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In Vitro Selection of Multiple Libraries Created by Genetic Code Reprogramming To Discover Macrocyclic Peptides That Antagonize VEGFR2 Activity in Living Cells

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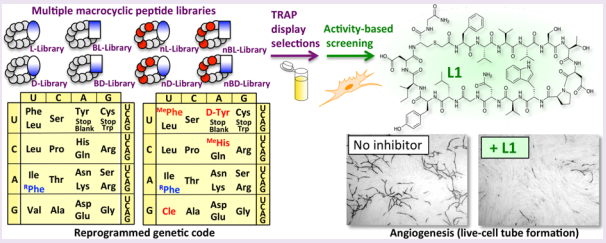
Supporting Information

ABSTRACT: We report the *in vitro* selection of thioether-macrocyclized peptides against vascular endothelial growth factor receptor 2 (VEGFR2) from multiple, highly diverse peptide libraries constructed utilizing genetic code reprogramming. The macrocyclic peptide libraries consisted of combinations of four types of amino acid linkers for cyclization and two types of elongator amino acid compositions, including four backbone-modified non-proteinogenic amino acids. Affinity selection from these libraries, using our recently developed TRAP (Transcription–translation coupled with Association of Puromycin-linker) display, yielded multiple anti-VEGFR2 macrocyclic peptide leads. Further antagonizing activity-based screening of the chemically synthesized lead peptides identified a potent macrocyclic peptide that inhibited VEGF-induced VEGFR2 autophosphorylation, proliferation, and angiogenesis of living vascular endothelial cells. The TRAP display-based selection from multiple, highly diverse peptide libraries followed by activity-based screening of selected peptides is a powerful strategy for discovering biologically active peptides targeted to various biomolecules.



Peptide selection by means of linkage between a genotype (DNA) and a phenotype (the encoded peptide) is a powerful method to discover novel functional peptides such as peptide aptamers,[1] peptidic inhibitors,[2] and peptide substrates of enzymes.[3] Furthermore, researchers have been developing methods for incorporating non-canonical structures into ribosomally synthesized peptides using post-translational modification[4] and/or expanding the amino acid substrates that can be used in a ribosomal translation system.[5] In particular, since a non-reducible macrocyclic structure gives peptides greater stability against peptidases and more conformational rigidity even in cellular environments, this non-canonical structure is an attractive platform for selection of *in vivo* functioning peptides such as therapeutic peptidic drugs.

In an example of selection from non-reducible macrocyclic peptide libraries, backbone-cyclized peptide libraries produced by circularly permuted inteins were used to select peptide inhibitors and modulators of protein–protein interactions inside bacterial cells.[6,7] Recently, this cyclization method, referred to as SICLOPPS, was combined with genetic code expansion to select a backbone-cyclized peptide inhibitor containing a *p*-benzoylphenylalanine that covalently bound to its target protease, demonstrating the utility of using non-proteinogenic amino acids.[8] In another example of non-reducible macrocyclic peptide selection, Heinis and co-workers used tris(bromomethyl)benzene to cross-link three cysteine residues to create bicyclic peptides displayed on phage, and the resulting peptide library was used to obtain bicyclic peptide inhibitors against several enzymes.[9−12] Although these *in vivo* macrocyclic peptide selections successfully generated novel functional macrocyclic peptides, the library size of *in vivo* selection is inherently limited by the transformation efficiency of *Escherichia coli*.[13] Furthermore, only one non-proteinogenic amino acid per library has incorporated into peptide libraries for *in vivo* selection to date,[8,14,15] which still restricts the functional and structural diversity of the library.

Conversely, *in vitro* peptide selection has the advantage of enabling the screening of larger peptide libraries, which increases the probability of isolating desired peptides. For example, Millward et al. used mRNA display (or *in vitro* virus)[16,17] to generate a highly diverse library of 10^{12} peptides cyclized with a bis-*N*-hydroxysuccimide homobifunctional cross-linker and selected a macrocyclic peptide with high affinity for the target Gαi1 protein.[18] Very recently, the *in vitro*

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peptide selection was developed into the selection of macrocyclic peptides containing non-proteinogenic amino acids by genetic code reprogramming,[19−21] in which, unlike genetic code expansions, multiple (more than three) non-proteinogenic amino acids can be simultaneously incorporated[22−28] to further increase the functional and structural diversity of the library.

In an example of macrocyclic peptide selection using genetic code reprogramming based on flexible tRNA acylation ribozymes (flexizymes)[21,29,30] and mRNA display, anti-Akt2 macrocyclic peptides with completely different sequences were obtained from the L- and D-isomer-initiated libraries, indicating that structurally different types of peptide libraries can be generated from the same mRNA library by simply altering the chirality of a cyclization linker amino acid.[31] Another example showed that all *N*-methyl group removal in the selected macrocyclic *N*-methyl-peptide resulted in the complete loss of affinity for its target E6AP, and all selected macrocyclic *N*-methyl-peptides in the same family had *N*-methylation at the same positions but had different side-chain structures between the corresponding *N*-methyl amino acid residues.[32] These results suggest that not only can structurally different peptide libraries be generated from the same mRNA library by altering the peptide backbone structure of the building blocks but also backbone alteration can increase the structural diversity of a macrocyclic peptide library more than side-chain alteration.

Using aminoacyl-tRNA synthetase-based genetic code reprogramming coupled with mRNA display, Szostak's group selected anti-thrombin macrocylclic peptides with different sequences from the library containing proteinogenic amino acid analogues and the library consisting of only proteinogenic amino acids, which demonstrates that the use of non-proteinogenic amino acid building blocks generates functionally different peptide libraries.[33] Using a similar approach, Hoffman et al. reported the mRNA-display selection of lantipeptides against sortase and showed that chirality reversal of only one amino acid in the selected lantipeptide rendered it completely inactive against sortase, demonstrating the importance of the chirality of endocyclic amino acids for the peptide activity.[34]

Taken together, these macrocyclic peptide selections demonstrate that changing the structure of the linker (e.g., the *N*-acyl group or chirality of an initiator amino acid) and/or modifying the backbone structure (e.g., *N*-methylation or chirality alteration of elongator amino acid building blocks) can generate libraries with distinct properties. Therefore, *in vitro* display selection combined with genetic code reprogramming enables us to discover desired peptides from "quantitatively" and "qualitatively" highly diverse libraries.

Here we report *in vitro* selection of macrocyclic peptides from multiple highly diverse peptide libraries generated using a combination of four types of amino acid linkers for cyclization and two types of elongator amino acid compositions (Figure 1). Although mRNA display can generate highly diverse peptide library (up to 10^{13} compounds), this is a laborious and time-consuming technique involving multiple steps[16,17] and is not suitable for parallel selection from multiple libraries. To overcome these disadvantages, we recently developed a new *in vitro* display method, the TRAP (Transcription−translation coupled with Association of Puromycin-linker) display, which greatly simplifies *in vitro* peptide selection, enabling selection from many libraries in parallel.[35] In the TRAP display, a puromycin molecule attached to the 3′-end of an oligo DNA linker, which is complementary to the 3′-end of mRNA

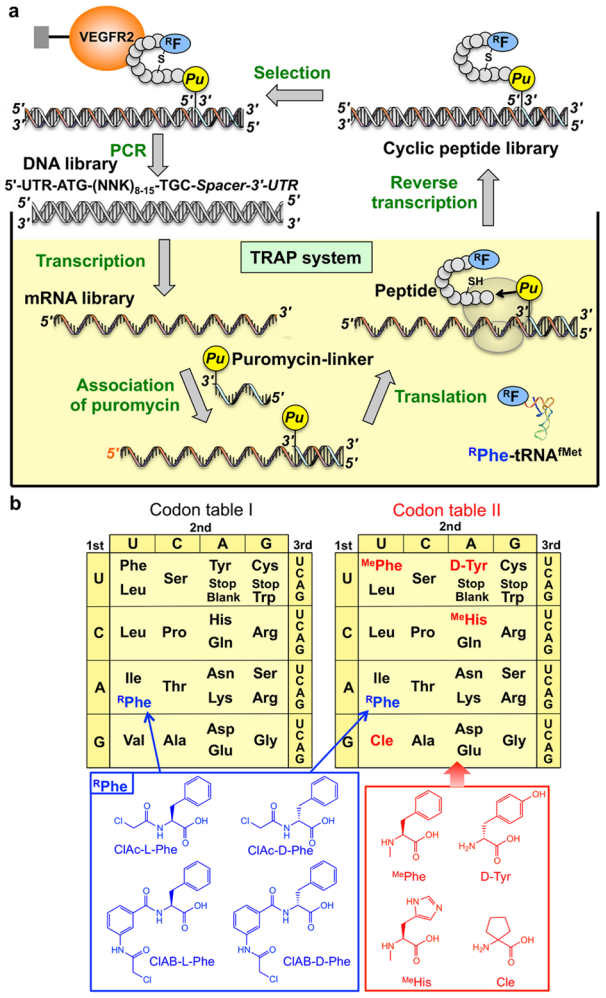

Figure 1. TRAP display selection of macrocyclic peptides against VEGFR2 from multiple libraries created by genetic code reprogramming. (a) Schematic representation of TRAP display selection. The DNA library containing randomized sequences, $(NNK)_{8-15}$, is transcribed and translated into the peptide library. The expressed peptides are spontaneously displayed on their encoding mRNAs through a puromycin linker and are cyclized between a chloroacetamido-modified phenylalanine analogue (^{R}Phe) used as the translation initiator and the C-terminal cysteine residue. The macrocyclic-peptide/mRNA complexes are then reverse transcribed to form macrocyclic-peptide/mRNA/cDNA complexes, and VEGFR2-binding macrocyclic peptides are isolated using VEGFR2-immobilized beads. The recovered cDNAs are amplified by PCR and used for the next round of selection. In this study, selection for VEGFR2 binders was performed using eight types of macrocyclic peptide libraries prepared with combinations of four ^{R}Phe and two sets of elongator amino acids shown in panel b. (b) Reprogrammed codon tables for the preparation of macrocyclic peptide libraries. Codon table 1 was used to prepare macrocyclic peptide libraries composed of 19 proteinogenic amino acids (without Met) as translation elongators. The ^{R}Phe used to initiate translation and cyclize the peptide libraries are shown in blue. ClAc-L-Phe, *N*-chloroacetyl-L-phenylalanine; ClAc-D-Phe, *N*-chloroacetyl-D-phenylalanine; ClAB-L-Phe, *p*-(*N*-chloroacetoamido)benzoyl-L-phenylalanine; ClAB-D-Phe, *p*-(*N*-chloroacetoamido)benzoyl-D-phenylalanine. Codon table 2 was used to prepare macrocyclic peptide libraries composed of 15 proteinogenic amino acids (without Met, Phe, Tyr, His, and Val) and four backbone-modified non-proteinogenic amino acids as translation elongators (red). ^{Me}Phe, *N*-methyl-L-phenylalanine; D-Tyr, D-tyrosine; ^{Me}His, *N*-α-methyl-L-histidine; Cle, cycloleucine.

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libraries, is directly added to a cell-free transcription−translation coupling system (Figure 1a). Inside this system, named the TRAP system, DNA libraries are continuously transcribed and translated into peptide libraries, and the peptide libraries are spontaneously displayed ("trapped") on their encoding mRNA libraries via the puromycin linker. Thus, the peptide−mRNA complex library can be produced from the corresponding template DNA library inside the TRAP system. In our previous study, we established the protocol for the TRAP display and demonstrated *in vitro* selection of one macrocyclic peptide library against human serum albumin as a model target protein.[35] In the present study, we extended the TRAP display method to prepare eight types of macrocyclic peptide libraries with various amino acid linkers for cyclization and elongator amino acid building blocks of different compositions, including multiple backbone-modified non-proteinogenic amino acids by using genetic code reprogramming (Figure 1). We used these libraries to select macrocyclic peptides against a therapeutically important vascular endothelial growth factor receptor 2 (VEGFR2) and demonstrated antagonist activities of the selected macrocyclic peptides against VEGFR2 expressed on the surface of living endothelial cells.

RESULTS AND DISCUSSION

Construction of Multiple Macrocyclic Peptide Libraries. To select macrocyclic peptides that bind to VEGFR2, we designed the TRAP display selection scheme shown in Figure 1. In this selection a DNA library was designed to encode a peptide library consisting of 8−15 random amino acids between an initiator amino acid residue and a Cys residue to be used for peptide cyclization. As the initiator amino acids, the L- and D-isomers of *N*-chloroacetyl-phenyalanine (ClAc-L-Phe, ClAc-D-Phe) and *N*-[3-(2-chloroacetamido)benzoyl]-phenyalanine (ClAB-L-Phe, ClAB-D-Phe) shown in Figure 1b were used to generate four types of thioether-macrocyclic peptide libraries. As described above, the configuration of a linker amino acid could generate different macrocyclic peptide structures even from the same set of the elongator amino acid sequences. Similarly, the insertion of the acetamidobenzoyl moiety in the linker generated different types of macrocyclic peptide libraries. We prepared the macrocyclic peptide libraries by incorporating the corresponding mRNA library into the TRAP system together with 19 proteinogenic amino acids (without Met) and any of the modified phenyalanine analogues charged on initiator tRNAs by flexizyme. These proteinogenic amino acid-based peptide libraries cyclized with ClAc-L-Phe, ClAc-D-Phe, ClAB-L-Phe, and ClAB-D-Phe were designated as the L-, D-, BL-, and BD-libraries, respectively (Figure 1b, codon table 1). The mRNA library rather than the DNA library was used in the TRAP system for the initial library construction to ensure a high diversity of macrocyclic peptide libraries since we previously found that peptide/mRNA complexes could be more efficiently formed when the mRNA templates were used instead of DNA templates.[35] We also estimated the libraries to contain up to 10^{13} peptides in 150 μL translation mixture since we found that approximately 10% of mRNA molecules in the random library could be translated to form peptide/mRNA complexes in our previous study.[35]

In addition to these proteinogenic amino acid-based libraries (Figure 1b, codon table 1), we constructed macrocyclic peptide libraries containing four types of backbone-modified non-proteinogenic amino acids (Figure 1b, codon table 2) in place of the corresponding proteinogenic amino acids (Val, His, Phe, Tyr). The backbone-modified non-proteinogenic amino acids were cycloleucine (Cle), *N*-α-methyl-L-histidine (^{Me}His), *N*-methyl-L-phenylalanine (^{Me}Phe), and D-tyrosine (D-Tyr), which were reported to be substrates for the corresponding *E. coli* wild-type aminoacyl-tRNA synthetases (aaRSs) and to be incorporated into polypeptide chains through a ribosomal translation system.[22,36−38] Because the results in some of these reports were not sufficient evidence for the use of these amino acids in peptide library construction, we first tested the compatibility of these non-proteinogenic amino acids with the *E. coli* reconstituted translation system[39] via *in situ* aaRS-catalyzed tRNA aminoacylation. In a previous report, D-Tyr incorporation was performed using an S-100 *Bacillus subtilis* lysate extract together with *E. coli* ribosomes and precharged D-Tyr-tRNA.[36] We here tested *in situ* synthesis of D-Tyr-tRNA and the incorporation of the D-Tyr into a peptide in the *E. coli* reconstituted translation system.[39] D-Tyr was added to the translation system in place of L-Tyr to express a radioisotope-labeled model peptide. Tricine-SDS-PAGE analysis showed that the D-Tyr was incorporated into the peptide with 49% of the efficiency of L-Tyr incorporation (Supplementary Figure 1b). The mobility of the expressed D-Tyr peptide differed from that of the L-Tyr peptide in tricine-SDS-PAGE, but the mass value of the D-Tyr peptide matched the calculated value (Supplementary Figure 1a). These results indicated that D-Tyr was incorporated into the peptide, which is consistent with our recent result using flexizyme-based tRNA aminoacylation.[40] *In situ* synthesis of ^{Me}Phe-tRNAs and ^{Me}His-tRNAs and the incorporation of these amino acids into a peptide were demonstrated in previous reports; however, contamination with an unmethylated amino acid derived from a commercial *N*-methyl amino acid was observed in the translated peptides.[22,37,38] Szostak's group developed a clever approach for removing unmethylated His, utilizing the fact that His is a more efficient substrate for HisRS than ^{Me}His.[22] However, multiple rounds of tRNA aminoacylation and ethanol precipitation were required for this purification procedure, which diminished the simplicity of *in situ* aaRS-based non-proteinogenic amino acid incorporation. We devised a simple approach for the selective acetyl-capping of contaminating His, utilizing the higher reactivity of the primary amine of His compared with that of the secondary amine of ^{Me}His. Commercial ^{Me}His was pretreated with a smaller amount of acetic anhydride to acetylate the primary amine of contaminating His. Subsequently, the ^{Me}His solution pretreated with the acetic anhydride was added directly to the translation reaction without any purification, yielding a ^{Me}His-containing peptide without misincorporation of contaminated His (Supplementary Figure 1a). Although misincorporation of contaminating Phe was observed in a previous report using ^{Me}Phe,[38] the direct addition of unpurified commercial ^{Me}Phe to the translation system produced a ^{Me}Phe-containing peptide as the sole product (Supplementary Figure 1a), presumably because of the higher purity of the commercial ^{Me}Phe we used. Furthermore, because the previous report demonstrated only the qualitative incorporation of ^{Me}Phe,[38] we quantified the incorporation efficiency of ^{Me}Phe and found it to be 99% of the Phe incorporation efficiency (Supplementary Figure 1b).

These four backbone-modified non-proteinogenic amino acids were reassigned to the corresponding codons in the mRNA libraries according to the reprogrammed genetic code (Figure 1b, codon table 2). Similarly to the proteinogenic amino acid-based libraries, the non-proteinogenic amino acid-

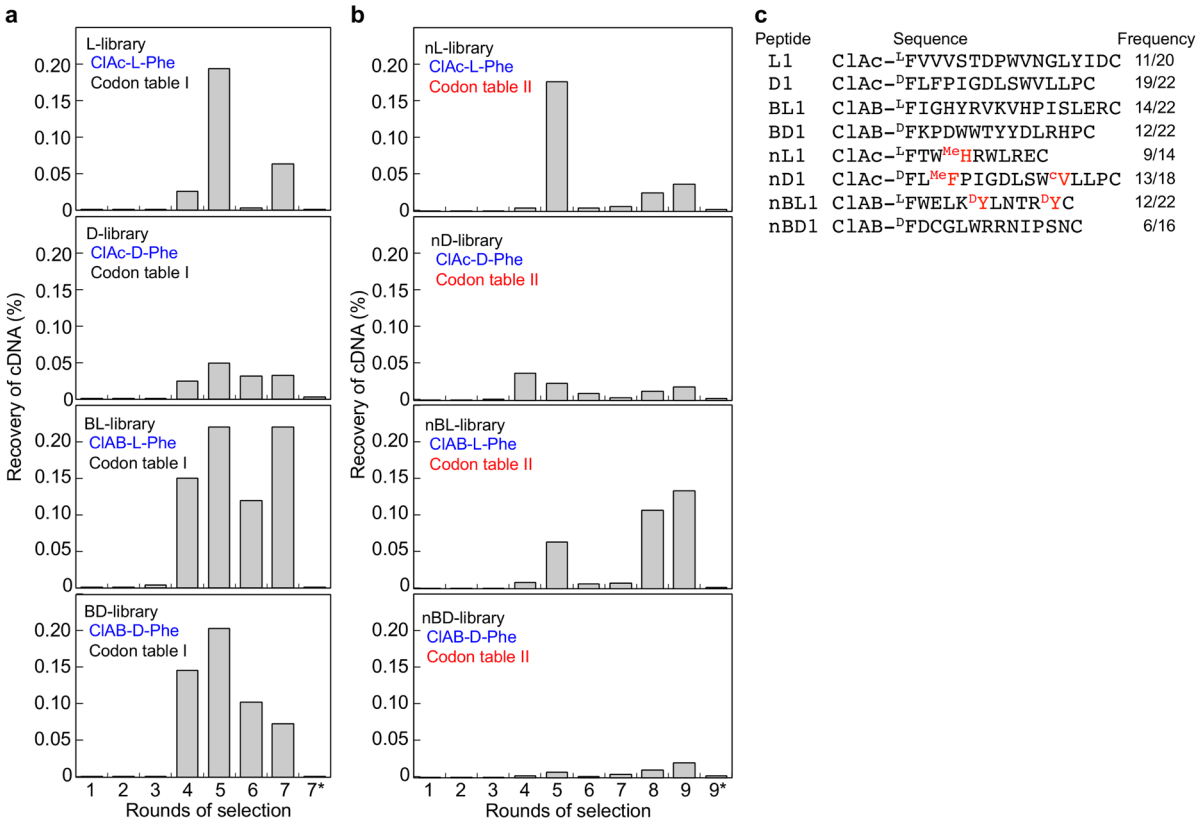

Figure 2. Selection progress for VEGFR2 binding from multiple macrocyclic-peptide libraries and the selected sequences from each library. Progress of TRAP display selection from multiple libraries prepared by alternating the linker and elongator amino acids sets containing either the 19 proteinogenic amino acids in codon table 1 (a) or the 15 proteinogenic and four non-proteinogenic amino acids in codon table 2 (b). Recovery of cDNAs in each round of selection was determined by quantitative PCR. Beginning with round six, the selection pressure was increased by incubating the libraries with immobilized VEGFR2 at 37 °C and washing more rigorously. The L-, D-, BL-, and BD-libraries represent proteinogenic amino acid-based peptide libraries (codon table 1) cyclized with ClAc-L-Phe, ClAc-D-Phe, ClAB-L-Phe, and ClAB-D-Phe, respectively. The nL-, nD-, nBL-, and nBD-libraries represent the non-proteinogenic amino acid-based peptide libraries (codon table 2) cyclized with ClAc-L-Phe, ClAc-D-Phe, ClAB-L-Phe, and ClAB-D-Phe, respectively. cDNA recoveries from round 7* in panel a and round 9* in panel b were determined with VEGFR2-free beads. (c) Sequences of the most abundant macrocyclic peptide selected from each library and their frequency of appearance in the clone sequencing. Elongator non-proteinogenic amino acid residues in the cloned peptide sequences are highlighted in red. ^{L}F, L-phenylalnine; ^{D}F, D-phenylalanine; ^{Me}F, *N*-methyl-L-phenylalanine; ^{D}Y, D-tyrosine; ^{Me}H, *N*-α-methyl-L-histidine; ^{C}V, cycloleucine.

based peptide libraries cyclized with ClAc-L-Phe, ClAc-D-Phe, ClAB-L-Phe, and ClAB-D-Phe were designated as the nL-, nD-, nBL-, and nBD-libraries, respectively.

Selection of VEGFR2-Binding Macrocyclic Peptides Using TRAP Display. The eight types of libraries displayed on mRNAs in the TRAP system were used in parallel for *in vitro* selection against the extracellular domain of VEGFR2 immobilized on magnetic beads (Figure 1a). VEGFR2-free magnetic beads were used throughout the selection to remove undesired bead-binding peptides. To monitor the VEGFR2-binding ability of the libraries in each round of selection, we determined the ratios of recovered cDNAs to input cDNAs in each round of selection using quantitative PCR (Figure 2a, b).

In the selections with proteinogenic amino acid-based libraries, the cDNA recovery rates increased during the fourth and fifth rounds (Figure 2a). To obtain higher-affinity macrocyclic peptides, we increased the selection stringency in the sixth and seventh rounds by raising the incubation and washing temperature of the libraries and VEGFR2-immobilized beads from 25 to 37 °C. For the seventh round, we measured cDNA recoveries from both VEGFR2-immobilized and VEGFR2-free magnetic beads and confirmed that the recovery of selected peptides was strongly dependent on the presence of VEGFR2 (Figure 2a, round 7 vs 7*). Similarly, in the selections with the non-proteinogenic amino acid-based libraries, cDNA recoveries increased in the fourth and fifth rounds (Figure 2b). After another four rounds of selection at 37 °C, VEGFR2 dependency was confirmed as above (Figure 2b, round 9 vs 9*).

The cDNAs obtained from the final round of the selection were cloned, sequenced, and translated according to the codon tables shown in Figure 1b. The most abundant sequence from each library and its frequency of appearance are shown in Figure 2c (see Supplementary Figure 2 for all selected sequences). A single specific sequence was selected from each library with high frequency, indicating that the serial selections were adequate for isolating the strongest VEGFR2-binding macrocyclic peptide from each library. The most abundant macrocyclic peptide clones in the proteinogenic amino acid-based L-, D-, BL-, and BD- libraries were designated as L1, D1, BL1, and BD1, respectively (Figure 2c). The most abundant clones from the non-proteinogenic amino acid-based nL-, nD-, and nBL-libraries, similarly designated as nL1, nD1, and nBL1, respectively, included one or two non-proteinogenic amino acids, whereas the most abundant clone in the nBD-library did not contain any non-proteinogenic amino acids in its random

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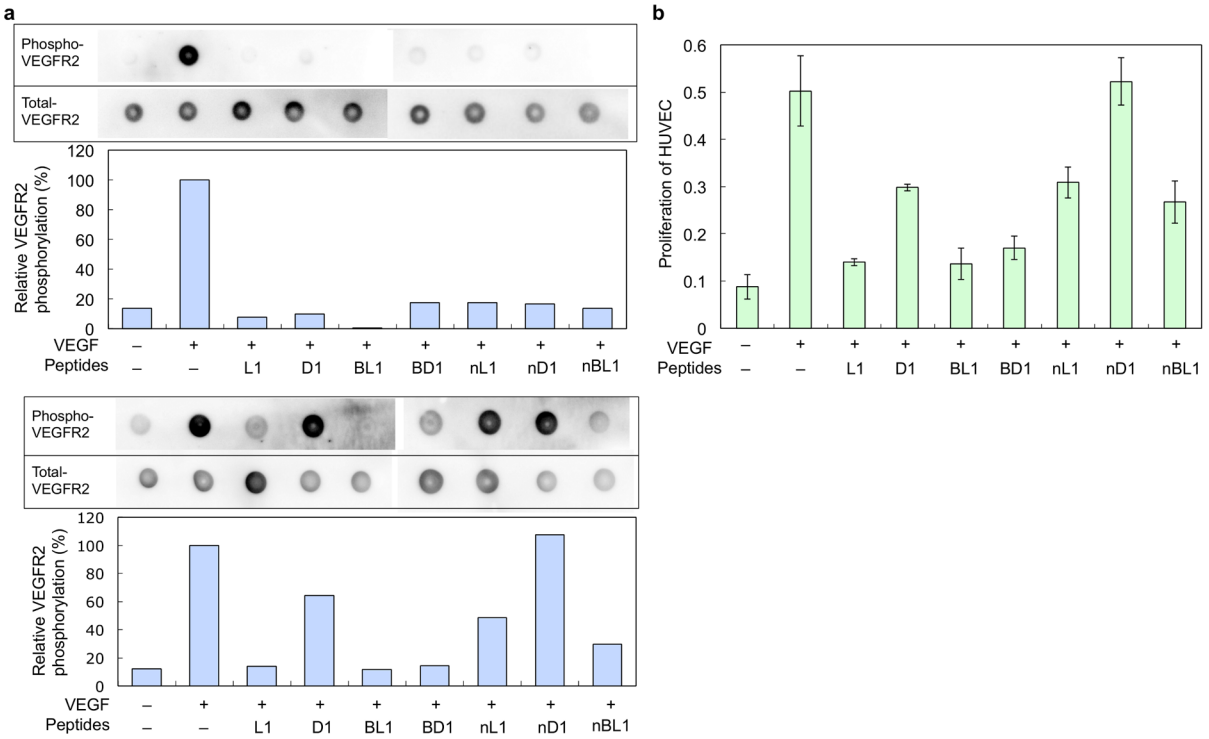

Figure 3. Screening of the selected cyclic peptides for VEGFR2 inhibition. (a) Dot-blot assays to determine the VEGF-induced autophosphorylation levels of VEGFR2 expressed on HUVEC. Cells were treated with 10 μM (upper panel) or 1 μM (lower panel) concentrations of each macrocyclic peptide for 15 min, followed by treatment with 10 ng/mL VEGF for 7 min. Total VEGFR2 and phosphorylated VEGFR2 were visualized by dot blotting of cell lysates with antibodies specific for VEGFR2 and phosphorylated VEGFR2, respectively. The signal ratio of phosphorylated VEGFR2 to total VEGFR2 with VEGF treatment alone was defined as 100% phosphorylation. (b) BrdU assays for inhibition of HUVEC proliferation by the selected peptides. HUVEC were treated with 10 μM concentration of each macrocyclic peptide for 30 min, followed by co-incubation with 10 ng/mL VEGF. After 24 h, the cells were labeled with BrdU in the presence of the macrocyclic peptide and VEGF for 4 h, fixed, and stained with peroxidase-conjugated anti-BrdU antibody. Peroxidase activity was detected with tetramethylbenzidine colorimetric substrate. Control cells were treated with or without VEGF in the absence of peptide. Assays were performed in triplicate. Error bars, ± 1 SD.

region. In addition, cDNA recovery from the nBD-library was low, indicating the absence of a high-affinity peptide in the nBD-library; therefore, nBD1 peptide was not used in further experiments. The VEGFR2-binding activities of each of the most abundant cloned peptides (L1, D1, BL1, BD1, nL1, nD1, and nBL1) were confirmed by pull-down assays (Supplementary Figure 3).

VEGFR2 Antagonist Activity-Based Screening of *in Vitro* Selected Macrocyclic Peptides. Because the macrocyclic peptides were selected by their ability to bind to VEGFR2, they were not necessarily VEGFR2 antagonists. Therefore, we chemically synthesized the selected macrocyclic peptides with C-terminal amidated glycines by Fmoc solid-phase peptide synthesis for use in activity-based screening. In the initial screening, we used sufficiently pure macrocyclic peptides without HPLC purification (Supplementary Figures 4 and 5 and Supplementary Table 1) to examine the effects on the VEGF-dependent autophosphorylation activity of VEGFR2 expressed on the surface of human umbilical vein endothelial cells (HUVEC). The VEGFR2 phosphorylation levels were determined by dot blot analysis of HUVEC lysates using anti-VEGFR2 and anti-phospho-VEGFR2 antibodies. The specificities of the antibodies were confirmed by Western blotting of HUVEC lysates with and without VEGF stimulation (Supplementary Figure 6). HUVEC treatment with each of the synthesized macrocyclic peptides (10 μM) almost completely blocked VEGF-induced VEGFR2 phosphorylation (Figure 3a, upper panel), whereas treatment with 10 μM concentration of a control macrocyclic peptide (rBL1, the reverse of the BL1 sequence) had no effect on the phosphorylation (data not shown). This indicated that all of the *in vitro* selected macrocyclic peptides had at least some antagonist activity. When the peptide concentration was reduced to 1 μM, four (L1, BL1, BD1, and nBL1) of the seven peptides retained their inhibitory activity (Figure 3a, lower panel). To determine whether this inhibition of autophosphorylation by the macrocyclic peptides inhibited HUVEC proliferation, the peptides were tested in a BrdU-labeled cell proliferation assay (Figure 3b). A similar trend of macrocyclic peptide inhibitory activity was observed. These results suggested that the L1, BL1, BD1, and nBL1 macrocyclic peptides inhibited HUVEC proliferation by blocking VEGF-induced phosphorylation of VEGFR2.

Characterization of the Macrocyclic Peptide Antagonists. The four macrocyclic peptides, L1, BL1, BD1, and nBL1, were purified by HPLC, and their affinities for VEGFR2 were determined using the ForteBio system. The dissociation constants of L1, BL1, BD1, and nBD1 were 94 nM, 8 nM, 2 nM, and 33 nM, respectively, demonstrating their high affinity for VEGFR2 (Supplementary Figure 7 and Supplementary Table 2).

Next, we determined the dose dependency of inhibition of VEGF-induced VEGFR2 autophosphorylation by the purified macrocyclic peptides. HUVEC were incubated with varying concentrations of the peptides, and VEGFR2 phosphorylation levels were assayed by dot blots. The macrocyclic peptides L1

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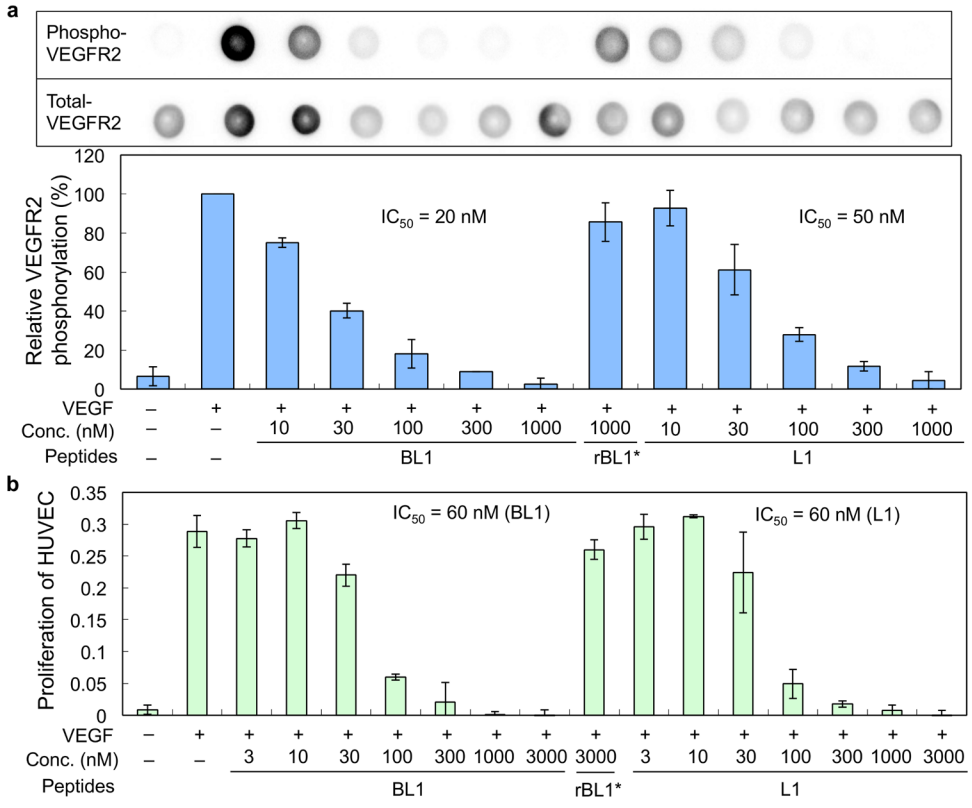

Figure 4. VEGFR2 inhibitory activity of the purified macrocyclic peptides L1 and BL1. (a) Inhibition of VEGF-induced VEGFR2 autophosphorylation on HUVEC was determined using dot blot assay as described in the legend to Figure 3a. HUVEC cells were treated with various concentrations of HPLC-purified L1 or BL1. rBL1*, the reverse of the BL1 sequence, was used as a control macrocyclic peptide. Experiments were performed in triplicate. Error bars, ± 1 SD. (b) Inhibition of HUVEC proliferation determined by BrdU assays. HUVEC were treated with various concentrations of each macrocyclic peptide, and cell proliferation was determined as described in the legend to Figure 3b. Experiments were performed in triplicate. Error bars, ± 1 SD.

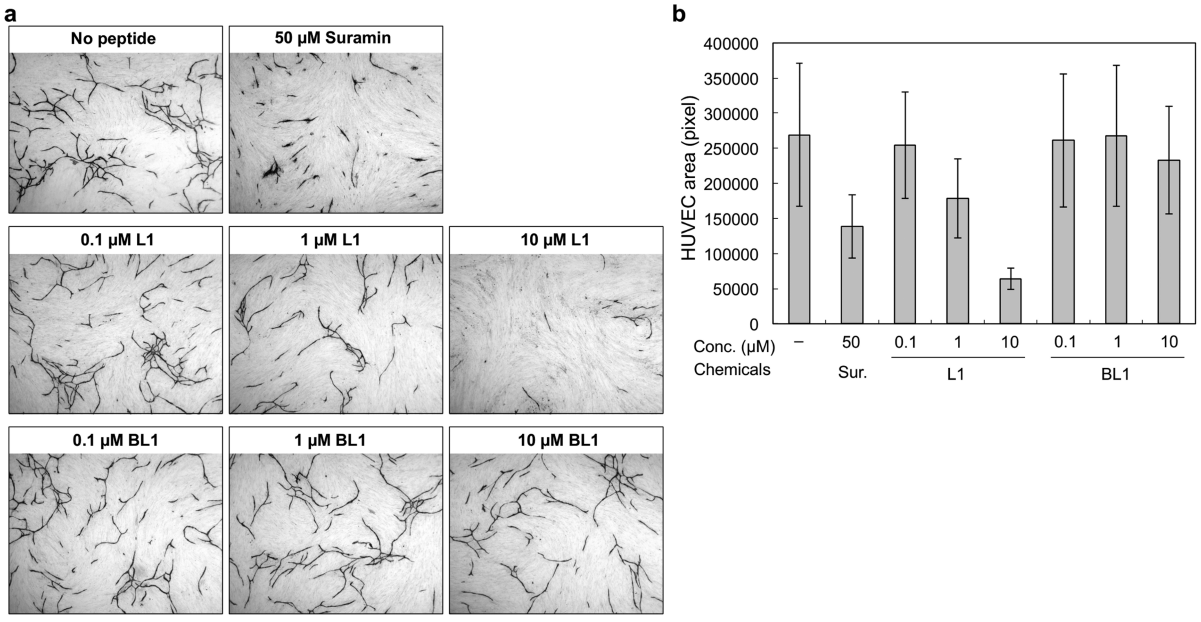

Figure 5. Effects of the macrocyclic peptides L1 and BL1 on angiogenesis as measured by HUVEC tube formation. Co-cultured HUVEC and fibroblasts were incubated with 10 ng/mL VEGF and various concentrations of the macrocyclic peptides. After 11 days, the cells were fixed and immunostained with anti-CD31 antibody. (a) Representative phase-contrast microscopic images of capillary structures formed by HUVEC are shown. The images were taken at 2.52× magnification. (b) HUVEC areas in 10 images from two different cell samples for each condition were quantified using an image analyzer and statistically analyzed. Error bars, ± 1 SD. Surinam (Sur.) is a small molecule control that is known to inhibit HUVEC angiogenesis.

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and BL1 inhibited VEGFR2 phosphorylation with IC_{50} values of 50 and 20 nM, respectively (Figure 4a). In contrast, BD1 and nBL1 only modestly inhibited VEGFR2 phosphorylation, with IC_{50} values between 300 and 1000 nM (Supplementary Figure 8). Therefore, the more potent L1 and BL1 macrocyclic peptides were further tested for inhibition of HUVEC cell proliferation. Both macrocyclic peptides had IC_{50} values of 60 nM in the VEGF-induced endothelial cell proliferation assay (Figure 4b). The control rBL1 macrocyclic peptide failed to inhibit VEGFR2 phosphorylation and HUVEC proliferation at 1 and 3 μM, respectively (Figure 4), suggesting that the amino acid sequence, rather than the amino acid composition alone, was important for VEGFR2 inhibition.

We compared the blocking of VEGF-dependent angiogenesis by L1 and BL1 using a HUVEC tube formation assay. VEGF and various concentrations of L1 or BL1 were added to the culture media, and HUVEC were co-cultured with fibroblasts for 11 days. Lumens were visualized by fixing the HUVEC cells and immunostaining with anti-CD31 antibody (Figure 5). Although BL1 did not significantly inhibit VEGF-dependent HUVEC angiogenesis, L1 clearly inhibited angiogenesis in a dose-dependent manner.

Discussion. Since late 2011, several functional macrocyclic peptides have been isolated by combining genetic code reprogramming with mRNA display selection of one or two kinds of macrocyclic peptide libraries.[31−34,41] These reports demonstrated the inhibitory activity of the macrocyclic peptides against target enzymes *in vitro* but not *in vivo*. In this study, we successfully demonstrated the activity of selected macrocyclic peptides against the target VEGFR2 expressed on the live endothelial cells. Furthermore, similarly to the macrocyclic peptides that were previously selected by mRNA display,[31,41] we found that the macrocyclic peptides L1 and BL1 selected by TRAP display had isoform-specificity for VEGFR2 over VEGFR1 and VEGFR3 (Supplementary Figure 9).

VEGFR2 consists of an extracellular domain, a transmembrane domain, and an intracellular tyrosine kinase domain.[42,43] It interacts with its dimeric protein ligand VEGF through the extracellular domain. The interaction of VEGFR2 with VEGF results in VEGFR2 dimerization, and the VEGFR2 dimers are activated through trans-phosphorylation by the intracellular tyrosine kinase domain. VEGFR2 activation stimulates several different endothelial cell functions such as migration, proliferation, and cell survival, all of which play important roles in angiogenesis. The disruption of the VEGFR2−VEGF interaction has been shown to be clinically important in the treatment of pathologic angiogenesis, such as retinal angiopathies and cancers with metabolic demands for oxygen and other nutrients that require increased vasculature.[44]

In this study, we obtained VEGFR2-binding macrocyclic peptides from various libraries constructed with combinations of four types of cyclization linkers and two types of elongator amino acid compositions (Figure 1b). Each library yielded a distinct macrocyclic peptide as the most abundant clone in the library through the TRAP display selection against VEGFR2 (Figure 2c and Supplementary Figure 2). This result suggests that multiple macrocyclic peptide libraries with distinct properties can be produced by altering the elongator amino acid compositions or the cyclization linker structures.

Of the selected peptides, four had nanomolar affinities and low IC_{50} values for VEGFR2. The macrocyclic peptide affinities for immobilized VEGFR2-Fc chimera and the IC_{50} values for VEGF-induced VEGFR2 autophosphorylation were not necessarily correlated (Figure 4, Supplementary Figure 8, and Supplementary Table 2): L1, K_d = 94 nM vs IC_{50} = 50 nM; BL1, K_d = 8 nM vs IC_{50} = 20 nM; BD1, K_d = 2 nM vs IC_{50} > 300 nM; and nBL1, K_d = 33 nM vs IC_{50} > 300 nM. This difference may be (1) because the structure of extracellular domain of VEGFR2 immobilized on beads through Fc-tag and VEGFR2 expressed in live HUVEC were not completely the same and/or (2) because the VEGFR2-binding site of the macrocyclic peptides was different. Furthermore, although L1 and BL1 had similar IC_{50} values in the VEGF-induced VEGFR2 autophosphorylation and cell proliferation assays, their inhibitory activities against VEGF-dependent angiogenesis of HUVEC differed (Figure 5). This may be because of the higher serum stability of L1 compared to that of BL1 (Supplementary Figure 10) since the angiogenesis assay was conducted in the serum-containing media, whereas the VEGFR2-phosphorylation assay and HUVEC proliferation assay were performed under the serum-starved condition. These results suggest that there are clear limitations to affinity-based selections for isolating molecules that inhibit the activity of target proteins in living cells. Thus, the following steps appear to constitute a good strategy for obtaining macrocyclic peptides with high inhibitory activity in live cells: preparation of various types of libraries, parallel selection for high-affinity binding to the target, and screening of the selected macrocyclic peptides for target inhibition using activity-based live-cell assays. The TRAP display facilitates this strategy by markedly simplifying the selection procedure using multiple libraries in parallel.

The L1 macrocyclic peptide was much more potent than previously reported small molecule ("monomer") antagonists obtained from combinatorial libraries.[45−48] In addition, because of their small size, these macrocyclic peptides have potentially significant advantages over protein-based antagonists for manufacturing and for tissue penetration. These small macrocyclic peptides may also be useful for blocking VEGFR2 on both the luminal and abluminal sides of endothelial cells particularly because VEGFR2 is predominately expressed on the abluminal surface.

Further optimization of the amino acid sequences in the macrocyclic peptide antagonists obtained in this study through affinity maturation should yield more potent antagonists, as observed in previous reports on affinity maturation of protein-based VEGFR2 antagonists.[49,50] Even when *in vitro* selection for affinity maturation is based on VEGFR2 binding rather than antagonism, the binding sites of the affinity-maturated peptides are expected to be the same as those of the progenitor peptides. Thus, binding-based *in vitro* selection that can rapidly survey highly diverse libraries, such as TRAP display selection, becomes a powerful approach in such studies. Potent small molecule antagonists have also been developed by the dimerization of ligands with modest inhibitory activity against dimeric VEGFR2.[45−48] For example, Udugamasooriya et al. generated a dimeric derivative of a small peptoid isolated from their chemical combinatorial library and elegantly demonstrated its VEGFR2 antagonistic activity not only in cultured endothelial cells but also in a murine model.[46] Therefore, our future work will involve the generation of more potent antagonists through similar dimerizations of macrocyclic peptides, together with affinity maturation and determination of the binding modes of the macrocyclic peptides to VEGFR2.

In conclusion, our TRAP display-based selection of macrocyclic peptides from multiple highly diverse libraries enabled us to isolate potent VEGFR2 antagonists. We demonstrated their

antagonistic activity in live endothelial cells using various functional assays including phosphorylation, HUVEC proliferation, and angiogenesis. We believe that our TRAP display-based selection can be extended to the discovery of macrocyclic peptide drug candidates for other clinically important extracellular proteins, and such efforts are currently underway in our laboratory.

METHODS

Preparation of Macrocyclic Peptide Libraries. The macrocyclic peptide libraries for the TRAP display selection were prepared by a similar procedure described elsewhere[35] with several modifications to prepare eight types of the libraries. For the preparation of random DNA templates encoding random peptides for TRAP display selection, T7SD8M2.F44 and SD8NNK$_{(8-15)}$ were annealed and extended by KOD-plus-neo DNA polymerase (TOYOBO, Japan), and then the resulting dsDNAs were amplified using T7SD8M2.F44 and G5S-4an16.R36 by Taq DNA polymerase (see the Supporting Information for the sequences of oligo DNA). Amplified DNAs were purified by extraction with phenol/chloroform and ethanol precipitation. The prepared random DNA templates were transcribed by runoff *in vitro* transcription using T7 RNA polymerase, and the random mRNAs were purified by isopropanol precipitation. An mRNA library was prepared by mixing the random mRNAs containing 8−15 repeated NNK sequences. Four kinds of libraries of a proteinogenic amino acid-based macrocyclic peptide/mRNA complex were prepared by translation in the Met-depleted TRAP system at a scale of 150 μL containing 2.5 μM of the mRNA library, 2.5 μM puromycin-linker (BEX, Japan), and either 10 μM ClAc-L-Phe-tRNA^{fMet}(CAU), 20 μM ClAc-D-Phe-tRNA^{fMet}(CAU), 10 μM ClAB-L-Phe-tRNA^{fMet}(CAU), or 20 μM ClAB-D-Phe-tRNA^{fMet}(CAU) for 25 min at 37 °C. Likewise, four kinds of libraries of a non-proteinogenic amino acid-based macrocyclic peptide/mRNA complex were prepared in the similar Met-deficient TRAP system except that ^{Me}His, ^{Me}Phe, D-Tyr, and Cle together with additional HisRS and native tRNA^{Tyr} were included instead of His, Phe, Tyr, and Val. Then, EDTA was added to the solution of the mRNA-displayed macrocyclic peptide library to dissociate ribosomes.

TRAP Display Selection of Anti-VEGFR2 Macrocyclic Peptides. The TRAP display selection was performed by a similar procedure described elsewhere[35] with several modifications. For the first round selection, VEGFR2-Fc (R&D Systems, amino acid residues 1−764 of the extracellular domain of human VEGFR2 fused to the hexa-histidine -tagged Fc of human IgG via the IEGRMD peptide) immobilized on Protein G magnetic beads (VERITAS) was used at a concentration of 30 nM VEGFR2-Fc and mixed with the solution of the mRNA-displayed macrocyclic peptide library at 25 °C for 30 min. After supernatant was removed, the beads were washed with HBS-T (50 mM HEPES-K pH 7.5, 300 mM NaCl, 0.05% tween 20) three times, and mRNA-displayed macrocyclic peptides on the VEGFR2 immobilized beads were reverse transcribed using G5S-4.R20 primer by M-MLV reverse transcriptase (Promega) at 42 °C for 30 min. The reaction mixture was diluted 20 times with the PCR premixture solution [10 mM Tris-HCl pH 8.4, 50 mM KCl, 0.1%(v/v) Triton X-100, 2 mM $MgCl_2$, 0.25 mM each dNTP, 0.25 μM T7SDM2.F44, 0.25 μM G5S-4.R36], and the selected cDNAs on the beads were eluted at 95 °C for 5 min. The amount of the eluted cDNAs was quantified by SYBR green-based quantitative PCR using T7SD8M2.F44 and G5S-4an16.R36 as primers and the eluted cDNAs were PCR-amplified using T7SD8M2.F44 and G5S-4an16.R36 by Taq DNA polymerase for the next round of selection. From the second round, libraries of mRNA-displayed macrocyclic peptide were prepared by transcription and translation in the TRAP system (10 μL for second round and 2.5 μL for the later rounds) containing 1 μM T7 RNA polymerase and 5 vol % of the crude PCR mixture containing cDNA library obtained from the previous round. The reverse transcription of mRNAs displaying macrocyclic peptides was performed using RNase H-inactivated reverse transcriptase (TOYOBO, ReverTra) at 42 °C for 30 min prior to the selection. After quenching the reverse transcription with EDTA and neutralizing the solution with HEPES, the mixture containing cDNA/mRNA-displayed macrocyclic peptides were incubated with Fc immobilized on Protein G magnetic beads at 25 °C for 5 min for negative selection. This negative selection was preformed five times to remove the peptides that bind to Fc and Protein G magnetic beads. Subsequently, the supernatant was mixed with the VEGFR2-immobilized beads at 25 °C for 30 min for positive selection, followed by thrice washing with HBS-T at 25 °C until 5 rounds. From 6 rounds, the positive selection was performed at 37 °C for 30 min, followed by washing with HBS-T at 37 °C. Similarly to the first round, the selected cDNAs on the beads were eluted at 95 °C for 5 min and quantified by quantitative PCR. The cDNAs were amplified by PCR for the next round of selection.

ASSOCIATED CONTENT

Supporting Information

Additional methods and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): Authors Hiroshi Murakami, Hiroaki Suga, Patrick C. Reid declare competing financial interests, as founders and shareholders in PeptiDream Inc, a peptide drug discovery company using similar technology.

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