coupled to the side chains of Dpr and 4-Apc, respectively. Although permutation of L2 library is relatively small (1,764 compounds), those in libraries L1 and L3 are rather high (74,088 library compounds for each library) and have a good chance to interact with some of the numerous receptors on the surface of the target cells. The chemical decoding can be readily achieved with an automated protein microsequencer as described above. Please note that decoding can also be accomplished with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) if a cleavable linker is added between the bead and coding tag [20–22], but the coding amino acids at each position of the coding tag cannot have identical molecular weight, and the cleaved analyst has to be extracted out from the single bead prior to MS decoding.

3.2.3 Synthesis of OB2C Peptide and Peptidomimetic Libraries—The OB2C-branched libraries L1, L2, and L3 were synthesized on trifunctional bilayer beads (**4**, as described in Subheading 3.1) using Fmoc-chemistry and "split-mix" method [1–3]. The synthetic approach of library L1 is shown in Scheme 1. Although Fmoc-4-Apc(Alloc)-OH (Scheme 1) is not commercially available, it can be easily synthesized from commercially available Fmoc-4-Apc-OH hydrochloride salt (Pharmacore, High Point, NC) with Alloc-OSu using a similar approach as previously reported (Scheme 1) [23].

The Fmoc on 6 g of beads **4** was removed with 20 % 4-methylpiperidine in DMF (45 mL), twice (first 5 min, second 15 min). After filtration, the beads were washed with DMF (3×45 mL), MeOH (3×45 mL), and DMF (3×45 mL); a mixture of D-biotin (175.9 mg, 0.72 mmol), HBTU (273 mg, 0.72 mmol), and DIEA (251 μL, 1.44 mmol) in NMP (45 mL) was added to the beads. The column was rotated until a Kaiser test was negative. The resin beads **5** were washed and subjected to Alloc deprotection with $Pd(PPh_3)_4$ (66.5 mg, 0.0576 mmol) and $PhSiH_3$ (355 μL, 2.88 mmol) in DCM (45 mL), for 45 min twice. Following deprotection, the beads were washed sequentially with DCM (6×45 mL), DMF (3×45 mL), 0.5 % DIEA in DMF (3×45 mL), 0.5 % sodium diethyldithiocarbamate in DMF (3×45 mL), 50 % DCM in DMF (3×45 mL), MeOH (3×45 mL), and DMF (3×45 mL). A solution of Fmoc-L-Dpr(Alloc)-OH (295.5 mg, 0.72 mmol), 6-Cl-HOBt (122 mg, 0.72 mmol), and DIC (111.5 μL, 072 mmol) in DMF (35 mL) was added to the beads. The coupling was carried out at room temperature for 2 h to generate beads **6**. After Fmoc deprotection, the beads were

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washed with DMF, MeOH, and DMF, respectively, three times. A solution of Fmoc-4-Apc(Alloc)-OH (324.4 mg, 0.72 mmol), 6-Cl-HOBt (122 mg, 0.72 mmol), and DIC (111.5 μL, 072 mmol) in DMF (35 mL) was added to the beads and rotated for 4 h (Kaiser test indicated negative) to give beads **7**.

The following procedure was used for synthesis of library L1: one-third of beads **7** (about 2 g) were subject to Fmoc deprotection as described above. After Fmoc deprotection and washing, a solution of acetic anhydride (22.7 μL, 0.24 mmol) and DIEA (83.6 μL, 0.48 mmol) in DMF (12 mL) was added to the beads. The acetylation was carried out at room temperature for 1 h. After washing with DMF (3×12 mL), MeOH (3×12 mL), and DCM (3×12 mL), respectively, the Alloc protecting groups in both Dpr and 4-Apc were removed as described above. Then the beads were treated with 50 % TFA in DCM (12 mL) for 30 min to remove Boc inside the beads to give beads **8**. The beads were neutralized with 5 % DIEA in DMF (2×12 mL) and washed with DMF (3×12 mL), MeOH (3×12 mL), and DMF (3×12 mL) before split-mix coupling of 42 amino acids. The beads were split into 42 equal portions in 42 disposable polypropylene columns with a polyethylene frit (5 mL). Forty-two different Fmoc-amino acids shown in Figs. 2 and 3 (4 eq. to bead total substitution) were separately dissolved in a solution of 6-Cl-HOBt (4 eq.) and DIC (4 eq.) in DMF (2 mL) and added to 42 columns, each column receiving one amino acid. The coupling was carried out at room temperature for 2 h. Four random tubes were chosen for a Kaiser test. After the Kaiser test was negative, the beads were pooled to a column, drained, and washed, and Fmoc was deprotected. Then the beads were coupled with two more cycles with the 42 Fmoc-amino acids as described above. After the last cycle of coupling, the beads were combined, and Fmoc was deprotected. After Fmoc deprotection, the beads were washed with DMF (3×12 mL), MeOH (3×12 mL), and DCM (3×12 mL), respectively, three times. The beads were then dried under vacuum. Side-chain deprotection was achieved using a TFA cocktail (a mixture of 82.5 % TFA, 5 % phenol, 5 % thioanisole, 5 % water, 2.5 % TIS, v/v) for 4 h. After neutralization with 5 % DIEA/DMF (2×12 mL), the beads were washed sequentially with DMF (3×12 mL), MeOH (3×12 mL), DCM (3×12 mL), DMF (3×12 mL), 50 % DMF/water (3×12 mL), water (3×12 mL), and 70 % ethanol/water (3×12 mL), respectively. The bead library was stored in 70 % ethanol/water and was ready for screening.

The synthetic approach of OB2C peptidomimetic library L2 and L3 is shown in Scheme 2. After Fmoc deprotection of the remaining two-thirds of beads **7** (about 4 g), the beads were split into 42 disposable columns with frit (5 mL) for coupling of 23 carboxylic acids, 16 isocyanates, and 3 sulfonyl chlorides as shown in Table 2. The coupling condition was as follows: the carboxylic acids (20 eq., each) were dissolved separately in a solution of HBTU (20 eq.) and DIEA (40 eq.) in DMF and added to the corresponding columns; each column only received one acid. The 16 isocyanates and 3 sulfonyl chlorides (20 eq., each) were separately dissolved in a solution of DIEA (40 eq.) in DMF (2 mL) and then added to the corresponding columns. The reaction was carried out at room temperature overnight. After filtration, the beads were kept in separate columns and washed with DMF (3×3 mL), MeOH (3×3 mL), and DCM (3×3 mL), respectively. The Boc-protecting groups inside the beads were removed with 50 % TFA in DCM (3 mL for each column) for 30 min. After filtration

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and neutralization with 5 % DIEA in DMF (2×3 mL), the beads were washed with DMF (3×3 mL), MeOH (3×3 mL), and DMF (3×3 mL). The beads in each column received one of the 42 Fmoc-amino acids (coding amino acids X_R from Figs. 2 and 3, 4 eq. to beads) which was preactivated with 6-Cl-HOBt (4 eq.) and DIC (4 eq.) in DMF (2 mL). The coupling was carried out at room temperature for 2 h. All beads from 42 columns were pooled into a big column, drained, and washed to give beads **9**. The Alloc-protecting groups in both Dpr and 4-Apc were removed with $Pd(PPh_3)_4$ as described above. The Fmoc inside the beads was deprotected to give beads **10** which were subjected to one cycle and two cycles of split-mix synthesis with 42 Fmoc-amino acids for library L2 and L3, respectively. The side-chain deprotection and final washing were the same as described in the synthesis of L1. The OB2C library L2 and L3 were stored in 70 % ethanol/water prior to screening.

3.3 Screening of OB2C Libraries for "Death Ligands" Against Molt-4 Lymphoma Cells

To screen OB2C libraries for specific cell signaling, immunocytochemistry offers some advantages over immunofluorescence. It is easier to perform and does not require a fluorescent microscope. To isolate visible dye-stained beads under a stereomicroscope is much faster than to isolate fluorescent beads in a dark room. Furthermore, the immunocytochemical-stained cell/beads, unlike fluorescent dyes, do not bleach and therefore can be kept in the refrigerator or dried and then isolated at a later date. Because caspase-3 is activated in both intrinsic and extrinsic apoptotic signaling pathways, we used commercially available horseradish peroxidase (HRP)-conjugated anti-cleaved caspase-3 antibody as a probe to screen OB2C libraries for proapoptotic molecules. LLP2A is a high-affinity (IC_{50} = 2 pM) and high-specificity ligand for α4β1 integrin of lymphoid cancer cells [12]. Therefore, LLP2A was chosen as the cell-capturing ligand for Molt-4 lymphoma cells and was introduced to the bead surface of OB2C libraries through ligand-biotin-neutravidin complexation (Fig. 4c). LLP2A will direct the suspension cells to attach to the bead surface, resulting in the display of random peptides in proximity to the cell surface. The hypothesis is that some of these random peptides or peptidomimetics will interact with a cell-surface receptor, resulting in the activation of the apoptotic pathway. Beads displaying library compounds that elicit apoptosis (cleaved caspase-3 positive) are considered as positive beads.

First, we reacted neutravidin with LLP2A-biotin [12] (Fig. 7) in 1:2 molar ratio for 20 min. Mixture of neutravidin and LLP2A-biotin was added onto the wells (12-well plate) containing 20 μL of settled down OB2C beads (~15,000 beads) and incubated for 20 min. We carefully removed the solution, and Molt-4 cells were seeded onto the well. After 20 min incubation at 37 °C, unbounded cells were washed out with PBS. Cell-bound beads were incubated for further 24 h, followed by fixation in 4 % paraformaldehyde for 20 min. For immunocytochemical assay on beads, nonspecific protein binding was blocked by adding 5 % BSA, and cell membranes were permeabilized by 0.5 % Triton X-100. We used rabbit antihuman cleaved caspase-3 (Cell Signaling Technology) as primary antibodies. Beads were incubated with primary antibody (1:100 in PBS) for overnight at 4 °C. After washing with PBS, beads were then incubated with the secondary antibody, an HRP-conjugated goat anti-rabbit IgG, for 1 h at room temperature. HRP activity was finally detected using diaminobenzidine (DAB) tetrahydrochloride as a substrate for 3 min in

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accordance with the manufacturer's instructions (Biogenex). The beads with brown cells were considered as positive beads (Fig. 8) and were manually isolated with a micropipette. Only one positive bead was identified from L2 and L3, respectively. No positive beads were identified from library L1.

3.4 Decoding of Positive Beads

The two isolated positive beads were treated with 6 M guanidine HCl (pH 1.0, three times), to remove the bound cells or any proteins or biomolecules produced by the cells, and washed thoroughly with water prior to chemical decoding with automatic Edman microsequencer using modified program [18]. The structures of the two ligands are shown in Fig. 9. Interestingly, the two ligands share a highly consensus sequence. They have identical urea moiety (out of 42 diversities, red part in Fig. 9) at the *N*-terminal of 4-Apc and have L-tyrosine (out of 42 diversities, in blue in Fig. 9) on the side chains of Dpr and 4-Apc. The urea moiety in the "death ligands" probably is critical for the proapoptotic activity because no positive beads were identified from library L1 which has an acetyl group at that position.

3.5 Validation of Apoptosis of Ligands on Beads

The death ligands were resynthesized on trifunctional bilayer beads for confirmation using the same approach as described in the OB2C library synthesis. The methods of preparation of beads conjugated with LLP2A and binding with Molt-4 cells are the same as mentioned in the screening of OB2C libraries. The validation of proapoptotic effect was done with both caspase-3 staining and PI staining. The method for caspase-3 staining is the same as in the screening of OB2C libraries. Cells bound to both S7-Y beads and S8-FY beads were shown undergoing apoptosis (Fig. 10). For PI staining, after 48 h cell-bound beads were stained with PI at 1 μg/mL for 10 min. After one wash with PBS, cells were photographed using a fluorescence microscope. The result is shown in Fig. 11. Dead cells on both S7-Y beads and S8-FY beads are clearly seen in red (Fig. 11). The result from both caspase-3 staining and PI staining verified the proapoptotic activity of those two "death ligands."

4 Summary

The OB2C combinatorial library approach is the most efficient and economical synthetic library format available for chemical libraries with discrete compounds (not compound mixtures). Although chemical decoding is needed in OB2C library method, it eliminates the need to have expensive storage and archive system to track every compound in the conventional chemical libraries. With the OB2C method, one can now rapidly probe living cells with a large number of discrete compounds for cell-surface acting molecules that can elicit specific biochemical or cellular functions such as proapoptotic activities. An OB2C library is bead based and therefore includes a built-in PEG linker between the library compound and the solid support. This linker can be used to link the "death ligand" to the cancer-cell-capturing ligand for efficient delivery of the "death ligands" to the tumor cells.

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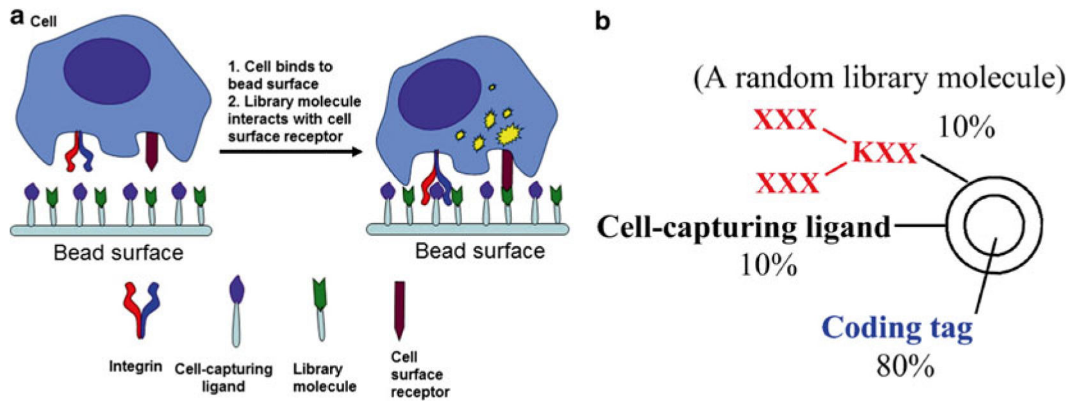

Fig. 1.

OB2C combinatorial library technology for the discovery of functional ligands. (**a**) A cartoon illustrates OB2C concept. (**b**) Example of the structure of an OB2C combinatorial library bead. The library in red is a branched peptide library derived from a lysine (K). X represents natural or unnatural amino acids

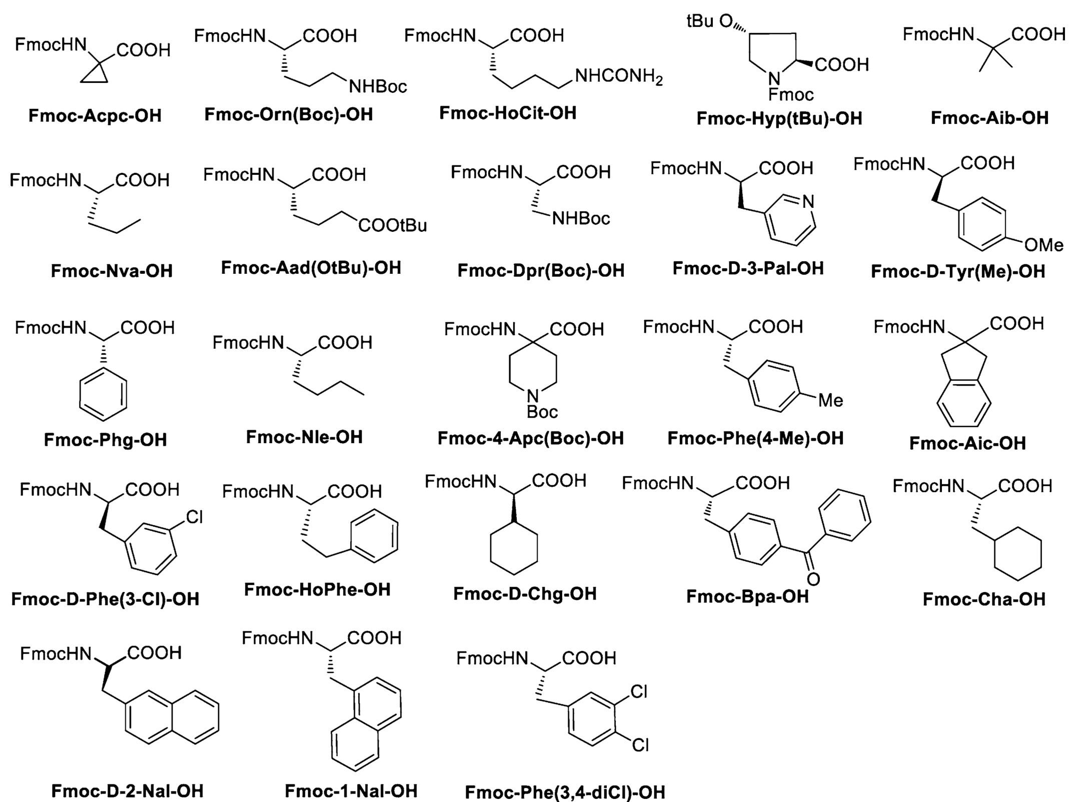

Fig. 2.

Structures of unnatural amino acids for OB2C peptide and peptidomimetic libraries

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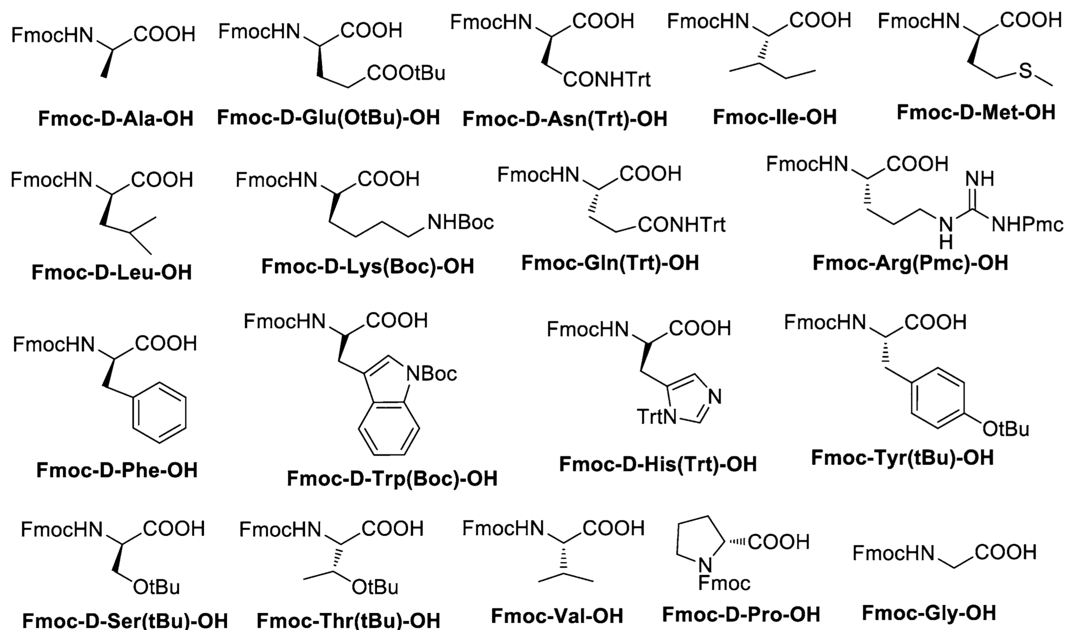

Fig. 3.

Structures of natural amino acids and their D-isomers for OB2C peptide and peptidomimetic libraries

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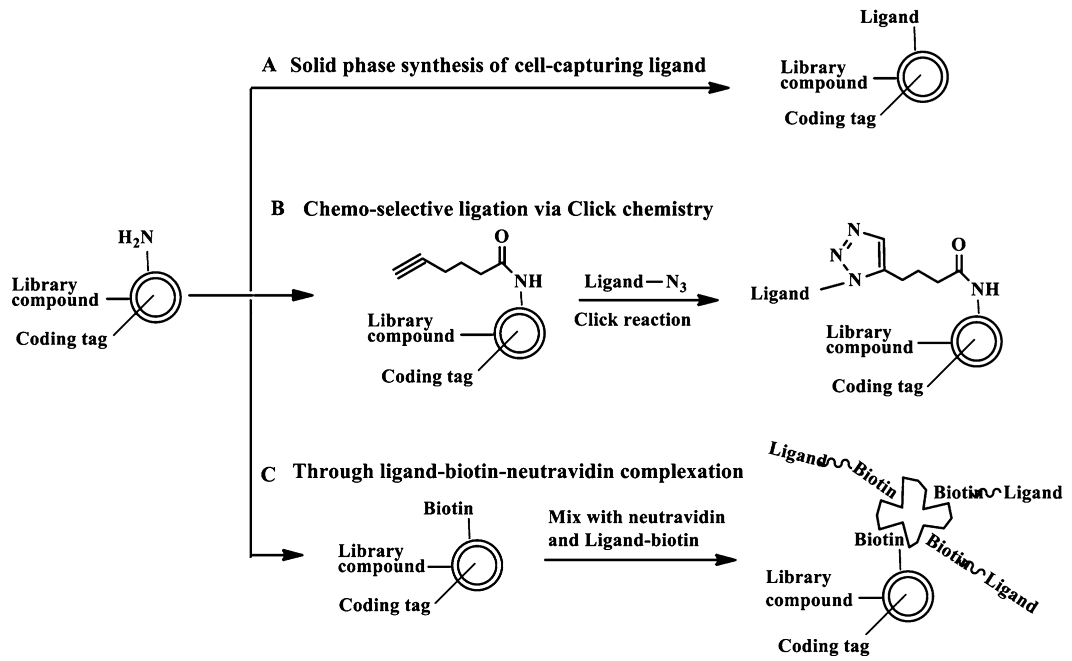

Fig. 4.

Approaches to introduce cell-capturing ligand on bead surface of OB2C library. Ligand stands for cell-capturing ligand

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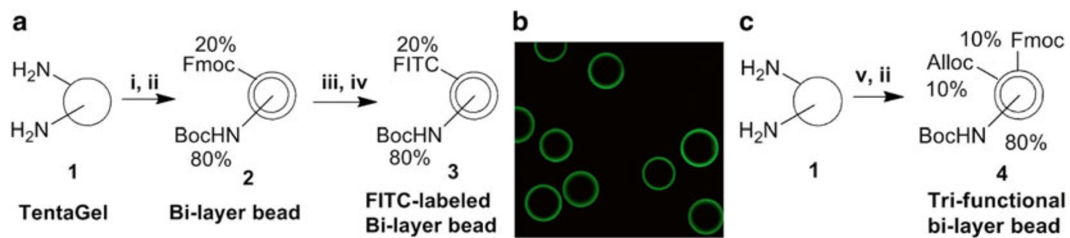

Fig. 5.

(**a**) Preparation of bilayer bead. The outer of bilayer beads was labeled with FITC (**3**). (**b**) Confocal photomicrograph of FITC-labeled bilayer **3**. (**c**) Preparation of trifunctional bilayer bead **4**. Reagents and conditions: (i) swell in water for 24 h; (ii) Fmoc-OSu (0.2 eq. to the bead total substitution) and DIEA (0.4 eq.), DCM/diethyl ether (55:45), 45 min; (iii) $(Boc)_2O$ (4 eq.) and DIEA (8 eq.), 1 h; (iv) 20 % N-Methylpiperidine in DMF, twice (fi rst 5 min, second 15 min); (v) FITC (4 eq.), DIEA (8 eq.), DMF, 2 h; (vi) Fmoc-OSu/Alloc-OSu (1:1, 0.2 eq. to the bead total substitution) and DIEA (0.8 eq.), DCM/diethyl ether (55:45), 45 min

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Fig. 6.
Structures of OB2C peptide and peptidomimetic libraries

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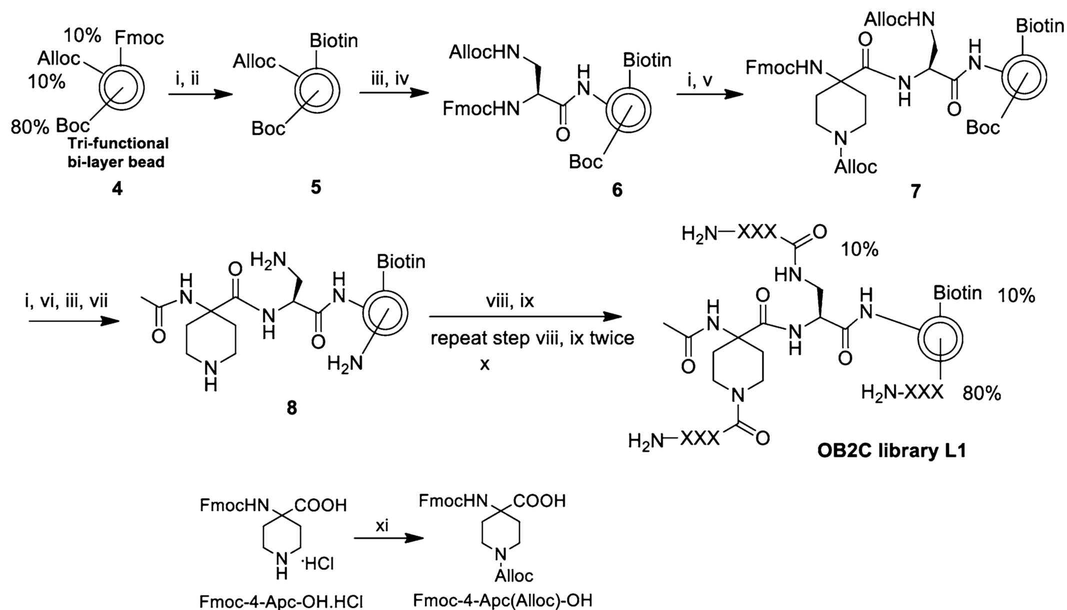

Scheme 1.

Synthetic scheme of OB2C peptide library L1. Reagents and conditions: (i) 20 % 4-methylpiperidine, fi rst 5 min, then 15 min; (ii) biotin (5 eq.), HBTU (5 eq.), DIEA (10 eq.), NMP, 5 h; (iii) $Pd(PPh_3)_4$ (0.4 eq.), $PhSiH_3$ (20 eq.), DCM, 45 min, twice; (iv) Fmoc-Dpr(Alloc) (5 eq.), 6-Cl-HOBt (5 eq.), DIC (5 eq.), 2 h; (v) Fmoc-4-Apc(Alloc)-OH (5 eq.), 6-Cl-HOBt (5 eq.), DIC (5 eq.), 4 h; (vi) Ac_2O (5 eq.), DIEA (10 eq.), 1 h; (vii) 50 % TFA/DCM, 30 min; (viii) split beads into 42 columns, then couple Fmoc-X-OH (5 eq.), 6-Cl-HOBt (5 eq.), DIC (5 eq.), overnight; (ix) combine beads, then de-Fmoc with 20 % 4-methylpiperidine; (x) TFA cocktail, 4 h; (xi) Alloc-OSu (1.1 eq.), $NaHCO_3$ aq, overnight

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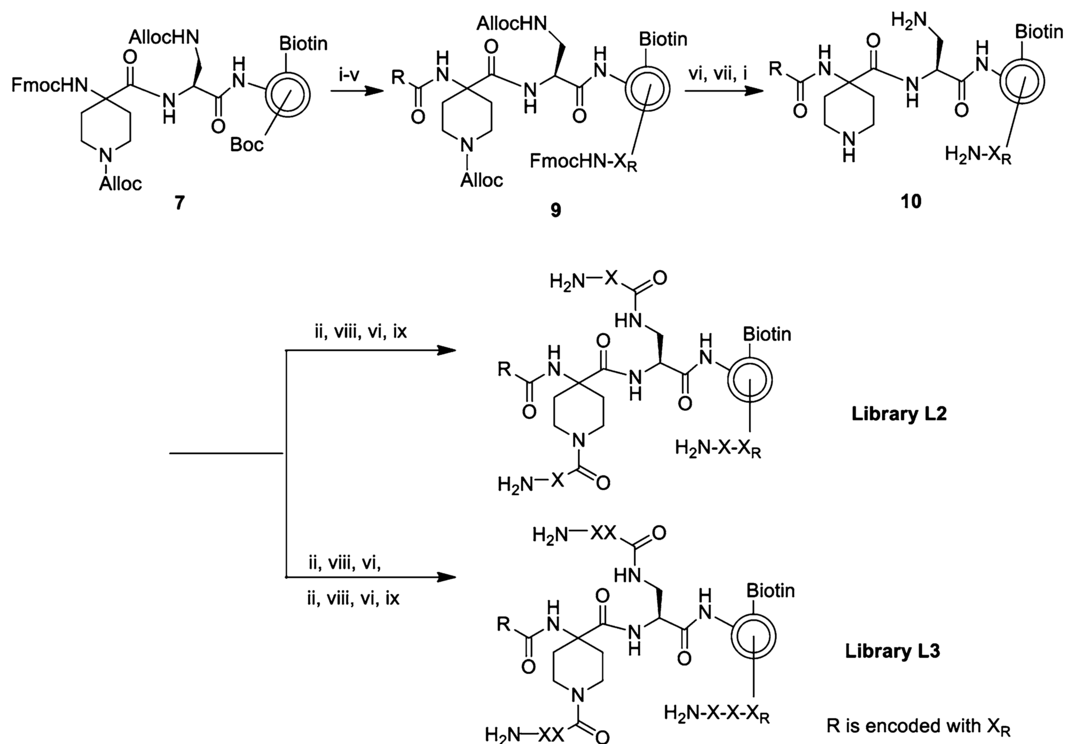

Scheme 2.

Synthetic approach of OB2C peptidomimetic library L2 and L3. Reagents and conditions: (i) 20 % 4-methylpiperidine, fi rst 5 min, second 15 min; (ii) split beads; (iii) RCOOH (20 eq.), 6-Cl-HOBt (20 eq.), DIC (20 eq.), or RNCO (20 eq.), DIEA (40 eq.), or RSO_2Cl (20 eq.), DIEA (eq.), overnight; (iv) 50 % TFA/DCM, 30 min; (v) Fmoc-X_R-OH (5 eq.), 6-Cl-HOBt (5 eq.), DIC (5 eq.), 4 h; (vi) combine beads; (vii) $Pd(PPh_3)_4$ (0.4 eq.), $PhSiH_3$ (20 eq.), 45 min, twice; (viii) Fmoc-X-OH (5 eq.), 6-Cl-HOBt (5 eq.), DIC (5 eq.), 4 h; (ix) TFA cocktail, 4 h

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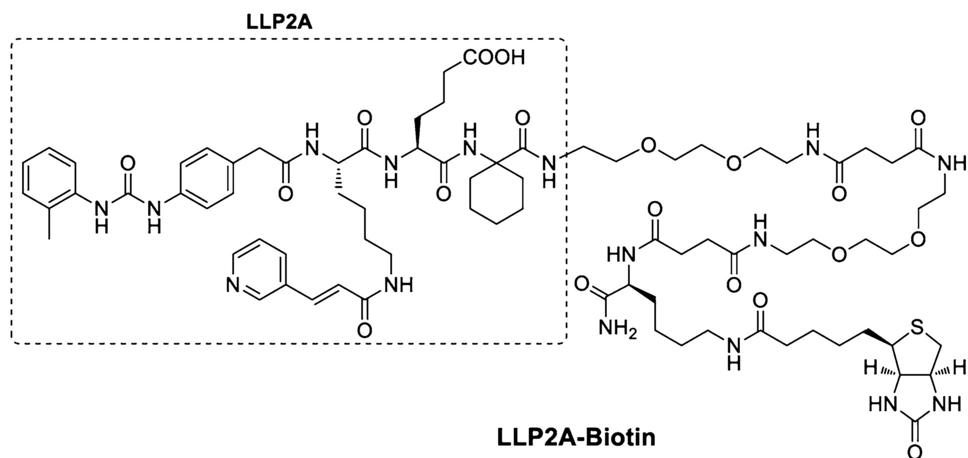

Fig. 7.
Structure of LLP2A-biotin

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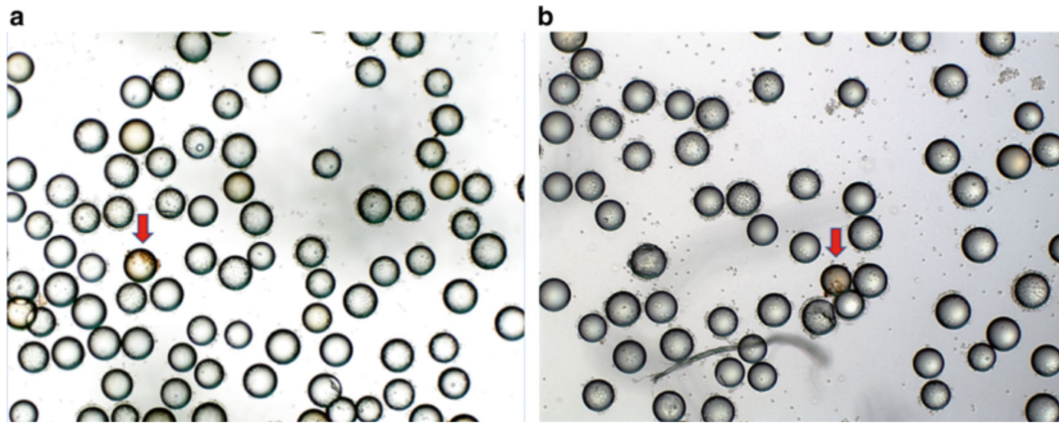

Fig. 8.

Screening of “death ligands” from OB2C libraries against Molt-4 cells. *Arrows* point to the positive beads. (**a**) Screening of library L2; (**b**) screening of library L3

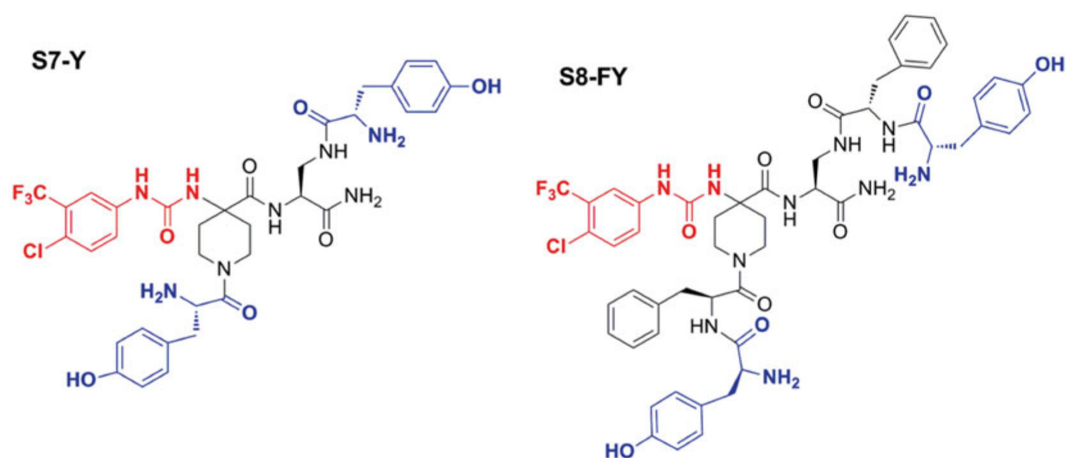

Fig. 9.

Chemical structures of “death ligands” against Molt-4 cells identifi ed from OB2C peptidomimetic library L1 (S7-Y) and L2 (S8-FY)

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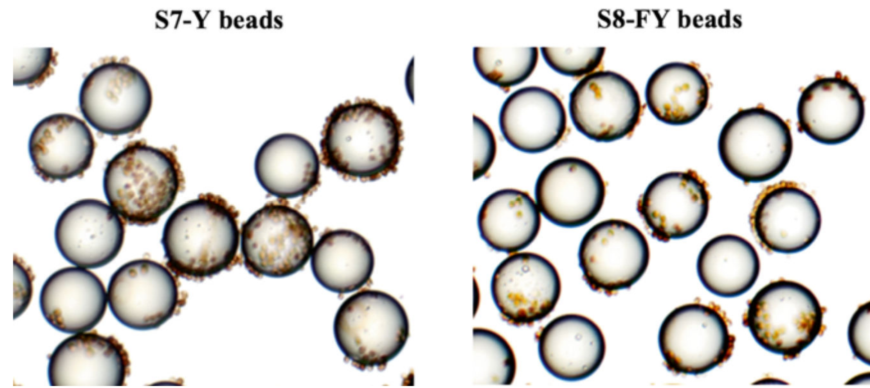

Fig. 10.

Immunocytochemistry (ICC) stain of bead-bound Molt-4 cells for caspase-3 activation. Beads displaying "death ligand" were incubated with Molt-4 cells for 24 h followed by ICC staining. Note: some cells were fallen off the beads during extensive washing. Cells in brown were undergoing apoptosis

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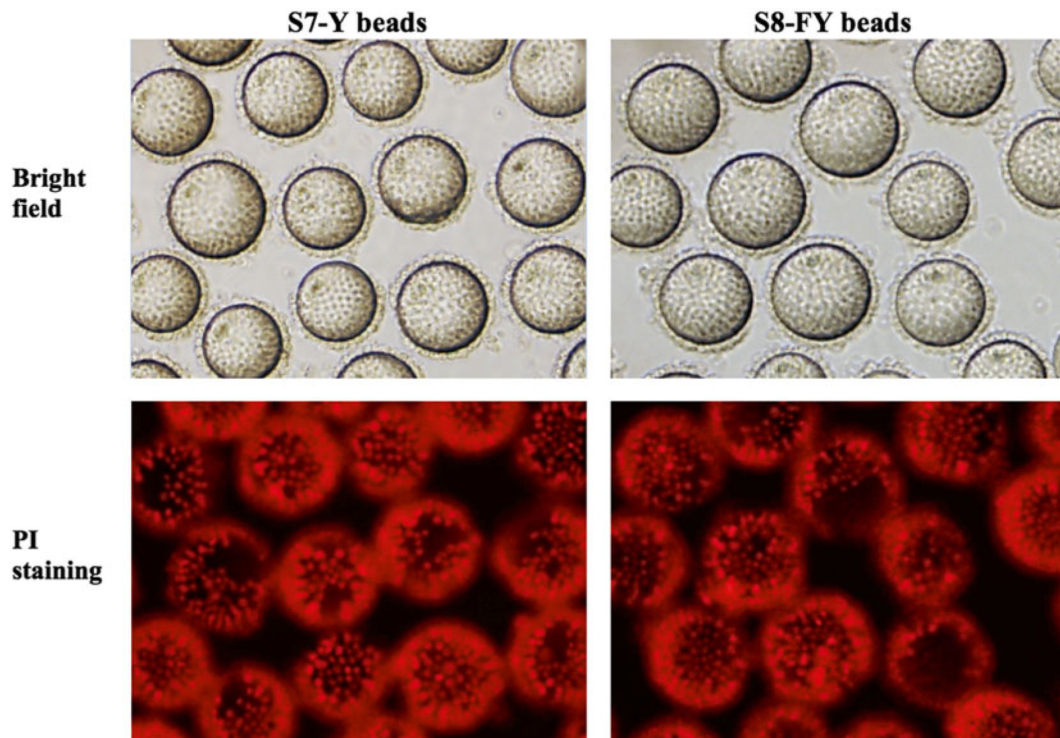

Fig. 11.

PI staining for dead cells on beads displaying proapoptotic ligands. Lymphoma Molt-4 cells were incubated with beads for 48 h. Dead cells were stained in *red*

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Table 1

Carboxylic acids, isocyanates, and sulfonyl chlorides used for R position in OB2C peptidomimetic libraries L2 and L3

#1 3-Pyridinepropionic acid	#2 4-(4-Pyridyl)benzoic acid	#3 4-(Dimethylamino) phenylacetic acid	#4 L-Pyroglutamic acid
#5 trans-3-(3-Pyridyl)acrylic acid	#6 5-Hydantoinacetic acid	#7 Cyclopropane carboxylic acid	#8 2-Pyrazinecarboxylic acid
#9 trans-4-Cotininecarboxylic acid	#10 (S)-(+)-Oxo-4-phenyl-3-oxazolidineacetic acid	#11 1-Methylindole-2-carboxylic acid	#12 5-Cyclopropyl isoxazole-4-carboxylic acid
#13 2-Thiopheneacetic acid	#14 tert-Butylacetic acid	#15 m-Tolylacetic acid	#16 5-Bromofuroic acid
#17 trans-3-Furanacrylic acid	#18 5-Bromo-2-thiophenecarboxylic acid	#19 1-Methylpyrrole-2-carboxylic acid	#20 2-Methoxypyridine-4-carboxylic acid
#21 6-(1-Piperidinyl) pyridine-3-carboxylic acid	#22 Quinoline-6-carboxylic acid	#23 4-Fluorobenzoic acid	#24 Methyl (*S*)-(−)-2-isocyanato-3-phenylpropionate

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#25 4-(Dimethylamino)phenyl isocyanate	#26 4-Chloro-3-(trifluoromethyl)phenyl isocyanate	#27 3-Methoxyphenyl isocyanate	#28 4-Methoxyphenyl isocyanate
#29 3-Chlorophenyl isocyanate	#30 Methyl isocyanatoformate	#31 Furfuryl isocyanate	#32 2-Chlorophenyl isocyanate
#33 3-Chloropropyl isocyanate	#34 ®-(+)-α-Methylbenzyl isocyanate	#35 Isopropyl isocyanate	#36 2-Methoxybenzyl isocyanate
#37 2-Fluorophenyl isocyanate	#38 1-(4-Isocyanatophenyl) piperidine	#39 Cyclopentyl isocyanate	#40 2,5-Dichlorothiophene-3-sulfonyl chloride
#41 Benzenesulfonyl chloride	#42 Cyclopentane sulfonyl chloride		

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Table 2

Retention time of 42 amino acids used for OB2C libraries on a microsequencer

Amino acid	Retention time (min)	Amino acid	Retention time (min)	Amino acid	Retention time (min)
Asp	4.17	Arg	10.13	D-Lys	18.05
Acpc	4.59	Tyr	10.50	D-Leu	18.36
D-Asn	4.70	Aib	10.90	Nle	18.88
D-Ser	5.34	D-Pro	13.02	4-Apc	19.22
Gln	5.61	D-Met	13.70	Phe(4-Me)	20.38
Thr	5.85	Val	14.03	Aic	20.89
Gly	6.11	Nva	14.82	D-Phe(3-Cl)	21.13
D-Glu	6.49	**DPTU**	**15.32**	HoPhe	21.66
HoCit	6.98	Dpr	15.75	D-Chg	21.86
Hyp	7.57	D-Tyr(Me)	16.66	Bpa	22.11
Aad	7.80	D-Trp	16.48	D-2-Nal	22.41
D-His	8.08	Orn	16.83	1-Nal	22.65
D-Ala	8.37	Phg	17.09	Phe(di Cl)	23.23
D-3-Pal	8.71	D-Phe	17.24	Cha	23.98
Hyp	8.75	Ile	17.76		

Note: Retention time refers to the HPLC peak of phenylthiohydantoin (PTH)-amino acid formed during Edman degradation on an ABI protein sequencer. Hyp has typical two peaks at ratio of 3:1. Diphenylthiourea (DPTU) is a by-product of Edman degradation and appears at each residue cycle; therefore, it can be used as an internal reference peak