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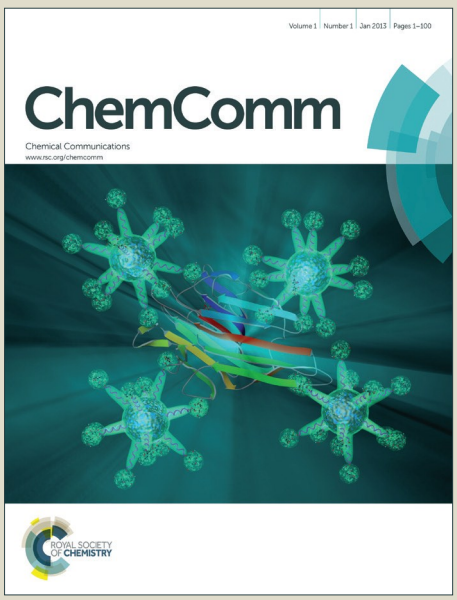





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# The first report of direct inhibitors that target the C-terminal MEEVD region on heat shock protein 90

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**Sixteen linear and cyclic peptides were designed *de novo* to target the C-terminus of heat shock protein 90 (Hsp90). Protein binding data indicates that three compounds directly block co-chaperone access to Hsp90's C-terminus and luciferase renaturation assays confirm Hsp90-mediated protein folding is disrupted. This is the first report of an inhibitor that binds directly to the C-terminal MEEVD region of Hsp90.**

Heat shock protein 90 (Hsp90) is molecular chaperone involved in the maintenance of protein homeostasis.[1-3] Hsp90 interacts with over 400 proteins, many of which are implicated in human diseases such as cancer.[4] As a result, inhibition of Hsp90 has been a rigorously investigated chemotherapeutic approach. Hsp90 exists as a homodimer, where each monomer contains three domains: (1) the N-terminus, (2) the middle domain, and (3) the C-terminus (Figure 1). The N-terminus contains an ATP-binding site, the middle domain contains binding sites for client proteins and some co-chaperones, and the C-terminus contains binding sites for co-chaperones whilst also serving as the dimerization domain. The C-terminus of Hsp90 also contains a highly conserved 5 amino acid sequence, MEEVD, which selectively interacts with co-chaperones that contain tetratricopeptide repeats (TPR domains) (Figure 1).[5] TPR domains are loosely conserved 34-amino acid structural motifs.[6] Hsp90 regulates numerous cellular pathways *via* these C-terminal co-chaperone interactions. Specifically, Hsp90-Cyp40 and Hsp90-FKBP51/FKBP52 interactions modulate hormone receptor activity; Hsp90-HOP regulates protein folding; and Hsp90-CHIP controls protein degradation.[7-11]

The classical Hsp90 inhibitors target the ATP-binding site located in the N-terminus. All of these classical inhibitors disrupt Hsp90's chaperone activity, and concurrently induce a cytoprotective response, referred to as a heat shock response (HSR). Induction of the HSR is responsible for the disappointing results observed with classical inhibitors in the clinic.[12-14] N-terminal Hsp90 inhibitors are still undergoing investigation and there are currently 32 active clinical trials involving these inhibitors (clinicaltrials.gov database). However, these studies utilise only three unique molecular structures and more than half of the clinical trials use these molecules in combination with other chemotherapeutics. This highlights a need for inhibitors that disrupt Hsp90 activity without inducing rescue mechanisms or drug resistance.

C-terminal Hsp90 inhibition has emerged as a promising alternative strategy for modulating Hsp90 activity as it overcomes the limitations associated with classical Hsp90 inhibitors. Specifically, recent work has shown that molecules that modulate the C-terminus of Hsp90 do not produce the HSR, and act *via* a mechanism that is distinct from the N-terminal inhibitors.[14-21] Recent evidence indicates that inhibiting co-chaperone binding at Hsp90's C-terminus actively decreases the cell protection mechanisms, e.g. the HSR, in contrast to the classical inhibitors that activate the HSR. Thus, targeting the C-terminus will likely produce a different outcome than modulating the N-terminus.[17, 22]

Recently reported C-terminal inhibitors, termed the SM series,[14-21] act allosterically by binding to the N-middle domain of Hsp90 and inhibiting access of co-chaperones to Hsp90's C-terminus. Developing effective molecules that act *via* allosteric mechanisms is highly challenging because it is difficult to establish predictable structure-activity relationships. Specifically, the SM series controls protein conformation, making it unpredictable. Herein we describe for the first time molecules that directly block access to the C-terminus of Hsp90's MEEVD region.

Kawakami and co-workers discovered a 12-amino acid sequence that disrupts Hsp90-HOP binding.[23-25] Heat shock

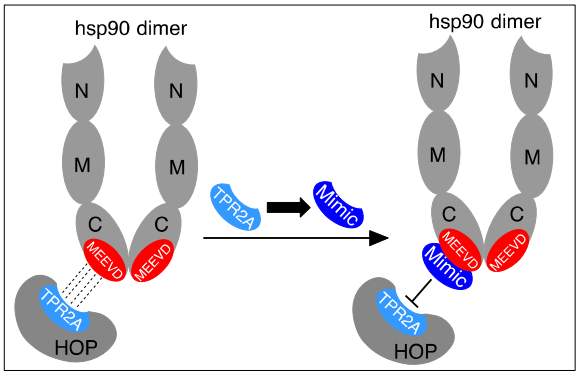


**Figure 1.** The MEEVD region on Hsp90's C-terminus interacts with the TPR2A domain on HOP. A 12 amino acid sequence was discovered to mimic the TPR2A and bind to Hsp90, thereby inhibiting the binding of HOP to Hsp90.

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organising protein (HOP) contains three TPR domains, where TPR2A interacts with the MEEVD region on Hsp90. This 12-amino acid sequence termed the "TPR Peptide", was derived from helix 3 of TPR2A and is based on the sequence between Lys-301 and Lys-312 (Figure 2). This peptide mimics the TPR2A domain and binds to Hsp90, thereby preventing HOP from binding (Figure 1). However, in order to use the 12-amino acid probe, the molecule must have a 16-amino acid sequence attached to promote receptor-mediated endocytosis. This makes the molecule a total of 28 amino acids in length. Thus, this compound is excellent for initial studies but has limited use as a molecular probe or drug lead. That is, the "entry sequence" likely inhibits some of the probe's functions and could generate false positive results by interacting with the targeted protein.

This report describes the design and synthesis of molecules that mimic the TPR2A domain, with the goal of inhibiting the binding event between the C-terminus of Hsp90 and TPR-containing co-chaperones. Based on the 12-amino acid sequence from Kawakami, we synthesized short amino acid variants of this peptide (Figure 2). Synthesizing sequences from both the C-terminus and the N-terminus, we generated linear and cyclic compounds that were five to eight amino acids in length (Figure 2). We then evaluated the ability of these compounds to: (1) block the interaction of Hsp90 with Cyp40 using a binding assay, (2) bind to the MEEVD region using NMR, and (3) impact Hsp90-mediated protein folding using a luciferase renaturation assay.

Starting from the C-terminus of the 12-amino acid lead, we designed four linear molecules five, six, seven or eight amino acids in length. These compounds were referred to as **5.1 LIN**, **6.1 LIN**, **7.1 LIN,** and **8.1 LIN**, respectively. Cyclic variants with identical sequences to these molecules were referred to as **5.1 CYC**, **6.1 CYC**, **7.1 CYC** and **8.1 CYC**. The corresponding sequences starting with the N-terminus of the 12-amino acid lead were referred to as **5.2 LIN**, **6.2 LIN**, **7.2 LIN** and **8.2 LIN**, respectively. Finally, the cyclic variants were referred to as **5.2 CYC**, **6.2 CYC**, **7.2 CYC** and **8.2 CYC** (Figure 2).

The synthesis of these compounds utilised an Fmoc solid-phase peptide synthesis approach. Briefly, a 2-chlorotrityl chloride (2-ClTrt) resin pre-loaded with the first amino acid underwent sequential coupling and amine deprotection reactions with the appropriate Fmoc-protected amino acids to generate a linear precursor between 5 and 8 residues in length. This precursor was then split into two groups, where the first was cleaved from the resin and globally deprotected to produce the linear peptide. The second batch was cleaved from the resin using mild conditions, cyclised and deprotected to yield the cyclic peptide (Supplementary Figure S1). HPLC purification produced clean final compounds and LC/MS, HRMS and NMR were used to confirm the final structures (supplemental material). The 12-amino acid lead compound and the MEEVD peptide were also synthesised. Starting with Lys(Boc)-2-ClTrt and Asp(Ot-Bu)-2-ClTrt resins respectively, Fmoc protected amino acids were sequentially coupled to generate the linear precursors that were then cleaved from the resin and universally deprotected. The linear peptides were precipitated in methanol and pelleted by centrifugation to produce the TPR Peptide and the MEEVD Peptide.

With 17 molecules in hand, protein-binding assays were performed to evaluate the ability of these compounds to inhibit Hsp90-Cyp40 binding. All compounds were initially screened against Hsp90β using a HSP90β (C-terminal) Inhibitor Screening Kit (BPS Biosciences, cat. 50314). Specifically, 50 μM of each compound was incubated with 24 nM of a C-terminal fragment of Hsp90β (UniProt P08238, a.a. 527-724) and 100 nM Cyp40. DMSO (1%) was used as a positive

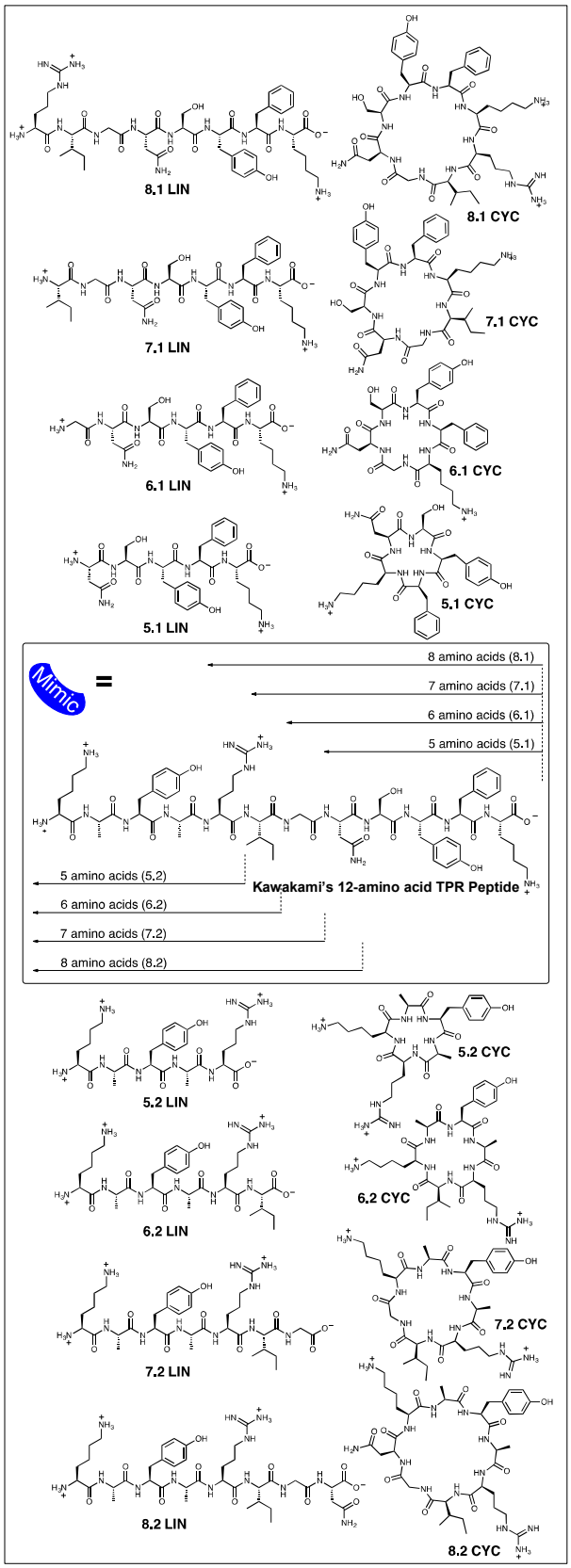
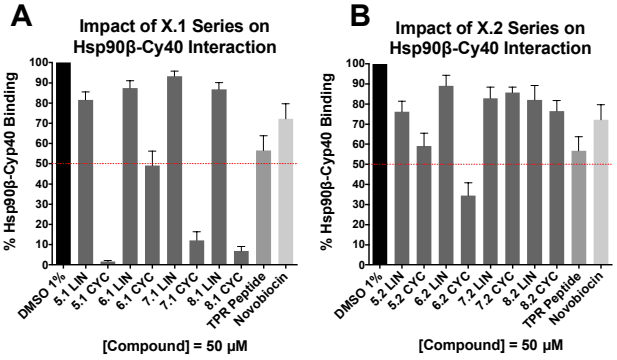



**Figure 2.** Structure of the TPR Peptide Mimic and novel analogues. Derivatives of this sequence were designed based on both sides of the molecule. The X.1 series was based on the right hand side and the X.2 series was based on the left hand side. All derivatives were between five and eight amino acids in length.

control, where it represents 100% binding between the C-terminus of Hsp90β and Cyp40. All compounds were compared to novobiocin, a well-investigated C-terminal Hsp90 inhibitor. At a concentration of 50 μM, novobiocin inhibited binding by approximately 30% and the TPR Peptide lead molecule showed approximately 40% inhibition (Figure 3, A & B). Analysis of the binding assay data for the X.1 series shows that the macrocycles were more effective at inhibiting Hsp90-Cyp40 binding than their linear counterparts, with each cyclic molecule displaying greater than 50% inhibition (Figure 3, A). This is logical since macrocycles have fewer conformations and a lower entropic cost of binding compared to linear peptides, which allows the cyclic peptide to bind with a high affinity.[26, 27] **5.1 CYC**, **7.1 CYC** and **8.1 CYC** were most effective at blocking Hsp90β-Cyp40 binding, where at 50 μM they inhibited the interaction by 98%, 88% and 93%, respectively (Figure 3, A). These molecules were significantly more effective than both novobiocin and the TPR peptide ($p<0.0001$).

The X.2 series were less effective inhibitors compared to the X.1 series, where only one compound, **6.2 CYC**, inhibited Hsp90β-Cyp40 binding by greater than 50% (Figure 3, B). Comparing the X.1 and X.2 series reveals some interesting structure-activity relationships: (1) the right-hand side of the TPR Peptide with sequence [Arg-Ile-Gly-Asn-Ser-Tyr-Phe-Lys] is critical for achieving tight binding to Hsp90, and (2) the inhibitory effects are directly related to the conformation of each individual molecule. That is, the active molecules must place the lysine and asparagine residues (the two residues considered critical for binding) into an appropriate conformation that allows them to disrupt the Hsp90β-Cyp40 binding event.[5, 28]

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| Compound | $IC_{50}$ / μM (Hsp90α-Cyp40) | $IC_{50}$ / μM (Hsp90β-Cyp40) |
|---|---|---|
| Novobiocin | 107 ± 1 | 1158 ± 1 |
| 5.1 CYC | 4 ± 1 | 4 ± 1 |
| 7.1 CYC | 44 ± 1 | 15 ± 1 |
| 8.1 CYC | 25 ± 1 | 18 ± 1 |

**Figure 3.** (A) Impact of X.1 series on Hsp90β-Cyp40 binding. (B) Impact of X.2 series on Hsp90α-Cyp40 binding. (C) $IC_{50}$ values of each compound. Graphs represent mean ± SEM, $n = 3$.

The three most effective molecules from the 50 μM screens were then taken on for concentration dependence studies. Testing **5.1 CYC**, **7.1 CYC**, **8.1 CYC** and novobiocin at multiple concentrations generated their $IC_{50}$ values. Compound **5.1 CYC** was the most effective at inhibiting Hsp90β-Cyp40 binding with an $IC_{50}$ value of approximately 4 μM. Compounds **7.1 CYC** and **8.1 CYC** had $IC_{50}$ values of 15 μM and 18 μM, respectively (Figure 3, C).

There are 2 possible mechanisms by which these compounds disrupt the Hsp90-Cyp40 interaction: (1) compounds are binding to Cyp40, or (2) compounds are binding to Hsp90. In order to confirm that these molecules were binding to Hsp90 and not Cyp40, a second series of binding assays between Hsp90α and Cyp40 were completed. Novobiocin, **5.1 CYC**, **7.1 CYC** and **8.1 CYC** were screened at multiple concentrations against Hsp90α using the HSP90α (C-terminal) Inhibitor Screening Kit (BPS Bioscience, cat. 50317). TPR-containing co-chaperones do not display Hsp90 isoform selectively and bind to Hsp90α and Hsp90β with the same affinity.[29, 30] In contrast, compounds binding to Hsp90 usually display isoform selectivity because Hsp90α and Hsp90β have different amino acid sequences. Specifically, the sequence alignment of the C-terminal proteins used in these binding assays shows that Hsp90α and Hsp90β are 85% identical. Thus, if the compounds are binding to Cyp40, they should have the same $IC_{50}$ values when assayed with Hsp90α or Hsp90β. However, if they are binding to Hsp90, it is likely they will have different $IC_{50}$ values for each isoform.

As reported by others, the results show that novobiocin has a different $IC_{50}$ value for each isoform, where it has a 10 fold lower $IC_{50}$ for Hsp90α. **7.1 CYC** and **8.1 CYC** also show isoform selectivity, where both preferentially bind to Hsp90β. Interestingly, **5.1 CYC** has approximately the same $IC_{50}$ value for both isoforms. Since **7.1 CYC** and **8.1 CYC** contain more amino acids than **5.1 CYC**, an explanation for our data is that the larger size allows **7.1 CYC** and **8.1 CYC** to bind not only to the MEEVD region, but also to additional positive interactions with the β isoform. However, **5.1 CYC** is a small macrocycle and as such it likely only interacts directly with the targeted MEEVD region, which is identical in both isoforms.

In order to prove that **5.1 CYC** was binding to the MEEVD region, we ran an experiment where **5.1 CYC** was titrated into a solution of MEEVD peptide, or TPR peptide (Figure 4). The controls involved the titration of: (A) Novobicin into MEEVD, and (B) TPR peptide into MEEVD (full spectra are included in the supplementary material). As reported by others, proton shifts and changes are subtle when two molecules are interacting, and they involve a peak decrease in height and definition and sometimes a small shift.[31, 32] The valine residue within MEEVD is known to play an important role when interacting with the TPR domain and as such will be impacted when binding occurs. There is a distinct broadening in the valine when **5.1 CYC** is titrated into the solution (Figure 4, A). In contrast, there is no change with novobiocin, nor is there a change in the same region of the TPR peptide (critical isoleucines).

The glutamic acid β-protons of MEEVD (2.00-2.05 ppm, Figure 4, B) are also impacted. Not only does this peak broaden, the height decreases as **5.1 CYC** is titrated into MEEVD. In contrast, the height and shape of this peak does not change with the titration of novobiocin. Thus, these glutamic acid protons are actively binding to **5.1 CYC.** Finally, the aspartic acid α-proton on MEEVD is impacted upon binding to **5.1 CYC** (Figure 4, C)**.** Not only does this peak decrease in height, it becomes significantly broader, and shifts 0.05 ppm. In contrast, the α-proton region does not change with titration of **5.1 CYC** into the TPR peptide. In summary, these data clearly indicate that **5.1 CYC** causes changes in the NMR spectrum of the MEEVD peptide by binding to this peptide sequence.

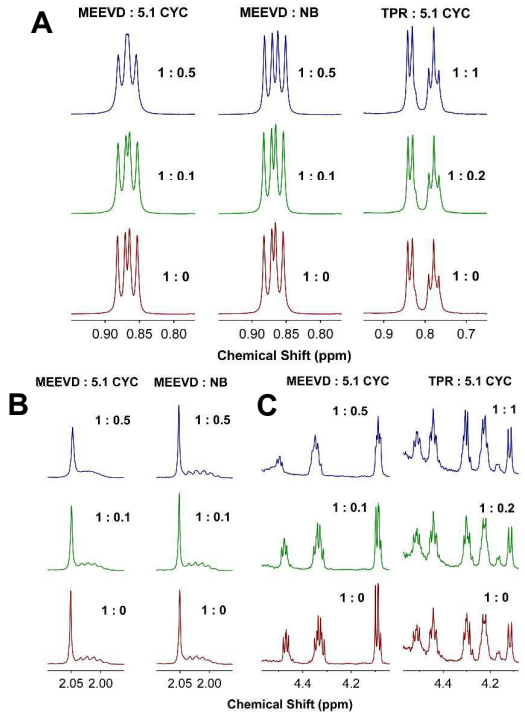


**Figure 4.** $^{1}$H NMR titrations. (A) 0.7 ppm – 0.9 ppm (B) 1.95 ppm – 2.05 ppm (C) 4.1 ppm – 4.6 ppm. NB = novobiocin. Ratios indicate relative concentrations of inhibitors and peptides.

Evaluating the impact of these compounds on Hsp90 function was accomplished using a luciferase protein renaturation assay involving rabbit reticulocyte lysate (RRL, Promega) and heat-denatured firefly luciferase (L9506, Sigma-Aldrich). Denatured luciferase was incubated in RRL, which had been pre-incubated with the test compounds. DMSO, novobiocin and AUY-922 (an N-terminal Hsp90 inhibitor currently in clinical trials) were used as controls, where DMSO (1%) represents 0% inhibition of luciferase activity. After 3 hours of incubation, **5.1 CYC** had the greatest impact on the refolding function of the Hsp90 machinery, inhibiting luciferase activity by 33% and 38% at concentrations of 25 µM and 50 µM, respectively (Figure 5). AUY-922 had little impact on protein folding, with less than 10% inhibition despite being tested at 50 fold of its $IC_{50}$ binding affinity for Hsp90. Novobiocin inhibited protein folding by only 17% at 100 µM. Thus, novobiocin was approximately half as effective as **5.1 CYC** was at 50 µM, despite being tested at double this concentration. These data show that **5.1 CYC** is clearly an effective inhibitor of Hsp90-mediated protein folding.

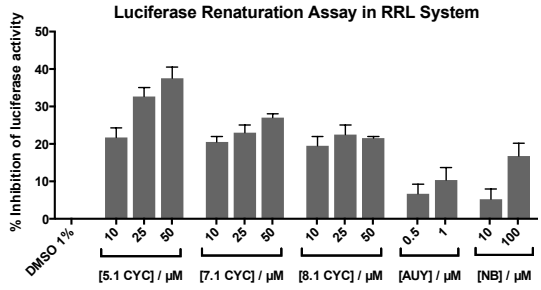


**Figure 5.** Luciferase renaturation assays. Graphs represent mean ± SEM, $n$ = 3. AUY = AUY-922 and NB = novobiocin.

In summary, we have generated sixteen novel derivatives that were designed *de novo* from the TPR domain of the co-chaperones that bind to Hsp90. Compound **5.1 CYC** showed extraordinary inhibition ($IC_{50}$ binding ~ 4 µM) considering that the molecule was a first generation compound, designed from a large insoluble peptide lead. Furthermore, we have shown that **5.1 CYC,** a small molecule containing only five amino acids was sufficiently effective at disrupting a large co-chaperone from binding and inhibiting Hsp90 function. The structure of **5.1 CYC** provides a solid foundation from which we can design new compounds that inhibit the C-terminus of Hsp90.

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