ChemPubSoc Europe

DOI: 10.1002/chem.201603464

CHEMISTRY A European Journal Full Paper

Antitumor Agents

Reinventing Hsp90 Inhibitors: Blocking C-Terminal Binding Events to Hsp90 by Using Dimerized Inhibitors**

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Abstract: Heat shock protein 90 (Hsp90) is a molecular chaperone (90 kDa) that functions as a dimer. This protein facilitates the folding, assembly, and stabilization of more than 400 proteins that are responsible for cancer development and progression. Inhibiting Hsp90's function will shut down multiple cancer-driven pathways simultaneously because oncogenic clients rely heavily on Hsp90, which makes this chaperone a promising anticancer target. Classical inhibitors that block the binding of adenine triphosphate (ATP) to the N-terminus of Hsp90 are highly toxic to cells and trigger a resistance mechanism within cells. This resistance mechanism comprises a large increase in prosurvival proteins, namely, heat shock protein 70 (Hsp70), heat shock protein 27 (Hsp27), and heat shock factor 1 (HSF-1). Molecules that modulate the C-terminus of Hsp90 are effective at inducing cancer-cell death without activating the resistance mechanism. Herein, we describe the design, synthesis, and biological binding affinity for a series of dimerized C-terminal Hsp90 modulators. We show that dimers of these C-terminal modulators synergistically inhibit Hsp90 relative to monomers.

Introduction

Molecular-targeted chemotherapies, which inhibit a single oncoprotein, are critical in the fight against cancer. Between 2005 and 2016, the Food and Drug Administration (FDA) approved more than 50 different molecular-targeted anticancer agents for use against more than 20 different types of cancer (http://www.centerwatch.com). Heat shock protein 90 (Hsp90) is a highly conserved molecular chaperone that participates in the folding, stabilization, and activation of more than 400 proteins involved in the 10 hallmarks of cancer.[1] Hsp90 makes up 1–2% of all cellular proteins in normal cells, whereas Hsp90 makes up 3–6% of all proteins in cancerous cells. The increase in Hsp90 protects mutated and overexpressed oncoproteins from degradation, thereby facilitating cancer-cell survival.[2] Hsp90 also plays a regulatory role in cellular-stress response pathways, thus increasing the prosurvival proteins heat shock protein 70 (Hsp70), heat shock protein 27 (Hsp27), and heat shock factor 1 (HSF-1), which facilitate drug resistance.[3] Thus, inhibiting the cellular function of Hsp90 blocks multiple roles of oncoproteins role in cancer-cell growth and prevents cellular-stress responses from promoting drug resistance and evading apoptosis.

Hsp90 consists of three domains: an amino (N) domain (25 kDa), a middle (M) domain (35 kDa), and a carboxy (C) domain (10 kDa).[4] Classical Hsp90 inhibitors bind to the N-terminal domain inside the ATP binding pocket. Although there have been significant efforts to produce molecules that target this chaperone (www.clinicaltrials.gov), the use of these molecules in clinical applications has been hampered by the induction of the side effects of the prosurvival proteins. Side effects of the drugs include a dramatic induction of HSF-1, Hsp70, and Hsp27. These proteins protect the cell from apoptosis and promote antiapoptotic pathways, thereby facilitating cancer growth.[5] In addition to these classical Hsp90 inhibitors, two other categories of inhibitor have been reported: 1) direct C-terminal inhibitors and 2) C-terminal modulators, which allosterically inhibit Hsp90 function by disrupting the interactions between Hsp90 and cochaperones that bind to the C-terminus.

C-terminal inhibitors of Hsp90 based on novobiocin have been developed.[6] Our group also recently developed a C-terminal inhibitor that targets the last five residues (MEEVD) at the C-terminal end of Hsp90.[7] C-terminal modulators (or allosteric C-terminal inhibitors) have been extensively studied.[8] Unlike classical inhibitors, both direct C-terminal inhibitors and allosteric inhibitors do not trigger a cell protection mechanism.[6,8] Instead, the C-terminal inhibitors and modulators act through a mechanism that appears to be more effective than that of the classical inhibitors. C-terminal modulators developed by our group bind between the amino and middle domains of Hsp90 and allosterically modulate binding between Hsp90 and the C-terminal clients or cochaperones.[8b–f,9] Four

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[**] Hsp90 = heat-shock protein 90.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201603464.

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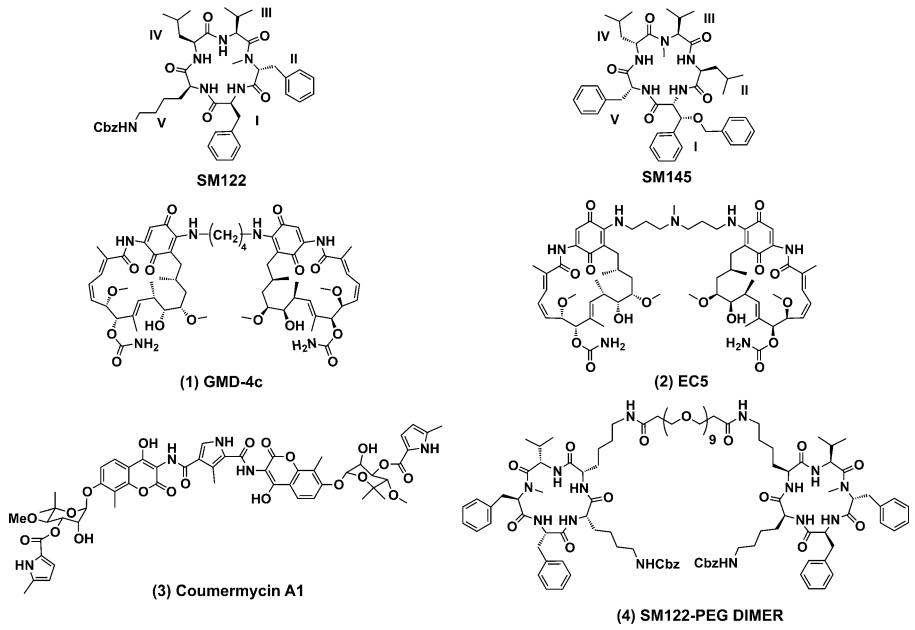

Figure 1. Structures of SM122, SM145, GMD-4c (**1**), EC5 (**2**), coumermycin A1 (**3**), and the SM122-PEG dimer (**4**).

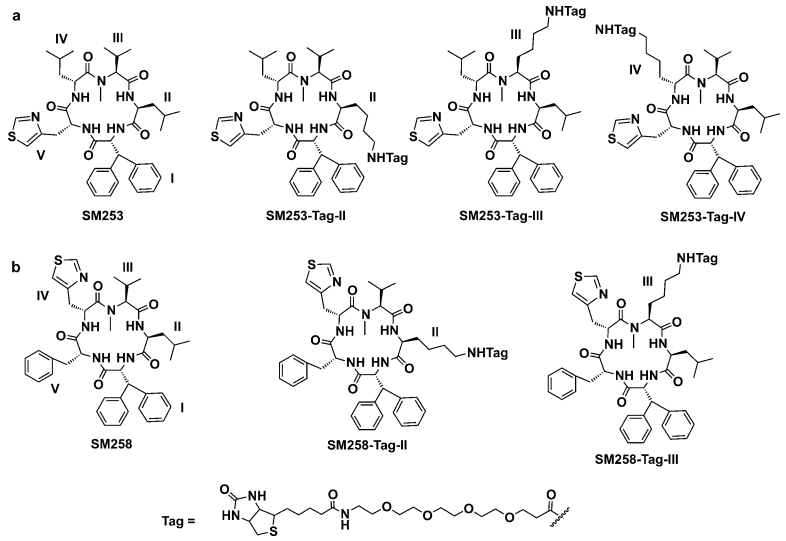

Figure 2. a) Structures of the SM253 molecules and their PEG-biotinylated analogues (SM253 tagged at positions II, III, and IV). b) Structures of SM258 and its PEG-biotinylated analogues (SM258 tagged at positions II and III).

analogues from this allosteric series are highly effective: SM122, SM145, SM253, and SM258 (Figures 1 and 2).[8a–d,9d,e] All four molecules bind to Hsp90 and block the binding of tetratricopeptide repeat (TPR) cochaperones, which interact at the Hsp90 C-terminus. These TPR proteins regulate pathways involved in cancer-cell growth, including hormone-receptor production and antiapoptosis. These C-terminal Hsp90 modulators, termed SM molecules, also block the cellular-stress response pathways of by inhibiting TPR protein access to Hsp90.

Hsp90 functions as a dimer, therefore dimerization of an Hsp90 inhibitor is one useful method in the generation of synergistic inhibitors. Zheng et al. employed a dimerization strategy in which the classical inhibitor geldanamycin (GM) was dimerized through several alkylamino chain lengths; in particular, the GMD-4c dimer (**1**; Figure 1) exhibited significant antiproliferative activity against tumor cell lines ($GI_{50} \approx 20$–100 nM).[10] A subsequent study by Yin et al. showed that dimerization of a classical ansamycin-based compound to produce EC5 (**2**; Figure 1) was effective at inhibiting the tumor growth of head and neck squamous cell carcinoma (HNSCC) cell lines ($GI_{50} <$ 200 nM) and was twofold more potent than the well-known classical inhibitor 17-AAG ($GI_{50} \approx 500$ nM).[11]

The C-terminal inhibitor coumermycin A1 was dimerized by using several linker lengths. Courmermycin A1 (**3**) exhibited

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a GI_{50} value of 200 nM against SKBr3 cell lines (Figure 1.[12] A report on the crystal structure of the Hsp90–Hsp organizing protein (HOP) complex revealed that the distance between two N-domains of Hsp90 in its open conformation is approximately 80 Å.[13] Thus, the appropriate linker length for C-terminal modulators that bind between the N-middle domain was estimated to be approximately 40–50 Å. Dimerized variants of SM122, a C-terminal modulator, with a poly(ethylene glycol) (PEG) chain (length of $(PEG)_9$: ≈46 Å) successfully blocked the protein-folding function of Hsp90 and disrupted the interaction between Hsp90 and TPR-containing proteins.[14] Dimerized SM122 (Figure 1) synergistically inhibited the binding interaction between Hsp90 and the TPR-containing proteins FKBP52 and HOP, in which the dimer was more than twice as effective than a single SM122 molecule.[14]

Herein, we describe the design and synthesis of four dimer molecules based on the C-terminal modulators SM122, SM145, SM253, and SM258. The linker placement was chosen by using pulldown assays to identify the optimal position for the linker of each molecule. The dimers were assessed by using Hsp90 binding assays for their ability to inhibit TPR proteins from binding to the Hsp90 C-terminus.

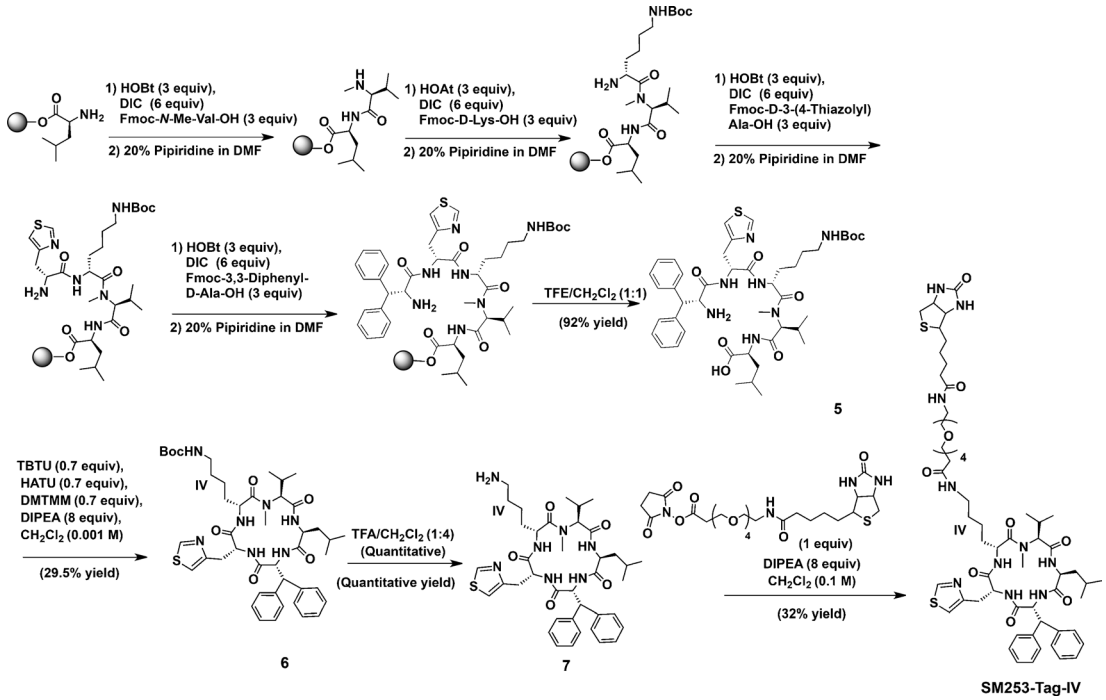
Results and Discussion

Determining the optimal linker placement on the monomers

A critical factor in the design of the PEG-linked SM dimer is to first identify the optimal site for the linker attachment that connects the two SM monomers together. Previous work on SM122-based dimer molecules had shown that the position for incorporating linkers to form dimers could impact their ability to inhibit Hsp90 activity.[9e] Our previously reported dimer **4** placed the PEG linker at position IV on SM122 because the pulldown assays that used Hsp90 and a biotinylated-tagged variant of SM122 had shown that this molecule was the most effective at pulling down Hsp90. However, our pulldown assays had also shown that the placement of a biotin tag at position III on SM122 was also very effective at binding Hsp90. Thus, the dimer can be generated by placing a linker at position III. Previous pulldown assays with tagged variants of SM145 had shown that SM145-Tag-II was highly effective at pulling down Hsp90.[8e] Thus, SM145 the linker would be placed at position II.

It was not clear which position of SM253 or SM258 would be the most effective for pulling down Hsp90. Thus, we synthesized all three tagged variants of SM253 (Figure 2), thus leaving the biphenyl residue (position I) and the thiazolyl moiety (position V) untouched. Both the biphenyl and thiazole residues have been established as critical for biological activity in structure–activity relationship (SAR) studies.[8b,15] We also synthesized two tagged variants of SM258, in which we maintained the thiazole (IV), phenyl (V), and biphenyl (I) residues by placing the pulldown tag at positions II and III (Figure 2).

The generation of the tagged molecules was accomplished by using solid-phase synthesis in which a lysine residue was substituted for the amino acid at the relevant position. The synthesis of SM253-Tag-IV is a representative example of how the molecules were generated (Scheme 1). By using the preloaded 2-chlorotrityl-leucine (Leu) resin, Fmoc-protected amino acids were sequentially coupled to the free amine group of the leucine residue. The amine group on each amino acid was deprotected after each coupling reaction. After the fourth amino acid residue was coupled, the pentapeptide unit was cleaved from the resin to produce the linear peptide **5**



Scheme 1. Solid-phase peptide synthesis followed by macrocyclization to afford the biotinylated macrocycle.

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(Scheme 1). Closing the macrocycle under our reported cyclization conditions[8b,d,9c,d] produced cyclic peptide **6**. Removal of the amino-protecting group *tert*-butyloxycarbonyl (Boc) produced **7** and coupling $(PEG)_4$–biotin in the presence of *N*,*N*-diisopropylethylamine (DIPEA) produced the tagged molecule (i.e., SM253–Tag-IV in this case; Scheme 1).

By using cell lysate and our reported pulldown assay conditions,[8d,e,9e] we evaluated how effectively the five tagged molecules (SM253-Tag-II, -III, and -IV and SM258-Tag-II and -III) pulled down Hsp90 (Figure 3). The most effective of each series would then be used to decide the placement of the dimer linker. Evaluation of SM253-Tag-II, -III, and -IV in the same gel (Figure 3 a) demonstrated that SM253-Tag-IV was the most effective molecule for binding to Hsp90. Thus, position IV on SM253 was used as the linker site. The comparison of SM258-Tag-II and -III (Figure 3 b) in pulldown assays revealed that SM258-Tag-III was the most effective at binding to Hsp90 in cell lysate. Thus, position III was chosen to link two SM258 molecules.

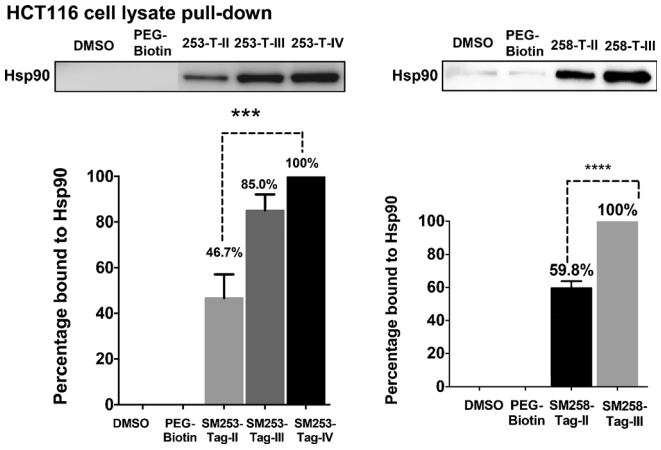

Figure 3. Hsp90 protein pulldowns in HCT116 cell lysates. Pulldown data for SM253 and the PEG-biotinylated analogues (left). Pulldown data for the SM258 molecules and their PEG-biotinylated analogues (right). Flow through the lanes are shown in the Supporting Information.

Synthesis of dimerized C-terminal modulators

With the optimal linker position for each monomer identified, we synthesized four dimers: SM122-Tag-III, SM145-Tag-II, SM253-Tag-IV, and SM258-Tag-III (Figure 4). Each dimer was constructed from the corresponding monomers. By starting from free amine **7** (Figure 4 b), which was generated by using solid-phase synthesis (Scheme 1), the dimer was produced using a 2:1 ratio of amine **7** to bis-*N*-succinimidyl-$(PEG)_9$ ($BS(PEG)_9$) in the presence of DIPEA. Upon purification by means of reversed-phase HPLC, the dimer structures were confirmed by using NMR spectroscopy and LC-MS and high-resolution (HR) MS. Ratios of the PEG peaks to individual protons in the monomer provided evidence that the compounds had dimerized.

Ability to block binding between Hsp90 and TPR-containing proteins HOP and FKBP52

Hsp90 interacts with 19 different C-terminal cochaperones and 14 of these molecules contain TPR domains.[16] These TPR-con-

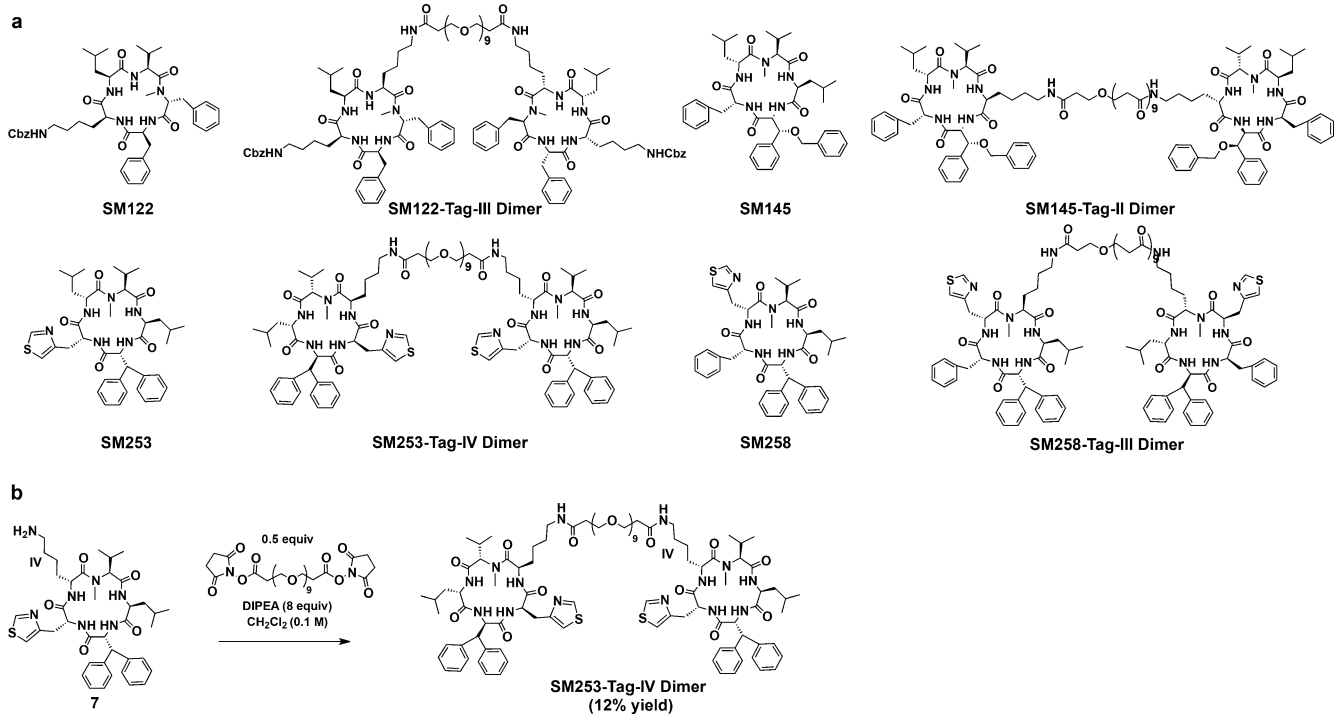

Figure 4. a) Structures of SM122, SM145, SM253, and SM258 and their dimerized variants. b) The synthesis of the SM253-Tag-IV dimer, which exemplifies the synthesis of the four dimers.

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taining proteins include HOP and the peptidyl–prolyl isomerase (PPIase) family members FKBP52, FKBP51, and Cyp40.[17] The monomers SM122, SM145, SM253, and SM258 block the interactions between Hsp90 and HOP and between Hsp90 and FKBP52.[8b–d,14] A structurally similar monomer to SM253 and SM258 was used as the negative control (SM271; see the Supporting Information) and improved the affinity between Hsp90 and HOP.[8b] We have also demonstrated that the linker $(PEG)_8$ had no impact on the binding affinity of the monomer.[14] Thus, the improved affinity of the dimer over the monomer would be purely due to the synergistic effect of the two monomers linked together.

To evaluate how effective the dimers were relative to the monomers, we performed pure-protein binding experiments with twice the concentration of the monomer relative to the dimer. Previous reports have shown that the use of the SM122-Tag-IV dimer (10 μM) was relatively effective at blocking the interaction between Hsp90 and HOP or Hsp90 and FKBP52 (blocking 56 and 40% of the binding affinity, respectively).[14] Thus, we evaluated these four dimers at 15 μM (two C-terminal modulators linked together) for which the monomer concentration was 30 μM, (e.g., a single C-terminal modulator). Although the same number of C-terminal modulators were present in the assay, the dimers were more effective than the monomers for inhibiting the binding between Hsp90 and HOP (Figure 5a). The two most effective dimers for inhibiting binding between these two proteins were SM122-Tag-III and SM258-Tag-III.

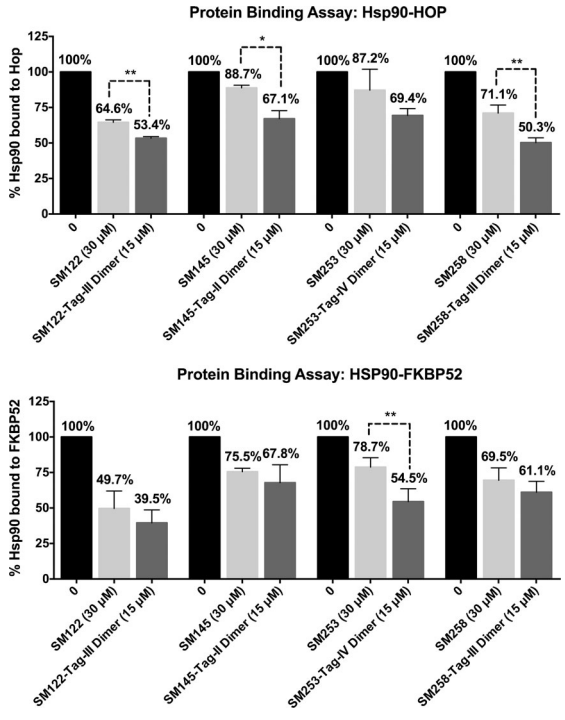

Figure 5. The inhibitory effects of SM122, SM145, SM253, and SM258 and the SM122-Tag-III, SM145-Tag-II, SM253-Tag-IV, and SM258-Tag-III dimers on the binding between Hsp90 and its cochaperones HOP (top) and FKBP52 (bottom). The significant differences between the indicated treatments are represented with *P* values (*$P \leq 0.05$ and **$P \leq 0.01$).

Binding assays between FKBP52 and Hsp90 also showed that the dimeric compounds were all synergistically able to disrupt the binding event. The most effective dimers were the SM122-Tag-III and SM253-Tag-IV dimers (Figure 5b). Because we established that the monomers bind to Hsp90 (Figure 3), it is most likely that these dimer molecules act by binding to the Hsp90 N-middle domain and synergistically modifying the conformation of Hsp90 to allow the binding of the second SM molecule on the dimers.

Despite the fact that the dimeric compounds provided a proof-of-concept demonstration for Hsp90 inhibition, these dimerized variants failed to show growth inhibition in HCT116 cancer cells (see the Supporting Information). These molecules were highly soluble in water, which was attributed to the PEG linker. Thus, the failure was most likely due to the large molecular size of the dimers ($MW \approx 2000\ g\,mol^{-1}$), which were approximately 2.8-fold larger than the size of the monomers that inhibited their entry into the cells through passive diffusion.

Conclusion

The design of dimeric C-terminal modulators has been described. The identification of the most effective linker placement for each compound was carried out by using pulldown assays. The identification was accomplished by quantifying the amount of Hsp90 that was pulled down by each tagged compound. The linker placement varied depending on the structure of the compound. The synthesis of four dimers, in which one molecule was composed of two C-terminal modulator (i.e., SM122, SM145, SM253, and SM258) with linkers placed at the appropriate positions, has been described. Evaluations that compared the monomer to the dimer in a binding assay by using Hsp90 and two different C-terminal cochaperones showed that the dimers synergistically inhibited the binding events. Although the number of C-terminal modulators were the same in both the dimer and monomer solutions, the results indicated that the dimers were more effective at blocking the Hsp90/cochaperone interactions than the monomers. These data have indicated that one approach to target Hsp90, and perhaps other dimeric proteins, would involve the dimerization of active monomers.

Experimental Section

General information

All moisture-sensitive reactions were performed in nitrogen gas and were monitored by using thin-layer chromatography (TLC) and liquid-chromatography mass spectrometry (LC-MS). TLC analysis was performed on sheets of aluminum silica gel (250 μm; Whatman® (4861-820) with UV light ($\lambda = 254$ nm) as the visualizing method. The developing agents for TLC analysis included potassium permanganate (general purpose) and ninhydrin (for amine-group detection). Flash column chromatography on silica gel was used to purify the crude materials from the synthesis. LC-MS analyses were performed on a LC-MS system connected to a trap running in positive electrospray ionization (ESI+) mode. The mobile phase consisted of doubly deionized water with 0.1% (v/v) formic

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acid (solvent A) and HPLC-grade acetonitrile with 0.1% (v/v) formic acid (solvent B) at a flow rate of 0.5 mL min^{-1}, starting at 70% solvent A, 30% solvent B. The gradient elutions were as follows: flow rate = 2 mL min^{-1}; initial: 70% solvent A, 30% solvent B held for 35 min; at 35 min: 100% solvent B, held for 18 min; at 53 min: 70% solvent A, 30% solvent B, held for 7 min. ^{1}H and ^{13}C NMR spectra were obtained and recorded at 25 °C on Bruker Avance III 500 and 600 MHz spectrometers.

General solid-phase peptide synthesis

Stepwise solid-phase peptide synthesis (SPPS) was performed in a polypropylene solid-phase extraction cartridge fitted with a polyethylene frit (20 μM) and preloaded 2-chlorotrityl resins with an approximate loading scale of 0.5 mmol g^{-1} were used. The resin was weighed, transferred to the cartridge, and swelled in DMF for 30 min prior to peptide coupling in the corresponding sequence.

General solid-phase peptide synthesis

Fmoc-protected amino acid coupling reactions were performed in DMF (0.2 M), amino acid (3.0 equiv), 1-hydroxybenzotriazole (HOBt; 3.0 equiv), and diisopropylcarbodiimide (DIC; 6.0 equiv). Coupling reaction mixture was shaken for a minimum of 4 h on a shaker and checked by using a ninhydrin test to confirm completion. Once completed, the coupling reaction mixture was drained, and the resin was subjected to removal of the Fmoc protecting group. (Note that 1-hydroxybenzotriazole was replaced with 1-hydroxy-7-azabenzotriazole for the peptide coupling between Fmoc and the *N*-methyl amino terminus and the coupling process was allowed to run overnight.)

General N-terminal solid-phase amine deprotection

After the peptide coupling process was completed, removal of the Fmoc protecting group was performed according to the following steps: DMF (3×1 min), 20% piperidine/DMF (1×5 min), 20% piperidine/DMF(1×10 min), DMF (2×1 min), isopropyl alcohol (IPA; 1×1 min), DMF (1×1 min), IPA (1×1 min), and DMF (3×1 min).

Cleavage of linear peptide

The eventual cleavage of a linear pentapeptide from the resin was carried out by swelling the resin in a solution of 2,2,2-trifluoroethanol (TFE)/CH_2Cl_2 (1:1 (v/v); 10 mL per gram of dried resin) and was allowed to stir for 24 h. The suspension was filtered through a Büchner filter, and the resin was washed repeatedly with additional CH_2Cl_2 to fully extract the cleaved peptide. The filtrate was evaporated and dried in vacuo overnight. The dried solid was eventually redissolved in CH_2Cl_2, coevaporated with CH_2Cl_2 several times to remove the entrapped TFE residue completely, and dried in vacuo overnight.

Macrocyclization procedure (syringe pump)

Macrocyclization of the double-deprotected linear pentapeptide by using a combination of three coupling agents 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholin-4-ium (DMTMM), (7-azabenzotriazol-1-yl)tetramethyluronium (HATU), and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU; 0.8 equiv each) with *N*,*N*-diisopropylethylamine (DIPEA; 8.0 equiv) in 75% of a calculated volume of anhydrous CH_2Cl_2 to generate an overall concentration of 0.001 M. The crude anhydrous double-deprotected linear peptide (DDLP) was dissolved in the remaining amount of CH_2Cl_2. DDLP in solution was added to the bulk solution dropwise by using a syringe pump over 2 h. After the addition of all of the DDLP, the reaction was monitored by using LC-MS after 1–2 h. The reaction was stirred overnight, if not complete within 2 h. Upon completion, the crude product was subjected to an acid/base wash to remove excess DIPEA and the coupling agents. The resulting crude product was first purified by flash column chromatography, followed by reversed-phase HPLC by using a gradient of acetonitrile and deionized water with 0.1% trifluoroacetic acid (TFA) to afford the final pure compounds.

Synthesis

Experimental methods for SM253-Tag-II

HO-D-Leu-D-3-(4-thiazolyl)-Ala-3,3-diphenyl-D-Ala-Lys(Boc)-*N*-Me-Val-NH: Resin-O-D-Leu-D-3-(4-thiazolyl)-Ala-3,3-diphenyl-D-Ala-Lys(Boc)-*N*-Me-Val-NH was synthesized by using resin-O-D-Leu-NH_2 (1.0 g, 0.5 mmol, 1.0 equiv) and the subsequent peptide coupling in the sequence by using an aliquot (1.5 mmol, 3.0 equiv) of each of the following: Fmoc-D-3-(4-thiazolyl)-Ala-OH (0.59 g), Fmoc-3,3-diphenyl-D-Ala-OH (0.70 g), Fmoc-Lys(Boc)-OH (0.70 g), and Fmoc-*N*-Me-Val-OH (0.53 g). Each peptide coupling was carried out in the presence of 1-hydroxy-7-aza-benzotriazole (HOAt; 0.20 g, 1.5 mmol, 3.0 equiv) or HOBt (0.20 g, 1.5 mmol, 3.0 equiv), DIC (0.47 mL, 3.0 mmol, 6.0 equiv), and DMF (2.5 mL) to generate a concentration of 0.20 M based on the amino acid. Each coupling reaction was run for 3 h and a negative ninhydrin test was used to confirm the reaction completion. The reaction mixture was drained to afford the Fmoc-protected resin-bound pentapeptide and the Fmoc protecting group was removed after completion of each coupling reaction. The linear pentapeptide was cleaved from the resin by using a solution of TFE (6 mL) and CH_2Cl_2 (6 mL). The resin-containing solution was filtered and dried in vacuo to yield the DDLP **SM253-Tag-II** as a white solid (140 mg, 33%). LC-MS (ESI): *m/z* calcd for $C_{44}H_{63}N_7O_8S$: 850.46 [$M+1$]; found: 850.10.

***cyclo*-D-Leu-D-3-(4-thiazoyl)-Ala-3,3-diphenyl-D-Ala-Lys(Boc)-*N*-Me-Val**: The product was afforded by using DDLP **SM253-Tag-II** (0.11 g, 0.13 mmol, 1.0 equiv), TBTU (0.021 g, 0.065 mmol, 0.50 equiv), HATU (0.017 g, 0.065 mmol, 0.50 equiv), DMTMM (0.019 g, 0.065 mmol, 0.50 equiv), and DIPEA (0.27 mL, 1.55 mmol, 12.0 equiv) in anhydrous CH_2Cl_2 (129 mL, 0.001 M) and following the macrocyclization procedure. The reaction was stirred overnight and the reaction was monitored by TLC and LC-MS. Upon completion, the reaction mixture was subjected to an acid/base wash to afford the crude product, which was purified by flash column chromatography on silica gel with an ethyl acetate/hexane gradient system as the eluent, followed by purification by HPLC to yield the Boc-protected macrocycle as a white solid (23 mg, 21.4%). LC-MS (ESI): *m/z* calcd for $C_{44}H_{61}N_7O_7S$: 832.44 [$M+1$]; found: 832.20.

The Boc protecting group of the macrocycle was removed by utilizing a mixture of TFA/CH_2Cl_2 (1:4, 0.1 M) and anisole (2.0 equiv) to generate free amine groups in the lysine residue. The free amine compound was used in the subsequent biotinylation reaction without purification. LC-MS (ESI): *m/z* calcd for $C_{39}H_{53}N_7O_5S$: 732.38 [$M+1$]; found: 732.00.

The biotinylated **SM253-Tag-II** was afforded by utilizing the deprotected macrocycle (23 mg, 0.031 mmol, 1.0 equiv), NHS-$(PEG)_4$-Biotin (25.9 mg, 0.044 mmol, 1.4 equiv), and DIPEA (43.8 μL, 0.25 mmol, 8.0 equiv) in CH_2Cl_2 (314 μL). The reaction mixture was stirred for 4 h and monitored by LC-MS. Upon completion, the crude product was purified by using preparative HPLC to generate pure the biotinylated compound as a white solid (12 mg, 33.1%).

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R_f = 0.41 (EtOAc/MeOH = 0.60:0.40); ^{1}H NMR (600 MHz, MeOD): δ = 9.34 (m, 1H), 7.28–7.04 (m, 11H), 5.19–5.16 (m, 1H), 4.54–4.48 (m, 2H), 4.43–4.41 (m, 1H), 4.25–4.19 (m, 2H), 4.07 (s, 1H), 4.02 (s, 1H), 3.26 (t, J = 6.4 Hz, 3H), 3.53–3.49 (m, 14H), 3.45–3.42 (m, 3H), 3.28–3.24 (m, 3H), 2.99–2.95 (m, 2H), 2.82 (s, 1H), 2.75 (s, 3H), 2.35–2.32 (m, 3H), 2.17–2.11 (m, 4H), 1.82–1.76 (m, 1H), 1.68–1.40 (m, 12H), 1.29–1.19 (m, 5H), 0.82–0.72 ppm (m, 12H); ^{13}C NMR (150 MHz, MeOD): δ = 174.8, 172.4, 171.6, 170.9, 170.2, 170.0, 161.9, 156.1, 148.3, 140.8, 140.6, 128.6, 128.5, 128.4, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 126.9, 126.6, 118.5, 70.6, 70.4, 70.3, 70.2, 70.1, 70.0, 69.9, 69.8, 69.2, 66.9, 63.7, 62.9, 58.0, 56.9, 56.8, 52.7, 48.5, 48.2, 39.0, 38.8, 36.2, 35.3, 34.9, 29.9, 29.1, 28.7, 28.5, 28.4, 28.1, 26.7, 25.5, 25.3, 25.1, 24.3, 23.1, 22.0, 21.2, 21.1, 18.8, 18.7, 17.8 ppm; LC-MS (ESI): m/z calcd for $C_{60}H_{88}N_{10}O_{12}S_2$: 1205.60 [$M$+1]; found: 1205.05; HRMS (ESI-TOF): m/z calcd for $C_{60}H_{88}N_{10}O_{12}S_2$: 1204.6025 [$M+Na^+$]; found: 1227.5926.

Experimental methods for SM253-Tag-III

HO-Leu-*N*-Me-Lys(Boc)-D-Leu-D-3-(4-thiazolyl)-Ala-3,3-diphenyl-D-Ala-NH$_2$: Resin-O-Leu-*N*-Me-Lys(Boc)-D-Leu-D-3-(4-thiazolyl)-Ala-3,3-diphenyl-D-Ala-NH$_2$ was synthesized by using resin-O-Leu-NH$_2$ (1.0 g, 0.5 mmol, 1.0 equiv) and the subsequent peptide coupling in the sequence by using an aliquot (1.5 mmol, 3.0 equiv) of each of the following: Fmoc-*N*-Me-Lys(Boc)-OH (0.72 g), Fmoc-D-Leu-OH (0.53 g), Fmoc-D-3-(4-thiazolyl)-Ala-OH (0.59 g), and Fmoc-3,3-diphenyl-D-Ala-OH (0.70 g). Each peptide coupling was carried out in the presence of HOAt (0.20 g, 1.5 mmol, 3.0 equiv) or HOBt (0.20 g, 1.5 mmol, 3.0 equiv), DIC (0.47 mL, 3.0 mmol, 6.0 equiv), and DMF (2.5 mL) to generate a concentration of 0.20 M based on the amino acid. Each coupling reaction was run for 3 h and a negative ninhydrin test was used to confirm the reaction completion. The resin-bound linear pentapeptide was cleaved from the resin by using a solution of TFE (6 mL) and CH_2Cl_2 (6 mL) The resin-containing solution was filtered and dried in vacuo to yield the DDLP **SM253-Tag-III** as a white solid (352 mg, 81.5%). LC-MS (ESI): m/z calcd for $C_{45}H_{65}N_7O_8S$: 864.46 [M+1]; found: 864.20.

***cyclo*-Leu-*N*-Me-Lys(Boc)-D-Leu-D-3-(4-thiazolyl)-Ala-3,3-diphenyl-D-Ala**: The product was afforded by using DDLP **SM253-Tag-III** (0.35 g, 0.41 mmol, 1.0 equiv), TBTU (0.092 g, 0.29 mmol, 0.70 equiv), HATU (0.15 g, 0.41 mmol, 1.0 equiv), DMTMM (0.079 g, 0.29 mmol, 0.70 equiv), DIPEA (0.57 mL, 3.26 mmol, 8.0 equiv) in anhydrous CH_2Cl_2 (407 mL, 0.001 M) and following the macrocyclization procedure. The reaction was stirred overnight and the reaction was monitored by TLC and LC-MS. Upon completion, the reaction mixture was subjected to an acid/base wash to afford the crude product, which was purified by flash column chromatography on silica gel with an ethyl acetate/hexane gradient system as the eluent, followed by purification by HPLC to yield the Boc-protected macrocycle as a white solid (105 mg, 30.5%). LC-MS (ESI): m/z calcd for $C_{45}H_{63}N_7O_7S$: 846.45 [M+1]; found: 846.50.

The Boc protecting group of the macrocycle was removed by utilizing a mixture of TFA/CH_2Cl_2 (1:4, 0.1 M) and anisole (2.0 equiv) to generate free amine groups in the lysine residue. The free amine compound was used in the subsequent biotinylation reaction without purification. LC-MS (ESI): m/z calcd for $C_{40}H_{55}N_7O_5S$: 746.40 [M+1]; found: 746.50.

The biotinylated **SM253-Tag-III** was afforded by utilizing (52 mg, 0.070 mmol, 1.0 equiv) of the deprotected macrocycle, NHS-(PEG)$_4$-Biotin (57.4 mg, 0.098 mmol, 1.4 equiv), DIPEA (97.1 μL, 0.56 mmol, 8.0 equiv) in CH_2Cl_2 (697 μL). The reaction mixture was stirred for 4 h and monitored by LC-MS. Upon completion, the crude product was purified by using preparative HPLC to generate the pure biotinylated compound as a white solid (13 mg, 15.3%). R_f = 0.45 (EtOAc/MeOH = 1:1); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.69 (d, J = 1.8 Hz, 1H), 8.20 (d, J = 5.6 Hz; N*H*), 7.77 (br; N*H*), 7.30–7.17 (m, 10H), 6.94 (br; N*H*), 6.86 (br; N*H*), 6.42 (m, 1H), 5.68 (br; N*H*), 5.23 (t, J = 10.2 Hz, 1H), 5.16 (dd, J = 10.5, 4.9 Hz, 1H), 4.67 (d, J = 10.9 Hz, 1H), 4.57 (br; N*H*), 4.52 (m, 1H), 4.41 (m, 1H), 4.17 (m, 1H), 3.74 (t, J = 6.0 Hz, 2H), 3.64 (m, 12H), 3.55 (t, J = 5.0 Hz, 2H), 3.37 (m, 1H), 3.29 (m, 2H), 3.20 (m, 2H), 2.95 (m, 1H), 2.91 (s, 3H), 2.81 (m, 1H), 2.46 (m, 2H), 2.30 (m, 6H), 2.10 (m, 1H), 1.81–1.70 (m, 7H), 1.61–1.43 (m, 8H), 1.31 (m, 2H), 0.94–0.81 ppm (m, 12H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 174.2, 173.3, 172.4, 171.9, 171.5, 171.2, 170.5, 163.9, 152.8, 152.6, 141.2, 140.5, 128.7, 128.6, 128.3, 128.2, 126.9, 126.8, 115.9, 70.6, 70.5, 70.4, 70.3, 70.2, 70.1, 70.0, 69.9, 67.4, 62.1, 62.0, 60.1, 57.7, 56.7, 55.9, 55.4, 52.0, 50.8, 49.5, 40.6, 39.6, 39.2, 39.1, 36.9, 36.0, 31.1, 30.6, 28.7, 28.4, 28.2, 25.7, 24.8, 24.7, 23.4, 23.2, 22.6, 22.4, 22.0 ppm; LC-MS (ESI): m/z calcd for $C_{61}H_{90}N_{10}O_{12}S_2$: 1219.62 [$M$+1]; found: 1219.00, 610.00 (half mass); HRMS (ESI-TOF): m/z calcd for $C_{61}H_{90}N_{10}O_{12}S_2Na$: 1241.6079 [$M+Na^+$]; found: 1241.6074.

Experimental methods for SM253-Tag-IV

HO-Leu-*N*-Me-Val-D-Lys(Boc)-D-3-(4-thiazolyl)-Ala-3,3-diphenyl-D-Ala-NH$_2$: Resin-O-Leu-*N*-Me-Val-D-Lys(Boc)-D-3-(4-thiazolyl)-Ala-3,3-diphenyl-D-Ala-NH$_2$ was synthesized by using resin-O-Leu-NH$_2$ (1.0 g, 0.50 mmol, 1.0 equiv) and the subsequent peptide coupling in the sequence with an aliquot (1.5 mmol, 3.0 equiv) of the following: Fmoc-*N*-Me-Val-OH (0.53 g), Fmoc-D-Lys(Boc)-OH (0.53 g), Fmoc-D-3-(4-thiazolyl)-Ala-OH (0.59 g), and Fmoc-3,3-diphenyl-D-Ala-OH (0.70 g). Each peptide coupling was carried out in the presence of HOAt (0.20 g, 1.5 mmol, 3.0 equiv) or HOBt (0.20 g, 1.5 mmol, 3.0 equiv), DIC (0.47 mL, 3.0 mmol, 6.0 equiv) and DMF (2.5 mL) to generate a concentration of 0.20 M based on the amino acid. Each coupling reaction was carried out for 3 h, and a negative ninhydrin test was used to confirm the reaction completion. The DDLP **SM253-Tag-IV** was cleaved from the resin by using a solution of TFE (6 mL) and CH_2Cl_2 (6 mL). The resin-containing solution was filtered and dried in vacuo to yield the DDLP **SM253-Tag-IV** as a white solid (430 mg, quantitative). LC-MS (ESI): m/z calcd for $C_{44}H_{63}N_7O_8S$: 850.45 [M+1]; found: 850.70.

***cyclo*-Leu-*N*-Me-Val-D-Lys(Boc)-D-3-(4-thiazolyl)-Ala-3,3-diphenyl-D-Ala**: The product was generated from DDLP **SM253-Tag-IV** (0.43 g, 0.51 mmol, 1.0 equiv) with TBTU (0.11 g, 0.35 mmol, 0.70 equiv), HATU (0.19 g, 0.51 mmol, 1.0 equiv), DMTMM (0.10 g, 0.35 mmol, 0.70 equiv), and DIPEA (0.70 mL, 4.05 mmol, 8.0 equiv) in anhydrous CH_2Cl_2 (506 mL, 0.001 M) and following the macrocyclization procedure. The reaction was stirred overnight and the reaction was monitored by TLC and LC-MS. Upon completion, the reaction mixture was subjected to an acid/base wash to afford the crude product, which was purified by flash column chromatography on silica gel with an ethyl acetate/hexane gradient system as the eluent, followed by purification by HPLC to yield the Boc-protected macrocycle as a white solid (124 mg, 29.5%). LC-MS (ESI): m/z calcd for $C_{44}H_{61}N_7O_7S$: 832.44 [M+1]; found: 831.95.

The Boc protecting group of the macrocycle was removed by utilizing a mixture of TFA/CH_2Cl_2 (1:4, 0.1 M) and anisole (2.0 equiv) to generate free amine groups in the lysine residue. The free amine compound was used in the subsequent biotinylation reaction without purification. LC-MS (ESI): m/z calcd for $C_{39}H_{53}N_7O_5S$: 732.38 [M+1]; found: 732.15.

The biotinylated **SM253-Tag-IV** was afforded by using utilizing the deprotected macrocycle (86.1 mg, 0.12 mmol, 1.0 equiv), NHS-(PEG)$_4$-Biotin (96.9 mg, 0.16 mmol, 1.4 equiv), and DIPEA (163.9 μL,

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0.94 mmol, 8.0 equiv) in CH_2Cl_2 (1.18 mL). The reaction mixture was stirred for 4 h and monitored by LC-MS. Upon completion, the crude product was purified by using preparative HPLC to generate pure the biotinylated compound as a white solid (25 mg, 17.3%). R_f = 0.38 (EtOAc/MeOH = 1:1); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.89 (s, 1H), 8.25 (br; N*H*), 7.57 (d, J = 8.6 Hz; N*H*), 7.44 (d, J = 7.5 Hz; N*H*), 7.36–7.14 (m, 10H), 7.07 (br; N*H*), 6.90 (m; N*H*), 6.76 (m; N*H*), 5.21 (t, J = 10.3 Hz, 1H), 4.83 (d, J = 10.6 Hz, 1H), 4.73 (d, J = 10.3 Hz, 1H), 4.59–4.51 (m, 3H), 4.40 (m, 2H), 4.02 (m, 1H), 3.74 (m, 3H), 3.64–3.61 (m, 13H), 3.42 (m, 3H), 3.27 (m, 2H), 3.20 (m, 3H), 3.12 (m, 2H), 2.96 (m, 1H), 2.83 (m, 5H), 2.47 (m, 2H), 2.32 (m, 1H), 2.24 (m, 2H), 1.72–1.61 (m, 6H), 1.50 (m, 4H), 1.29 (m, 1H), 0.83–0.72 ppm (m, 12H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 173.3, 171.4, 170.9, 170.5, 170.1, 169.5, 164.4, 152.7, 152.5, 140.9, 140.7, 128.8, 128.6, 128.2, 127.9, 127.1, 127.0, 115.9, 70.6, 70.5, 70.4, 70.3, 70.2, 70.1, 69.8, 67.5, 63.4, 62.2, 62.1, 60.2, 58.4, 57.5, 55.9, 55.6, 51.9, 50.5, 49.5, 40.8, 39.3, 37.1, 35.9, 32.1, 31.8, 30.9, 29.1, 28.2, 26.0, 25.6, 24.8, 23.0, 22.8, 22.5, 22.4, 19.8, 19.1 ppm; LC-MS (ESI): *m/z* calcd for $C_{60}H_{88}N_{10}O_{12}S_2$: 1205.60 [$M$+1]; found: 1205.00 [$M$+1], 603.00 (half mass); HRMS (ESI-TOF): *m/z* calcd for $C_{60}H_{88}N_{10}O_{12}S_2Na$: 1227.5922 [$M+Na^+$]; found: 1227.5918.

Experimental methods for SM253-Tag-IV dimer

SM253-Tag-IV dimer: The product was synthesized by utilizing *cyclo*-Leu-*N*-Me-Val-D-Lys-D-3-(4-thiazolyl)-Ala-3,3-diphenyl-D-Ala (31.2 mg, 0.043 mmol, 1.0 equiv), BS(PEG)$_9$ (15.1 μg, 0.021 μmol, 0.5 equiv), and DIPEA (59.4 μL, 8.0 equiv) dissolved in CH_2Cl_2 (426 μL). The crude material was purified by using HPLC to obtain the product (5 mg, 6%). ^{1}H NMR (600 MHz, DMSO): δ = 8.93 (m, 2H), 8.39 (m; 2N*H*), 7.56–7.16 (m, 24H), 6.87 (m, 2H), 6.67 (m; 2N*H*), 5.06 (m; 2N*H*), 4.87 (m; 2N*H*), 4.62 (m; 2N*H*), 4.46 (m, 2H), 4.21 (m; 2N*H*), 3.64 (m, 34H), 3.22 (m, 6H), 2.80 (m, 6H), 2.49 (m, 8H), 1.48 (m, 10H), 1.25 (m, 14H), 0.99–0.74 ppm (m, 24H); ^{13}C NMR (150 MHz, DMSO): δ = 171.6, 171.5, 171.4, 171.3, 170.8, 170.6, 152.2, 141.1, 140.5, 128.9, 128.8, 128.6, 128.2, 127.1, 126.9, 116.5, 70.6, 70.5, 70.4, 70.3, 70.2, 67.4, 59.5, 58.1, 49.4, 39.1, 39.0, 38.2, 37.0, 31.9, 31.6, 31.2, 29.8, 28.8, 26.1, 22.8, 22.7, 19.8, 19.7 ppm; LC-MS (ESI): *m/z* calcd for $C_{100}H_{144}N_{14}O_{21}S_2$: 1942.01 [$M$+1]; found: 993.00 (half mass); HRMS (ESI-TOF): *m/z* calcd for $C_{100}H_{144}N_{14}O_{21}S_2$ 1942.0072 [$M+H^+$]; found: 1943.0162.

Experimental methods for SM258-Tag-II

HO-Lys(Boc)-*N*-Me-Val-D-3-(4-thiazolyl)-Ala-D-Phe-3,3-diphenyl-D-Ala-NH$_2$: Resin-O-Lys(Boc)-*N*-Me-Val-D-3-(4-thiazolyl)-Ala-D-Phe-3,3-diphenyl-D-Ala-NH$_2$ was synthesized by using resin-O-Lys(Boc)-NH$_2$ (1.0 g, 0.5 mmol, 1.0 equiv), and the subsequent peptide coupling in the sequence was carried out by using an aliquot (1.5 mmol, 3.0 equiv) of each of the following: Fmoc-*N*-Me-Val-OH (0.53 g), Fmoc-D-3-(4-thiazolyl)-Ala-OH (0.59 g), Fmoc-D-Phe-OH (0.58 g), and Fmoc-3,3-diphenyl-D-Ala-OH (0.70 g). Each peptide coupling was carried out in the presence of HOAt (0.20 g, 1.5 mmol, 3.0 equiv) or HOBt (0.20 g, 1.5 mmol, 3.0 equiv), DIC (0.47 mL, 3.0 mmol, 6.0 equiv), and DMF (2.5 mL) to generate a concentration of 0.20 M based on the amino acid. Each coupling reaction was carried out for 3 h, and a negative ninhydrin test was used to confirm the reaction completion. The Fmoc protecting group was removed after the completion of each coupling reaction. The DDLP **SM258-Tag-II** was cleaved from the resin by using a solution of TFE (6 mL) and CH_2Cl_2 (6 mL). The resin-containing solution was filtered and dried in vacuo to yield the DDLP **SM258-Tag-II** as a white solid (400 mg, 90.5%). LC-MS (ESI): *m/z* calcd for $C_{47}H_{61}N_7O_8S$: 884.43 [M+1]; found: 884.10.

***cyclo*-Lys(Boc)-*N*-Me-Val-D-3-(4-thiazolyl)-Ala-D-Phe-3,3-diphenyl-D-Ala**: The product was generated by using DDLP **SM258-Tag-II** (0.40 g, 0.45 mmol, 1.0 equiv), TBTU (0.10 g, 0.32 mmol, 0.70 equiv), HATU (0.21 g, 0.54 mmol, 1.20 equiv), DMTMM (0.063 g, 0.23 mmol, 0.50 equiv), and DIPEA (0.63 mL, 3.62 mmol, 8.0 equiv) in anhydrous CH_2Cl_2 (452 mL, 0.001 M) and following the macrocyclization procedure. The reaction was stirred overnight and the reaction was monitored by TLC and LC-MS. Upon completion, the reaction mixture was subjected to an acid/base wash to afford the crude product, which was purified by flash column chromatography on silica gel with an ethyl acetate/hexane gradient system as the eluent, followed by purification by HPLC to yield the Boc-protected macrocycle as a white solid (82 mg, 20.9%). LC-MS (ESI): *m/z* calcd for $C_{47}H_{59}N_7O_7S$: 866.42 [M+1]; found: 866.05.

The Boc protecting group of the macrocycle was removed by utilizing a mixture of TFA/CH_2Cl_2 (1:4, 0.1 M) and anisole (2.0 equiv) to generate free amine groups in the lysine residue. The free amine compound was used in the subsequent biotinylation reaction without purification. LC-MS (ESI): *m/z* calcd for $C_{42}H_{51}N_7O_5S$: 766.37 [M+1]; found: 766.00.

The biotinylated **SM258-Tag-II** was afforded by using the deprotected macrocycle (40 mg, 0.052 mmol, 1.0 equiv), NHS-(PEG)$_4$-Biotin (43 mg, 0.073 mmol, 1.4 equiv), and DIPEA (72.8 μL, 0.42 mmol, 8.0 equiv) in CH_2Cl_2 (522 μL). The reaction mixture was stirred for 4 h and monitored by LC-MS. Upon completion, the crude product was purified by using preparative HPLC to generate pure biotinylated compound as a white solid (7.8 mg, 12.1%). R_f = 0.38 (EtOAc/MeOH = 1:1); ^{1}H NMR (600 MHz, DMSO): δ = 8.96 (d, J = 1.9 Hz, 1H), 8.27 (d, J = 8.2 Hz; 2N*H*), 7.84 (t, J = 5.6 Hz; N*H*), 7.76 (m; 2N*H*), 7.74 (t, J = 5.4 Hz; N*H*), 7.23–7.15 (m, 13H), 6.88 (m, 2H), 6.42 (s, 1H), 6.36 (s, 1H), 5.15 (m, 1H), 4.99 (dd, J = 15.0, 8.0 Hz, 1H), 4.52 (d, J = 11.3 Hz, 1H), 4.31 (m, 2H), 4.13 (m, 1H), 4.05 (m, 1H), 3.59 (t, J = 6.4 Hz, 2H), 3.50 (m, 14H), 3.40 (t, J = 5.9 Hz, 2H), 3.19 (dd, J = 11.7, 5.8 Hz, 1H), 3.10 (m, 1H), 2.91 (m, 4H), 2.82 (dd, J = 12.4, 5.1 Hz, 1H), 2.69 (dd, J = 14.0, 6.3 Hz, 1H), 2.62 (m, 3H), 2.59 (m, 2H), 2.29 (t, J = 6.7 Hz, 2H), 2.07 (t, J = 7.5 Hz, 2H), 1.61 (m, 2H), 1.51 (m, 4H), 1.31 (m, 4H), 0.92 (m, 2H), 0.81 (d, J = 6.4 Hz, 3H), 0.59 ppm (d, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, DMSO): δ = 172.6, 170.6, 170.2, 170.1, 169.9, 169.8, 168.9, 163.2, 154.0, 153.5, 141.7, 141.1, 137.7, 129.2, 129.1, 129.0, 128.8, 128.6, 128.4, 127.1, 126.9, 126.8, 115.4, 70.3, 70.2, 70.1, 70.0, 69.9, 69.6, 67.4, 67.3, 62.9, 61.5, 60.2, 59.7, 57.3, 56.7, 55.9, 53.9, 53.7, 49.4, 40.5, 40.3, 38.9, 37.7, 36.6, 35.6, 33.5, 30.2, 29.2, 28.7, 28.5, 25.7, 25.3, 23.6, 21.2, 20.0, 19.8 ppm; LC-MS (ESI): *m/z* calcd for $C_{63}H_{86}N_{10}O_{12}S_2$: 1239.59 [$M$+1] [$M$+1]; found: 606.00 (half mass) [M+1]; HRMS (ESI-TOF): *m/z* calcd for $C_{63}H_{86}N_{10}O_{12}S_2Na$: 1261.5766 [$M+Na^+$]; found: 1261.5763.

Experimental methods for SM258-Tag-III

HO-Leu-*N*-Me-Lys(Boc)-D-3-(4-thiazolyl)-Ala-D-Phe-3,3-diphenyl-D-Ala-NH$_2$: Resin-O-Leu-*N*-Me-Lys(Boc)-D-3-(4-thiazolyl)-Ala-D-Phe-3,3-diphenyl-D-Ala-NH$_2$ was synthesized by using resin-O-Leu-NH$_2$ (1.0 g, 0.5 mmol, 1.0 equiv) and the subsequent peptide coupling in the sequence by using an aliquot (1.5 mmol, 3.0 equiv) of each of the following: Fmoc-*N*-Me-Lys(Boc)-OH (0.72 g), Fmoc-D-3-(4-thiazolyl)-Ala-OH (0.59 g), Fmoc-D-Phe-OH (0.58 g), and Fmoc-3,3-diphenyl-D-Ala-OH (0.70 g). Each peptide coupling was carried out in the presence of HOAt (0.20 g, 1.5 mmol, 3.0 equiv) or HOBt (0.20 g, 1.5 mmol, 3.0 equiv), DIC (0.47 mL, 3.0 mmol, 6.0 equiv), and DMF (2.5 mL) to generate a concentration of 0.20 M based on the amino acid. Each coupling reaction was carried out for 3 h, and a negative ninhydrin test was used to confirm the reaction comple-

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tion. The Fmoc protecting group was removed after the completion of each coupling reaction. HO-Leu-*N*-Me-Lys(Boc)-D-3-(4-thiazolyl)-Ala-D-Phe-3,3-diphenyl-D-Ala-NH_2 was obtained from the resin cleavage by using a solution of TFE (6 mL) and CH_2Cl_2 (6 mL). The resin-containing solution was filtered and dried in vacuo to yield the DDLP **SM258-Tag-III** as a white solid (352 mg, 78.4%). LC-MS (ESI): *m/z* calcd for $C_{48}H_{63}N_7O_8S$: 898.45 [$M+1$]; found: 898.10.

***cyclo*-Leu-*N*-Me-Lys(Boc)-D-3-(4-thiazolyl)-Ala-D-Phe-3,3-diphenyl-D-Ala**: The product was synthesized by using DDLP **SM258-Tag-III** (0.35 g, 0.39 mmol, 1.0 equiv), TBTU (0.088 g, 0.27 mmol, 0.70 equiv), HATU (0.15 g, 0.39 mmol, 1.0 equiv), DMTMM (0.076 g, 0.27 mmol, 0.70 equiv), and DIPEA (0.55 mL, 3.13 mmol, 8.0 equiv) in anhydrous CH_2Cl_2 (392 mL, 0.001 M) and following the macrocyclization procedure. The reaction was stirred overnight and the reaction was monitored by TLC and LC-MS. Upon completion, the reaction mixture was subjected to an acid/base wash to afford the crude product, which was purified by flash column chromatography on silica gel with an ethyl acetate/hexane gradient system as the eluent, followed by purification by HPLC to yield the Boc-protected macrocycle as a white solid (150 mg, 43.7%). LC-MS (ESI): *m/z* calcd for $C_{48}H_{61}N_7O_7S$: 880.44 [$M+1$]; found: 880.10.

The Boc protecting group of the macrocycle was removed by utilizing a mixture of TFA/CH_2Cl_2 (1:4, 0.1 M) and anisole (2.0 equiv) to generate free amine groups in the lysine residue. The free amine compound was used in the subsequent biotinylation reaction without purification. LC-MS (ESI): *m/z* calcd for $C_{43}H_{53}N_7O_5S$: 780.38 [$M+1$]; found: 780.45.

The biotinylated **SM258-Tag-III** was afforded by using the deprotected **SM258-Tag-III** (52 mg, 0.067 mmol, 1.0 equiv), NHS-$(PEG)_4$-Biotin (54.9 mg, 0.093 mmol, 1.4 equiv), and DIPEA (92.9 μL, 0.25 mmol, 8.0 equiv) in CH_2Cl_2 (522 μL). The reaction mixture was stirred for 4 h and monitored by LC-MS. Upon completion, the crude product was purified by using preparative HPLC to generate pure biotinylated compound as a white solid (18 mg, 21.4%). R_f = 0.35 (EtOAc/MeOH = 0.30:0.70); ^{1}H NMR (600 MHz, DMSO): δ = 8.99 (d, J = 1.9 Hz, 1H), 8.35 (d, J = 6.7 Hz; N*H*), 8.14 (br; N*H*), 7.98 (d, J = 8.9 Hz; N*H*), 7.85 (t, J = 5.6 Hz; N*H*), 7.76 (m; N*H*), 7.52 (d, J = 6.4 Hz; N*H*), 7.26–7.15 (m, 15H), 6.95 (d, J = 5.7 Hz, 1H), 6.80 (s, 1H), 6.70 (s, 1H), 5.06 (m, 1H), 4.85 (dd, J = 14.6, 6.9 Hz, 1H), 4.81 (dd, J = 10.3, 5.7 Hz, 1H), 4.46 (m, 1H), 4.34 (m, 2H), 3.97 (dd, J = 14.6, 8.2 Hz, 1H), 3.71 (dd, J = 14.2, 6.7 Hz, 1H), 3.59 (m, 2H), 3.50 (m, 14H), 3.40 (t, J = 5.9 Hz, 2H), 3.35 (dd, J = 12.8, 1.7 Hz, 1H), 3.24 (m, 1H), 3.19 (m, 2H), 3.04 (dd, J = 14.1, 6.0 Hz, 1H), 2.97 (m, 2H), 2.93 (m, 1H), 2.69 (dd, J = 13.7, 8.7 Hz, 1H), 2.58 (s, 3H), 2.29 (m, 2H), 2.09 (t, J = 7.4 Hz, 1H), 1.73 (m, 2H), 1.55 (m, 2H), 1.35 (m, 8H), 0.93 (m, 2H), 0.69 (m, 3H), 0.57 ppm (m, 3H); ^{13}C NMR (150 MHz, DMSO): δ = 172.6, 171.3, 170.8, 170.7, 170.3, 170.2, 170.1, 161.7, 153.9, 153.5, 141.8, 141.3, 138.2, 129.3, 128.8, 128.7, 128.6, 128.5, 128.4, 128.1, 126.9, 126.7, 116.0, 70.5, 70.4, 70.3, 70.2, 70.1, 70.0, 69.9, 69.6, 67.3, 59.1, 57.5, 57.4, 57.1, 56.3, 53.2, 52.9, 52.8, 50.4, 38.9, 38.7, 37.1, 36.6, 35.4, 33.2, 30.2, 29.1, 27.1, 26.9, 25.6, 25.5, 24.7, 24.2, 23.1, 23.0, 22.4, 22.1 ppm; LC-MS (ESI): *m/z* calcd for $C_{64}H_{88}N_{10}O_{12}S_2$: 1253.60 [$M+1$]; found: 1253.00; HRMS (ESI-TOF): *m/z* calcd for $C_{64}H_{88}N_{10}O_{12}S_2Na$: 1275.5922 [$M+Na^+$]; found: 1275.5920.

Experimental methods for SM258-Tag-III dimer

SM258-Tag-III dimer: The product was synthesized by utilizing *cyclo*-Leu-*N*-Me-Lys-D-3-(4-thiazolyl)-Ala-D-Phe-3,3-diphenyl-D-Ala (10 mg, 0.013 mmol, 1.0 equiv), BS$(PEG)_9$ (4.54 mg, 0.0064 μmol, 0.5 equiv), and DIPEA (17.9 μL, 8.0 equiv) dissolved in CH_2Cl_2 (128 μL). The crude material was purified by using HPLC to yield the product (2 mg, 7.6%). ^{1}H NMR (600 MHz, DMSO): δ = 9.03 (m, 2H), 8.36 (m; 2N*H*), 8.06 (m; 2N*H*), 7.91(d, J = 8.6 Hz; 2N*H*), 7.78 (m; 2N*H*), 7.52 (d, J = 6.2 Hz; 2N*H*), 7.29–7.13 (m, 28H), 6.95 (d, J = 5.8 Hz, 4H), 5.06 (t, J = 10.4 Hz, 2H), 4.85 (dd, J = 14.1, 7.1 Hz, 2H), 4.81 (dd, J = 10.2, 5.7 Hz, 2H), 4.37 (d, J = 11.6 Hz, 4H), 3.97 (dd, J = 14.1, 7.9 Hz, 2H), 3.72 (m, 2H), 3.58 (m, 8H), 3.50 (m, 26H), 3.24 (dd, J = 14.2, 8.3 Hz, 2H), 3.05 (dd, J = 14.2, 6.0 Hz, 2H), 2.97 (m, 6H), 2.72 (m, 2H), 2.58 (s, 6H), 2.29 (m, 4H), 1.71 (m, 2H), 1.56 (m, 2H), 1.48 (m, 2H), 1.31 (m, 9H), 1.11 (m, 2H), 0.93 (m, 3H), 0.67 (d, J = 6.5 Hz, 6H), 0.58 ppm (d, J = 6.5 Hz, 6H); ^{13}C NMR (150 MHz, DMSO): δ = 175.2, 173.5, 172.3, 171.7, 170.3, 170.0, 159.9, 153.6, 142.0, 141.5, 138.6, 129.8, 129.4, 128.9, 128.6, 127.3, 127.1, 126.8, 126.7, 125.0, 116.1, 72.0, 70.2, 67.4, 59.4, 57.4, 56.9, 52.8, 50.4, 38.9, 37.0, 36.7, 36.3, 33.2, 30.0, 29.1, 26.9, 25.5, 24.8, 23.2, 23.0 ppm; LC-MS (ESI): *m/z* calcd for $C_{108}H_{144}N_{14}O_{21}S_2$: 2038.01 [$M+1$]; found: 1020.00; HRMS (ESI-TOF): *m/z* calcd for $C_{108}H_{144}N_{14}O_{21}S_2$: 2059.9970 [$M+Na^+$]; found: 2060.9998.

Experimental methods for SM145-Tag-II dimer

HO-D-Phe-racemic-β-OH-Phe-Lys(Boc)-*N*-Me-Val-D-Leu-NH_2: Resin-O-D-Phe-racemic-β-OH-Phe-Lys(Boc)-*N*-Me-Val-D-Leu-NH_2 was synthesized by using resin-O-D-Phe-NH_2 (1.5 g, 0.74 mmol, 1.0 equiv) and the subsequent peptide coupling in the sequence by using an aliquot (2.2 mmol, 3.0 equiv) of each of the following: Fmoc-(2*S*, 3*R*)/(2*R*, 3*S*)-β-OH-Phe-OH (0.89 g), Fmoc-Lys(Boc)-OH (1.03 g), Fmoc-*N*-Me-Val-OH (0.78 g), and Fmoc-D-Leu-OH (0.78 g). Each peptide coupling was carried out in the presence of HOAt (0.30 g, 2.2 mmol, 3.0 equiv) or HOBt (0.30 g, 2.2 mmol, 3.0 equiv), DIC (0.69 mL, 4.4 mmol, 6.0 equiv), and DMF (3.68 mL) to generate a concentration of 0.20 M based on the amino acid. Each coupling reaction was carried out for 3 h, and a negative ninhydrin test was used to confirm the reaction completion. The Fmoc protecting group was removed after completion of each coupling reaction. The DDLP **SM145-Tag-II** was generated by using a solution of TFE (7 mL) and CH_2Cl_2 (7 mL). The resin-containing solution was filtered and dried in vacuo to yield the DDLP as a white solid (263 mg, 45.4%). LC-MS (ESI): *m/z* calcd for $C_{41}H_{62}N_6O_9$: 783.46 [$M+1$]; found: 783.20.

***cyclo*-D-Phe-racemic-β-OH-Phe-Lys(Boc)-*N*-Me-Val-D-Leu**: The product was generated by using DDLP **SM145-Tag-II** (0.26 g, 0.34 mmol, 1.0 equiv), TBTU (0.086 g, 0.27 mmol, 0.80 equiv), HATU (0.10 g, 0.27 mmol, 0.8 equiv), DMTMM (0.074 g, 0.27 mmol, 0.80 equiv), and DIPEA (0.47 mL, 2.69 mmol, 8.0 equiv) in anhydrous CH_2Cl_2 (336 mL, 0.001 M) and following the macrocyclization procedure. The reaction was stirred overnight and the reaction was monitored by TLC and LC-MS. Upon completion, the reaction mixture was subjected to an acid/base wash to afford the crude product, which was purified by flash column chromatography on silica gel with an ethyl acetate/hexane gradient system as the eluent, followed by purification by HPLC to yield **SM145-Tag-II** as a white solid (150 mg, 43.7%). LC-MS (ESI): *m/z* calcd for $C_{41}H_{60}N_6O_8$: 765.45 [$M+1$]; found: 765.15.

***cyclo*-D-Phe-β-benzoxy-Phe-Lys(Boc)-*N*-Me-Val-D-Leu**: The product was synthesized following the benzylation procedure by using a mixture of the macrocycle (84.4 mg, 0.11 mmol, 1.0 equiv), NaH (60% in mineral oil; 7.9 mg, 0.33 mmol, 3.0 equiv), BnBr (37.7 μL, 0.22 mmol, 2.0 equiv), and anhydrous THF/DMF (1:1; 112 μL, 0.1 M). Upon completion, as determined by using LC-MS, the reaction mixture was extracted with deionized water and dichloromethane. The organic layer was collected, dried, and concentrated in vacuo. The residue was subjected to flash column chromatography for preliminary purification. Further purification of the resulting crude prod-

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uct was performed by RP-HPLC to yield *cyclo* D-Phe-β-benzoxy-Phe-Lys(Boc)-*N*-Me-Val-D-Leu (8 mg, 8.5%).

The Boc protecting group of the macrocycle was removed by following the Boc-removal procedure, in which a mixture of TFA/CH_2Cl_2 (1:4, 0.1 M) and anisole (2.0 equiv) were used to generate free amine groups in the lysine residue. The free amine compound was used in the subsequent dimerization without purification. LC-MS (ESI): *m/z* calcd for $C_{43}H_{53}N_7O_5S$: 755.45 [$M+1$]; found: 755.15.

SM145-Tag-II dimer: The product was synthesized by utilizing *cyclo* D-Phe-β-benzoxy-Phe-Lys-*N*-Me-Val-D-Leu (8 mg, 0.011 mmol, 1.0 equiv), $BS(PEG)_9$ (3.75 mg, 0.0053 μmol, 0.5 equiv), and DIPEA (14.8 μL, 8.0 equiv) dissolved in CH_2Cl_2 (106 μL). The crude material was purified by HPLC to yield the product (1.5 mg, 6.9%). ^{1}HNMR (600 MHz, DMSO): $\delta=7.58$ (m; 2N*H*), 7.50 (m; 2N*H*), 7.33–7.11 (m, 36H; 2N*H*), 4.80 (m, 2H; 2N*H*), 4.62 (m; N*H*), 4.42 (m, 3H; N*H*), 4.14 (m, 1H), 3.98 (m, 1H), 3.65 (m, 34H), 3.26–3.02 (m, 10H), 2.96 (m, 2H), 2.80 (m, 4H), 2.46 (m, 4H), 2.35 (m, 2H), 2.20 (m, 2H), 1.85 (m, 12H), 1.57 (m, 4H), 1.42 (m, 4H), 1.30 (m, 2H), 1.03–0.86 ppm (m, 24H); ^{13}CNMR (150 MHz, DMSO): $\delta=174.4$, 173.5, 171.9, 171.6, 169.7, 168.3, 138.5, 137.5, 136.8, 130.1, 129.9, 129.3, 129.1, 128.7, 128.4, 128.1, 127.6, 127.3, 79.9, 79.5, 70.5, 70.0, 67.2, 63.5, 61.6, 59.0, 57.4, 48.2, 42.2, 41.8, 41.2, 40.2, 36.9, 30.1, 29.2, 28.9, 26.3, 24.9, 22.8, 21.8, 19.6, 18.8, 17.9 ppm; LC-MS (ESI): *m/z* calcd for $C_{108}H_{154}N_{12}O_{23}$: 1988.12 [$M+1$]; found: 995.00 (half mass); HRMS (ESI-TOF): *m/z* calcd for $C_{108}H_{154}N_{12}O_{23}Na$: 2011.1100 [$M+Na^+$]; found: 2011.1174.

Experimental methods for SM122-Tag-III

HO-Phe-D-Phe-*N*-Me-Lys(Boc)-Leu-Lys(Cbz)-NH_2: Resin-O-Phe-D-Phe-*N*-Me-Lys(Boc)-Leu-Lys(Cbz)-NH_2 was synthesized by utilizing resin-O-Phe-NH_2 (1.5 g, 0.96 mmol, 1.0 equiv) and the subsequent peptide coupling in the sequence using an aliquot (2.88 mmol, 3.0 equiv) of each of the following: Fmoc-*N*-Me-D-Phe-OH (1.156 g), Fmoc-Lys(Boc)-OH (1.35 g), Fmoc-Leu-OH (1.02 g), and Fmoc-Lys(Cbz)-OH (1.447 g). Each peptide coupling was carried out in the presence of HOAt (0.392 g, 2.88 mmol, 3.0 equiv) or HOBt (0.389 g, 2.88 mmol, 3.0 equiv), DIC (0.90 mL, 3.0 mmol, 6.0 equiv), and DMF to generate a concentration of 0.20 M based on the amino acid. Each coupling reaction was performed for 3 h, and a negative ninhydrin test was used to confirm the reaction completion. The Fmoc protecting group was removed after the completion of each coupling reaction. HO-Phe-D-Phe-*N*-Me-Lys(Boc)-Leu-Lys(Cbz)-NH_2 was obtained from the resin cleavage by using a solution of TFE (10 mL) and CH_2Cl_2 (10 mL). The resin-containing solution was filtered and dried in vacuo to yield the DDLP **SM122-Tag-III** as a white solid (708 mg, 79%). LC-MS (ESI): *m/z* calcd for $C_{50}H_{72}N_7O_{10}$: 930.53 [$M+1$]; found: 930.00.

***cyclo*-Phe-D-Phe-*N*-Me-Lys(Boc)-Leu-Lys(Cbz)**: The product was synthesized by using DDLP **SM122-Tag-III** (0.203 g, 0.22 mmol, 1.0 equiv), TBTU (0.056 g, 0.18 mmol, 0.8 equiv), HATU (0.067 g, 0.18 mmol, 0.8 equiv), DMTMM (0.048 g, 0.18 mmol, 0.8 equiv), and of DIPEA (0.23 mL, 6.0 equiv) in anhydrous CH_2Cl_2 (220 mL, 0.001 M) and following the macrocyclization procedure. The reaction was stirred overnight, and the reaction was monitored by TLC and LC-MS. Upon completion, the reaction mixture was subjected to an acid/base wash to afford the crude product, which was purified by flash column chromatography on silica gel with an ethyl acetate/hexane gradient system as the eluent, followed by purification by HPLC to yield the Boc-protected macrocycle as a white solid (110 mg, 55%). LC-MS (ESI): *m/z* calcd for $C_{50}H_{70}N_7O_9$: 912.52 [$M+1$]; found: 912.00.

The Boc protecting group of the macrocycle was removed by utilizing a mixture of TFA/CH_2Cl_2 (1:4, 0.1 M) and anisole (2.0 equiv) to generate free amine groups in the lysine residue. The free amine compound was used in the subsequent biotinylation reaction without purification. LC-MS (ESI): *m/z* calcd for $C_{45}H_{62}N_7O_7$: 812.47 [$M+1$]; found: 812.00.

SM122-Tag-III dimer: The product was synthesized by utilizing *cyclo* Phe-D-Phe-*N*-Me-Lys-Leu-Lys(Cbz) (110 mg, 0.135 mmol, 1.0 equiv), $BS(PEG)_9$ (95.4 μL, 67.3 μmol, 0.5 equiv), and DIPEA (0.31 mL, 8.0 equiv) dissolved in dichloromethane (2.7 mL). The crude material was purified by using column chromatography on silica gel with ethyl acetate/methanol as the eluent to yield the product (127 mg, 90%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=8.16$–8.06 (m, 2H; N*H*), 7.64–7.55 (d, $J=8.71$ Hz, 2H; N*H*), 7.37–7.13 (m, 30H), 7.12–7.03 (d, $J=10.16$ Hz, 2H; N*H*), 6.94 (m, 2H; N*H*), 6.76–6.67 (m, 2H; N*H*), 5.36–5.26 (m, 2H), 5.09 (s, 4H), 5.09 (s, 4H), 4.80–4.68 (m, 2H), 4.74–4.64 (m, 2H), 4.23–4.13 (m, 2H), 3.75 (t, $J=12.1$, 6.7 Hz, 4H), 3.66 (m, 28H), 3.67–3.57 (m, 2H), 3.28–3.18 (m, 4H), 3.17–2.99 (m, 4H), 3.28–3.17 (m, 2H), 2.96–2.87 (m, 2H), 3.08–2.85 (m, 4H), 2.78 (s, 6H), 2.55–2.45 (t, $J=12.1$, 6.7 Hz, 4H), 1.82–1.72 (m, 4H), 1.88–1.65 (m, 8H), 1.71–1.61 (m, 2H), 1.82–1.57 (m, 4H), 1.58–1.36 (m, 4H), 1.33-.123 (m, 4H), 0.99–0.93 (d, $J=6.4$ Hz, 6H), 0.93–0.86 (m, 4H), 0.91–0.85 ppm (d, $J=6.4$ Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=172.3$, 172.1, 172.0, 171.6, 169.6, 156.6, 136.6, 136.5, 136.1, 129.3, 128.8, 128.7, 128.6, 128.5, 128.1, 127.1, 126.9, 70.4, 70.3, 70.2, 67.4, 66.6, 56.6, 55.6, 54.2, 49.5, 40.7, 40.3, 39.1, 38.0, 37.0, 32.6, 31.7, 29.8, 29.1, 28.6, 25.2, 23.2, 22.9, 22.7, 21.3 ppm; LC-MS (ESI): *m/z* calcd for $C_{112}H_{162}N_{14}O_{25}$: 2103.18 [$M+2$]; found: 1052.00 (half mass); HRMS (ESI-TOF): *m/z* calcd for $C_{112}H_{161}N_{14}O_{25}Na$: 2125.17 [$M+Na^+$]; found: 2125.13.

Biological methods

Hsp90 pulldown assays: Hsp90 pull-down was completed on NeutrAvidin agrose resin (Thermo Scientific Pierce) with biotin-tagged compounds (400 μM) incubated with crude cell lysate (2.5 mg) from human colorectal carcinoma cells (HCT 116) for 24 h at 4 °C. The unbound supernatant was removed. The beads were washed four times with wash buffer (20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl), 100 mM NaCl, 1% Triton X-100, pH 7.4) and subsequently boiled for 5 min at 95 °C in 5×Laemmli sample buffer (62.5 mM Tris–HCl, 2% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 100 mM dithiothreitol). The samples were analyzed by means of 4–20% SDS polyacrylamide gel electrophoresis (SDS-PAGE), followed by using the standard Western blot protocol to detect Hsp90. The amount of Hsp90 was analyzed by Image J and transformed to give a percentage of Hsp90 bound to each tagged compound, where 100% was set to the most effective tagged compound. Each experiment was completed with $n=3$, and the data shown are an average of these three experiments.

Protein binding assay: The pure protein binding assay to measure the binding affinity between Hsp90 and HOP/FKBP52 was completed by using human native protein Hsp90 (final concentration = 200 nM) and human-recombinant his-tagged HOP/FKBP52 (final concentration = 100 nM). The experiments were carried out with concentrations of 30 μM for monomeric molecules and 15 μM for dimeric molecules. The protein pulldown was completed on TALON metal affinity resin (Clontech; cat. no. 635501), followed by three washes of the beads in binding buffer and finally boiling the beads with 5×Laemmli sample buffer. The samples were analyzed by means of 4–20% SDS-PAGE, followed by using the standard Western blot protocol to detect Hsp90 and HOP/FKBP52. The re-

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spective ratio of Hsp90 to its cochaperones were analyzed by Image J and transformed to give a percentage of Hsp90 bound to cochaperone or client proteins. Each experiment was completed with $n=3$.

Cytotoxicity assay: HCT116 cells (ECACC) were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen/Life Technologies). The cells were propagated in a humidified chamber at 37°C with 5% CO_2 seeded into 96-well plates at 2000 cells per well and allowed to adhere overnight. The cells were treated with at a concentration of 30 μM, with a constant dimethyl sulfoxide (DMSO) concentration of below 1% for 72 h. After 72 hours, the media was removed and replaced with DMEM (100 μL) with the cell-counting kit 8 reagent (Dojindo; 10 μL). The cells were left in the incubator at 37°C with 5% CO_2 for 2–4 h. The absorbance was read according to the manufacturer's protocol by using a chromate plate reader.

Acknowledgements

We thank UNSW for support of S.R.M. and Y.-C.K. We also thank the Australian government for providing the Endeavour scholarship fund to Y.-C.K. Finally, we thank the NHMRC for funding (grant GNT1043561).

Keywords: antitumor agents · cancer · dimerization · inhibitors · proteins

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Received: July 21, 2016
Published online on ■■ ■■, 0000

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FULL PAPER

Antitumor Agents

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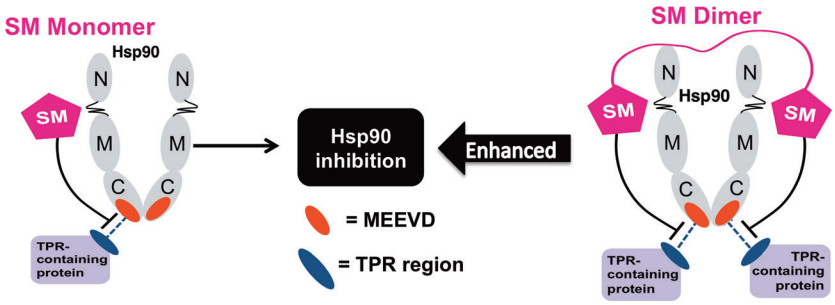
Reinventing Hsp90 Inhibitors: Blocking C-Terminal Binding Events to Hsp90 by Using Dimerized Inhibitors



Two are better than one: Heat-shock protein 90 (Hsp90) functions as a dimer and performs molecular-chaperone duties, thus stabilizing more than 400 proteins that are responsible for cancer development and progression. Molecules that modulate the C-terminus of Hsp90 effectively induce cancer cell death. Herein, we describe the design, synthesis, and binding affinity of dimerized C-terminal Hsp90 modulators, which are synergistically better at binding to Hsp90 than monomers (see picture; SM = Hsp90 C-terminal modulator, TPR = tetratricopeptide repeat.).

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