Supplemental information for:

Construction of crown ether-like supramolecular library by conjugation of genetically-encoded peptide linkers displayed on bacteriophage T7

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Supplemental figures S2-S9
Materials and methods S10-S19
Supplemental references S20
**Figure S1.** Tandem mass spectrometric analysis of the crown analogue displayed on T7 phage. (A) A model T7 phage monoclonal modified by the 10BASE₄-T was separated into subunits by SDS-PAGE. After Coomassie brilliant blue staining (left panel), the protein band corresponding to the crown analogue-fused gp10 (magenta asterisk) was excised and subjected to in-gel lysyl endopeptidase (Lys-C) digestion followed by LC-MS/MS analysis. A blue arrow indicates the Lys-C cleavage site. (B) MS spectrum (left panel). A series of multiple charged ions (green circles) were detected, and consistent with calculated m/z values of the crown analogue. MS/MS spectrum (right panel).
Figure S2. Infectivity of modified T7 phage. A T7 phage library (-S-G-G-X$_3$-C-X$_7$-C-X$_3$; X represents any randomized amino acid) was treated with or without EBB (500 μM) in the presence of TCEP (500 μM) under standard conditions. The number of plaque forming units was determined by a serial dilution method and plaque assay. The graph summarizes the results of three independent experiments. Error bars represent standard deviations. Statistical analysis was performed by unpaired Student’s $t$-test. n.s., not significant.
Figure S3. Biopanning against Hsp90 using the crown ether-like supramolecule candidate library (-S-G-G-G-X$_3$-C*-X$_7$-C*-X$_3$; Cys are conjugated with EBB). (A) T7 phage polyclones after each round (R) of biopanning were modified with EBB. Both the modified and unmodified T7 phage polyclones were subjected to ELISA. Bovine serum albumin (BSA) served as a mock protein. (B) T7 phage monoclones were chosen from the phage pool after 6 rounds of biopanning, and then subjected to DNA sequencing (see Fig. S4A). Sequence logo was generated by WebLogo program.
Figure S4. (A) DNA sequencing of a T7 phage monoclone that was isolated from the phage pool after 6 rounds of biopanning shown in Fig. S3. Unexpectedly, in addition to a couple of designated restriction enzyme sites (EcoRI/HindIII), an additional EcoRI site appeared in the middle of the insert DNA ligated to a T7Select vector. The DNA sequence data was generated by 4peaks software (http://nucleobytes.com/index.php/4peaks). (B) Design of the insert DNA fragment for the ligation into the T7Select-10 vector by using unique restriction enzyme sites (i.e.,
EcoRI and HindIII). The codon after the designated EcoRI site in the insert DNA was 5’-TCT-GGT-GGT-GGT-(NNK)₃-TGT-(NNK)₇-TGC-(NNK)₃-TAA-3’, which encodes the corresponding peptide (-S-G-G-G-X₃-C-X₇-C-X₃; X represents randomized amino acid). To encode all of the twenty amino acids in the randomized region, NNK codon was used and the corresponding mixed nucleotide is shown in lower panel. (C) A possible mechanism of a misligation during the construction of the T7 phage library. Restriction sites such as EcoRI can unintentionally be produced in the randomized region of the PCR product (upper panel). This DNA fragment is doubly-digested by EcoRI to afford a short DNA fragment 1 (upper-middle panel). The short fragment 1 possessing two EcoRI sites can be ligated into a T7Select vector tandemly with another fragment 2 (lower-middle panel). The ligated fusion DNA is translated as a lengthy peptide-fused gp10 (lower panel). This assumption was supported by the experimental data represented in Fig. S4A: (i) two EcoRI and one HindIII sites were found in the peptide encoding region (i.e., within the cloning site) of a T7 phage monoclone gene. (ii) After the second appearance of the EcoRI site, the DNA sequence of the fragment 2 (i.e., 5’-TCT-GGT-GGT-GGT-(NNK)₃-TGT-(NNK)₇-TGC-(NNK)₃-TAA-3’ encoding -S-G-G-G-X₃-C-X₇-C-X₃) was fully conserved.
Figure S5. Biopanning against Hsp90 using the naïve T7 phage library (-S-G-G-G-X₃- C-X₇-C-X₃). (A) ELISA result shows enrichment of Hsp90-binding phage through 6 rounds of biopanning. (B) Sequence alignment of Hsp90 binder (upper column) and non-binder (lower column). The alignment was generated by ESPript program (http://espript.ibcp.fr).² Consensus sequences are highlighted. Frequency of each amino acid sequence is shown on the left side, and summarized into a sequence logo by WebLogo program.¹ Hsp90-binding avidities are shown on the right. The consensus sequence was completely different from what was discovered in the Hsp90 NTD-binding crown analogue (Fig. S3B); only N-terminal arginine and C-terminal hydrophobic aromatic amino acid (tryptophan) were identical.
Figure S6. CD spectra of synthetic peptide linker and the corresponding Hsp90 NTD-binding crown analogue. The amino acid sequence of the linker is H$_2$N–R-S-W-C-R-K-S-R-K-N-S-G-G-G-L-V-W-C-F–OH.
**Figure S7.** (A) Determination of Hsp90 N-terminal domain (NTD)-binding affinity of fluorophore-conjugated geldanamycin (GA) by FP assay. Middle domain (MD) and C-terminal domain (CTD) of Hsp90 were used as mock proteins for negative controls. Error bars represent standard deviations. The binding affinity of geldanamycin to Hsp90 NTD was close to a previously reported one.³ (B) Competitive binding assay. Non-labeled GA and mock peptide (H₂N-G-C-D-P-E-T-G-T-C-G-OH) served as a positive and negative control, respectively. The Hsp90 NTD-binding crown analogue did not compete with GA-FITC, or possibly might enhance binding of GA-FITC to Hsp90.
Materials and methods

Synthesis of EBB

\[\text{H}_3\text{N} \overset{\text{O}}{\text{O}} \overset{\text{O}}{\text{O}} \overset{\text{NH}_2}{\text{O}} + \overset{\text{Br}}{\text{O}} \overset{\text{Br}}{\text{O}} \overset{\text{K}_2\text{CO}_3}{\rightarrow} \overset{\text{Br}}{\text{O}} \overset{\text{N}}{\text{O}} \overset{\text{O}}{\text{O}} \overset{\text{Br}}{\text{O}} \overset{\text{H}}{\text{H}} \]

\(N,N'\text{-}[1,2\text{-ethanediyl-oxyl-2,1-ethanediyl}]\text{bis}(2\text{-bromoacetamide})\) (EBB; \(1\)) was prepared as previously reported\(^4\) with minor modifications. Briefly, 2,2’-\((\text{ethylenedioxy})\text{bis(ethylamine)}\) (25 mmol; cat. No. 385506, Sigma-Aldrich) and potassium carbonate (60 mmol) were mixed in 100 mL of \(\text{H}_2\text{O}/\text{AcOEt} = 1:1\) solution. Then, bromoacetyl bromide (75 mmol; cat. No. B56412, Sigma-Aldrich) was added, and the mixture was stirred for 4 hours at room temperature. The organic layer was collected, and then the solvent was evaporated (0.86 g: 81% yield). The crude reaction product (0.37 g) was dissolved in pure water, and purified by reverse-phase middle pressure liquid chromatography (Yamazen ODS column 26 \(\times\) 300 mm, flow rate 20 mL/min with gradient 5-100% MeOH in pure water over 20 min). The fractionated sample was lyophilized (55% yield). ESI-IT-MS and \(^1\text{H}\) NMR (300 MHz, CDCl\(_3\)) spectrum were shown in below (Fig. S8).
Construction of a crown ether-like supramolecule candidate library via the 10BASE<sub>d</sub>-T

Synthesis of the crown analogue on T7 phage was performed as described previously.<sup>5, 6</sup> Standard reaction conditions of the gp10 based-thioetherification (10BASE<sub>d</sub>-T) are the following: T7 phage particles (approximately \(1.0 \times 10^{11}\) plaque-forming units) were well suspended by sonication or vortex in 700 μL of phosphate buffered saline (PBS) supplemented with 400 mM NaCl. After centrifugation at 12,000 rpm for 5 minutes at room temperature, the supernatant was mixed with neutralized TCEP aqueous solution at a final concentration of 500 μM at 4 °C: optimal molar concentration of EBB was estimated at 0.5 to 1.0 mM by LC-MS-based quantification.
of an intact peptide. Conversion yield to a crown analogue was maximum 80% (data not shown). EBB aqueous solution was added at a final concentration of 500 μM, and the mixture was incubated at 4 °C for 3 hours in the dark. To inactivate the unreacted EBB, 2-mercaptoethanol was added to the mixture at a final concentration of 5 mM, and further incubated at 4 °C for several minutes. The T7 phage particles were precipitated with a mixture of polyethylene glycol 6000 and sodium chloride to final concentrations of 5% w/v and 0.5 M, respectively. After centrifugation at 15,000 rpm for 10 minutes at 4 °C, the precipitate was dissolved in an appreciate buffer.

Figure S9. Western blotting against peptide-fused gp10. Trace amounts of gp10 dimer were formed in an EBB-concentration dependent manner.

**Mass spectrometric analysis**

Mass spectrometric analysis was performed as described previously.\textsuperscript{5, 6} The gel was stained with rapid stain CBB kit (Nacalai, Japan), and then the stained protein band was excised from the gel. The protein samples were reduced with 25 mM DTT at 55 °C for 30 min, and then alkylated with 55 mM iodoacetamide at room temperature for 30 min in the dark. Digestion was carried out with modified trypsin (Trypsin Gold, Promega) or lysyl endopeptidase (Wako, Japan) at 37 °C overnight. The resulting peptides were analyzed using an Agilent 1100 HPLC system (Agilent Technologies) equipped with a C18 reverse-phase column (Hypersil GOLD, 2.1 × 100 mm, Thermo Fisher Scientific) connected to a LCQ-Fleet ion trap mass spectrometer. The peptides were separated
using a 0-50% gradient of acetonitrile containing 0.1% formic acid during 40 min at a flow rate of 300 μL per minute, and then eluted peptides were directly sprayed into the mass spectrometer. The mass spectrometer was operated in the data-dependent mode and externally calibrated. Survey MS scans were acquired in the 100-2000 or 400-2000 m/z range. Multiply charged ions of high intensity per scan were fragmented with CID in the ion trap. A dynamic exclusion window was applied within 30 seconds. All tandem mass spectra were collected using a normalized collision energy of 40%. Data were acquired and analyzed with Xcalibur software v. 2.07 (Thermo Fisher).

**Biotinylation of Hsp90**

Porcine Hsp90 (a gift from the late Dr. Yasufumi Minami, Maebashi Institute of Technology)\(^7\,8\) was biotinylated and purified with a kit (Biotin Labeling Kit –NH\(_2\), DOJINDO, Japan) according to the manufacturer’s instruction. The biotinylation of Hsp90 was confirmed by Western blotting (Fig. S10A). From densitometric quantification, it was estimated that approximately 9 molecules of biotin were conjugated to an Hsp90 single molecule. Using FP assay (see FP assay section), we confirmed that the ATP binding pocket of biotinylated-Hsp90 almost remained intact (see below; Fig. S10B).

**Figure S10.** (A) Quantification of biotin conjugation to Hsp90. (B) Comparison of geldanamycin (GA)-binding avidity of Hsp90 and biotinylated-Hsp90 by FP assay.

**Biopanning**
Biopanning was performed as described previously\textsuperscript{5, 6} with minor modifications. Biotinylated-Hsp90 (20 pmol) was immobilized on streptavidin-coupled magnetic beads (FG beads,\textsuperscript{9} Tamagawa Seiki, Japan). For biopanning, approximately $1.0 \times 10^{11}$ pfu of T7Select10 library (-S-G-G-G-X\textsubscript{3}-C-X\textsubscript{7}-C-X\textsubscript{3}; X represents any randomized amino acid)\textsuperscript{10, 11} was modified via the 10BASE\textsubscript{d}-T. After modification, the T7 phage library was dissolved in selection buffer (PBS supplemented with 1% v/v TritonX-100 and 1% w/v BSA). To remove non-specific binders (i.e., beads and streptavidin binders), the modified T7 phage library was pre-incubated with streptavidin-coupled FG beads for 2 hours at 4 °C, and then the supernatant was further incubated with the Hsp90-immobilized beads for 12 hours. The latter beads were washed three times with 200 μL of the selection buffer. Whole binding and washing processes were performed using an automated machine (Target Angler 8, Tamagawa Seiki, Japan). Hsp90-bound phage was directly infected and amplified with \textit{E. coli} BLT5403 strain. Stringent conditions were applied stepwise to each round by shortening the binding time and by increasing the washing frequency. After 6 rounds of biopanning, randomly chosen T7 phage monoclones were subjected to DNA sequencing.

**Enzyme-linked immunosorbent assay (ELISA)**

ELISA was performed as described previously with minor modifications.\textsuperscript{5, 6} Each well of streptavidin-conjugated 96-well plates (Nunc Immobilizer Streptavidin F96, Thermo Scientific) was coated with blocking buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% v/v Tween-20, and 0.5% w/v BSA) at 4 °C overnight. After washing with Tris-buffered saline, biotinylated-Hsp90 (3 pmol) was immobilized on each well. Approximately $2.0 \times 10^{10}$ pfu of T7 phage in TBST was applied to the well and incubated for 1 hour at 25 °C with shaking using a maximizer (MBR-022UP, TAITEC, Japan). The plate was washed three times with Tris-buffered saline supplemented with 0.5% v/v TritonX-100, and then Hsp90-bound phage was incubated with T7 tail fiber monoclonal antibody (1:5,000 dilution, Merck Millipore) and anti-mouse IgG HRP-linked antibody (1:5,000 dilution, Cell Signaling). After washing with TBST, \textit{o}-phenylenediamine dihydrochloride substrate (SigmaFast OPD, Sigma Aldrich) was
added, and the absorbance was quantified using a microplate reader equipped with a 450 nm band-pass filter (Bio-Rad).

**Preparation of GST-fused Hsp90 domains and GST pull-down assay**

Three domains of human Hsp90α, N-terminal (NTD: 9-236), middle (MD: 272-617), and C-terminal domains (CTD: 629-732) were prepared as described previously.\(^{12, 13}\) Briefly, pGEX-4T-3 vector encoding fragments of HSP90 gene (a gift from Dr. Franz-Ulrich Hartl, Max-Planck-Institute of Biochemistry)\(^ {14}\) was introduced into *E. coli* BL21 (DE3) strain. Transformants were precultured overnight at 37 °C in 2 mL of LB medium supplemented with 100 μg/mL ampicillin, and then transferred to a 150 mL of fresh LB medium. After incubation for 4 hours at 37 °C, isopropyl β-D-1-thiogalactopyranoside was added at a final concentration of 0.2 mM, and the cells were further cultured for 20 hours at 20 °C. The cells were harvested, and suspended in ice-cold lysis buffer (50 mM Tris-HCl, pH 8, 300 mM NaCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, 0.5 % w/v Triton X-100, and 1 × complete protease inhibitor cocktail minus EDTA). After cell disruption by ultrasonication, the crude cell extract was cleared by centrifugation at 20,000 × g for 10 min at 4 °C. Using a 0.42 μm membrane filter, the extract was further cleared. Supernatant was incubated with glutathione sepharose 4B (GE Healthcare) for 2 hours at 4 °C. After several washings with the lysis buffer, the sepharose was suspended in stock buffer (20 mM Tris-HCl, pH 8, and 50% v/v glycerol) and stored at -80 °C until use.

For GST pull-down assay, 20 μL (50% slurry) of the GST-Hsp90-immobilized sepharose was dissolved in 200 μL of binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% v/v Tween-20, and 0.5% w/v BSA), and then 200 μL of the same buffer containing modified-T7 phage monoclonal was added. After incubation for 2 hours at 4 °C, the sepharose was washed three times with Tris-buffered saline supplemented with 0.5% v/v Triton X-100. GST-fusion proteins were eluted with elution buffer (50 mM HEPES-KOH, pH 7.4, 100 mM NaCl, 1 mM DTT, and 50 mM reduced glutathione). After addition of 4 × sample buffer (250 mM Tris-HCl, pH 6.8, 40% glycerol, 8% SDS, 20% 2-mercaptoethanol, and 0.008% bromophenol blue), the
solution was incubated at 95 °C for 5 min.

**SDS-PAGE and Western blot analysis**

SDS-PAGE and Western blot analysis were performed as reported previously. Proteins were resolved by 10% polyacrylamide gel. For Western blotting, proteins in the gel were transferred onto a polyvinylidene difluoride membrane (Bio-Rad). The blots were incubated with primary antibody, followed by incubation with anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology). After several washes, the blots were incubated with ECL plus reagent (GE Healthcare Life Sciences), and detected using ChemiDoc XRS+ (Bio-Rad). Image contrast and brightness was adjusted in Photoshop CS4 (Adobe). Primary antibodies: anti-T7 tag mouse monoclonal antibody (Merck Millipore) and anti-FLAG mouse monoclonal antibody (M2, Sigma Aldrich). Note that antigen of the anti-T7 tag mouse monoclonal antibody is N-terminal region of gp10, which is a component of bacteriophage T7.

**Synthesis of Hsp90 NTD-binding crown analogue**

A peptide linker (H$_2$N-R-S-W-C-R-K-S-R-K-N-S-G-G-G-L-V-W-C-F-OH) was synthesized and characterized by HiPep Laboratories (Japan). Purity of the peptide was estimated to be above 90%. For cyclization of EBB, the peptide was dissolved in phosphate buffer (10 mM phosphate-KOH, pH 7.4) at a final concentration of 100 μM, and then EBB (500 μM) and neutralized TCEP (500 μM) were added. The mixture was incubated overnight at 37 °C in the dark with shaking, and then lyophilized to reduce the solution volume. The lyophilizate was dissolved in 1% formic acid aqueous solution, and the crown analogue was purified with reverse-phase HPLC (Shimadzu, Japan) equipped with a XTerra Prep MS C18 column (10 × 50 mm, Waters). The crown analogue was separated using a 0-100% linear gradient of acetonitrile containing 0.1% trifluoroacetic acid during 12 min at a flow rate of 4 mL per minute. The crown analogue was lyophilized and characterized by LC-MS (see below; Fig. S11).
**Circular dichroism (CD) spectroscopy**

CD spectroscopy was performed as reported previously. All compounds were dissolved in phosphate buffer (20 mM phosphate-KOH, pH 7.2) at a final concentration of 50 μM. Circular dichroism spectra were recorded on a CD spectrometer (J-720W, JASCO, Japan) at 25 °C using a 0.2 cm path-length quartz cell (see below; Fig. S10). For reduction of disulfide-bonds, TCEP was added at a final concentration of 1 mM.

**Isothermal titration calorimetry (ITC)**

ITC experiment was performed using MicroCal iTC200 (GE Healthcare). GST-tagged Hsp90 NTD was enriched using an ultrafiltration column (vivaspin column 500 MWCO 10 kDa, GE Healthcare), and the buffer was changed to phosphate buffer (20 mM phosphate-KOH, pH 7.2, 50 mM NaCl, 1 mM TCEP). Protein concentration was determined by Bradford assay. For titration experiment, GST-Hsp90 NTD and compounds were diluted into 5 μM and 200 μM, respectively. Titrations were performed at 25°C. Injection parameters were the following: 2 μL volume, 4 sec duration, 4 sec spacing, and 5 sec filter period. Reference power was set to 10 μcal/s. Data were analyzed with Origin software 7.0 (MicroCal). Curve fitting was performed using 1:1 interaction model.
Fluorescence polarization (FP) assay

Fluorescence polarization was measured using HYBRID-3000ES system (Photoscience, Tokyo, Japan) equipped with appropriate filters (Ex. 480 nm and Em. 535 nm). The instrument was operated in static mode. FITC-labeled geldanamycin \(^1\) (GA-FITC; 4 pmol, 20 nM) was incubated with various concentrations of GST-fused Hsp90 domains (NTD, MD, and CTD) in phosphate-buffered saline supplemented with 1 mM TCEP for 5 min at 30°C, and fluorescence polarization was measured. In the TCEP-containing solution, geldanamycin (GA) bound to Hsp90 in a short incubation time (ca., 2 min; data not shown), although it is reported that Hsp90-binding affinity of GA is improved in a time-dependent manner.\(^2\) This is consistent with a previous report that TCEP cancels the time-dependent effect on binding.\(^3\) Klotz plot was generated by GraphPad Prism software 6.0 (GraphPad Software, San Diego, CA, USA), and the sigmoid curve was fitted with non-linear least squares analysis for \(K_D\) determination.

For competition assay, various concentrations of compounds were pre-incubated with GST-Hsp90 NTD (110 pmol, 550 nM) in phosphate-buffered saline supplemented with 1 mM TCEP for 10 min at room temperature. After addition of GA-FITC (4 pmol, 20 nM), the mixture was incubated for 5 min at 30 °C, and then fluorescence polarization was measured. GA-FITC and geldanamycin were purchased from Enzo Life Sciences (cat No. BML-EI361-0001, Farmingdale, NY, USA) and StressMarq (cat No. SIH-111A/B, Victoria, BC, Canada), respectively. Concentrations of GA-FITC and geldanamycin were determined by absorption coefficient at 336 nm.

Abbreviations

10BASE, \(d\)-T, gp10 based-thioetherification; ATP, adenosine triphosphate; BSA, bovine serum albumin; CID, collision induced dissociation; DNA, deoxyribonucleic acid; DTT, dithiothreitol; EBB, \(N,N'\)-[1,2-ethanediyl-oxy-2,1-ethanediyl]bis(2-bromoacetamide); ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; FP, fluorescence polarization; GA, geldanamycin; GST, glutathione S-transferase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; HSP, heat shock
protein; MS, mass spectrometry; ODS, octadecylsilyl; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; TCEP, Tris(2-carboxyethyl)phosphine; TMS, tetramethylsilane; Tris, tris(hydroxymethyl)aminomethane.
Supplemental references