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Synthesis and Structure−Activity Relationships of Inhibitors That Target the C-Terminal MEEVD on Heat Shock Protein 90

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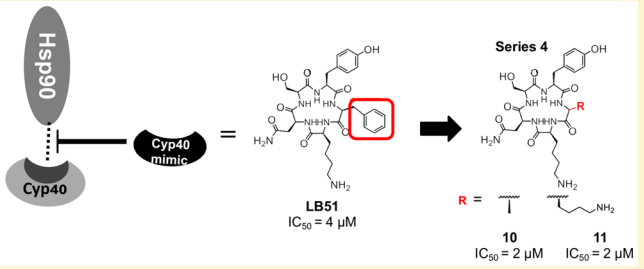
Supporting Information

ABSTRACT: Herein, we describe the synthesis and structure−activity relationships of cyclic peptides designed to target heat shock protein 90 (Hsp90). Generating 19 compounds and evaluating their binding affinity reveals that increasing electrostatic interactions allows the compounds to bind more effectively with Hsp90 compared to the lead structure. Exchanging specific residues for lysine improves binding affinity for Hsp90, indicating some residues are not critical for interacting with the target, whereas others are essential. Replacing L- for D-amino acids produced compounds with decreased binding affinity compared to the parent structure, confirming the importance of conformation and identifying key residues most important for binding. Thus, a specific conformation and electrostatic interactions are required in order for these inhibitors to bind to Hsp90.



KEYWORDS: *Heat shock protein 90 (Hsp90), MEEVD, C-terminus, macrocycle, cyclic peptide*

The evolutionarily conserved molecular chaperone heat shock protein 90 (Hsp90) is essential for the survival of eukaryotic cells and is involved in many cellular processes including signal transduction, protein folding, and protein degradation.[1] Hsp90 performs these cell maintenance tasks by dynamically coordinating with a diverse family of cochaperones. Forming complexes with cochaperones allows Hsp90 to individually regulate over 400 client proteins, including kinases, nuclear receptors, transcription factors, and mitochondrial proteins.[2,3] In cancer cells, the regulatory pathways modulated by Hsp90 are hijacked to support oncogenic processes. As a result, many Hsp90 client proteins are directly involved in driving malignancy.[4] Thus, Hsp90 is a powerful therapeutic target for anticancer drug development.

Hsp90 consists of three domains: an N-terminal domain (NTD), which contains an ATP-binding pocket; a middle domain (MD), where client proteins and some cochaperones dock; and a C-terminal domain (CTD), which includes the dimerization domain and binding sites for multiple cochaperones. Previous strategies aimed at blocking the function of Hsp90 utilized molecules that were bound to the highly conserved ATP-binding pocket, located in the NTD.[5] Inhibitors that target the NTD induced a cell protection mechanism, which led to drug resistance and activation of other defensive pathways.[6] Although there is some debate as to whether this cell protection mechanism is a result of Hsp90 inhibition or general cytotoxicity produced from off-target effects of these drugs,[6−9] all members of this class of inhibitors have, to date, failed as a single agent treatments in clinical trials (www.clinicaltrials.gov). Thus, there is general agreement that an effective drug must modulate Hsp90 through an alternative mechanism, one that does not induce a cytoprotective response. Targeting the CTD of Hsp90 is one such promising strategy.[6,10−13]

One Hsp90 inhibitor class, the SM series, was developed by McAlpine and co-workers to modulate the CTD via allosteric control[14,15] and decrease the cytoprotective response.[9] While a promising approach, the unpredictable structure−activity relationship (SAR) of this allosteric mechanism led to challenges in producing a highly potent molecule.[14,15] Another approach was taken by Kawakami and co-workers, who developed a peptide sequence that directly blocks the interaction between Hsp90 and the cochaperone heat-shock organizing protein (HOP) (Figure 1).[16,17] Kawakami's HOP-based peptide was designed to bind the acidic residues located at the end of the CTD, specifically MEEVD (Met-Glu-Glu-Val-Asp). The MEEVD region on Hsp90 binds to basic residues on HOP that are located within the TPR2A domain (Figure 1). This TRP2A domain is also located in other cochaperones that bind to Hsp90s MEEVD site.

We recently reported the development of truncated linear and cyclic variants of the TPR peptide in an effort to improve the drug-like properties of this molecule. We found that the

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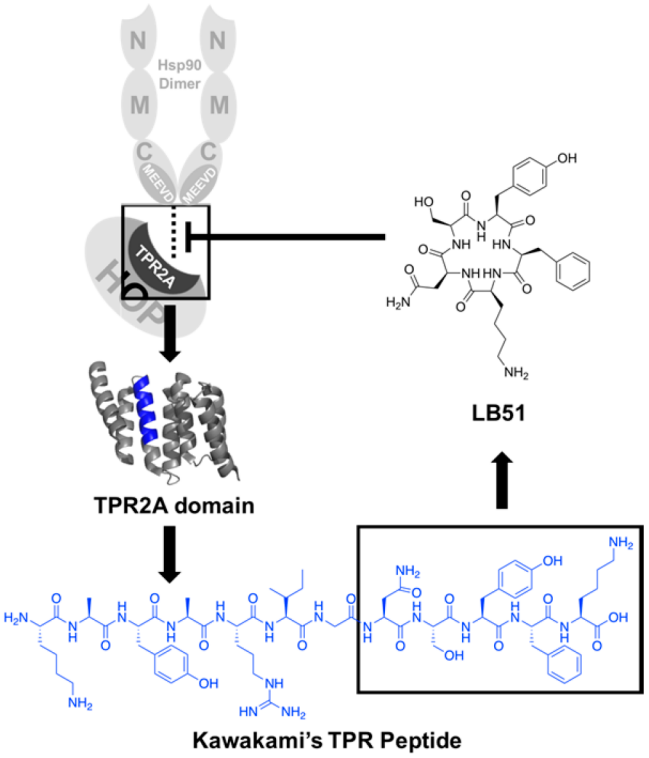


Figure 1. Lead inhibitors: 12-amino acid TPR peptide that binds to Hsp90 and LB51 lead scaffold derived from truncated sequence of TPR peptide.

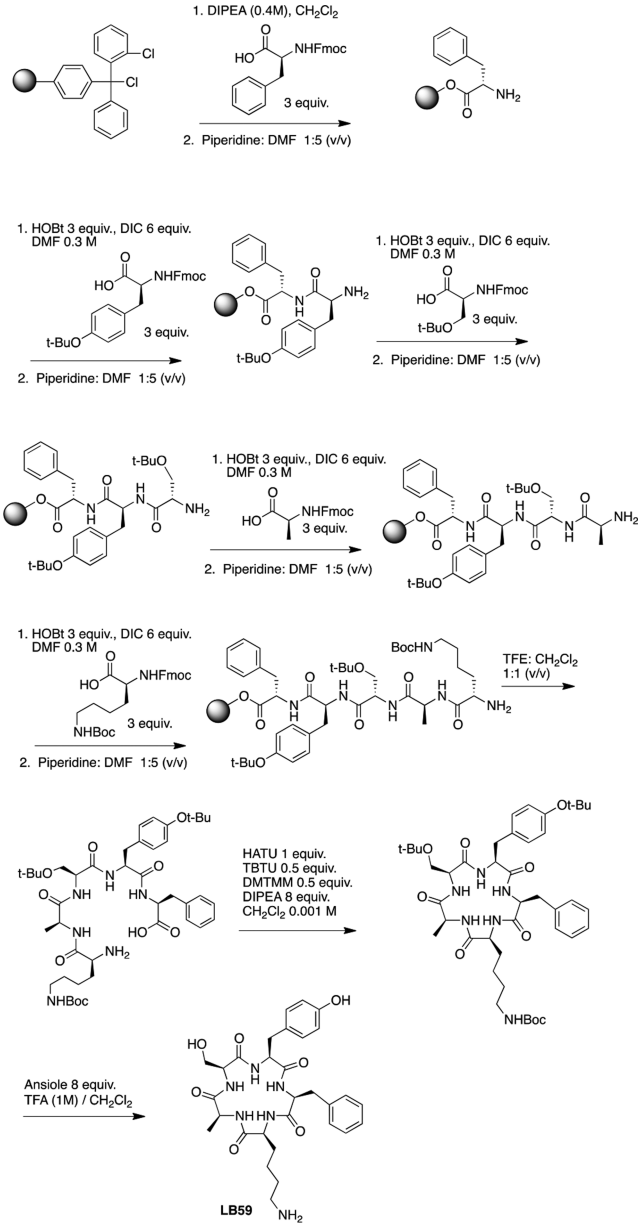
cyclic peptides were significantly more active than their linear counterparts.[18] The most active molecule, cyclic pentapeptide LB51 (Figure 1), binds to the MEEVD region of Hsp90s C-terminus and blocks interactions between the CTD and the cochaperone Cyp40 with an IC_{50} = 4 μM; in contrast, Kawakami's peptide has an IC_{50} = 50 μM.[18] This LB51 was >10-fold more effective than the lead.

Herein, we describe SAR studies that are based on the lead compound LB51. We generated five series of analogs (1−5), where each series represented a change to a single amino acid on the lead LB51 cyclic scaffold. At each amino acid, an alanine, lysine, or D-amino acid was substituted into the backbone. Examining how effectively these molecules blocked the interaction between Hsp90 and Cyp40, identified the essential and nonessential residues within these inhibitors.

All analog synthesis was completed using Fmoc solid-phase peptide chemistry (Scheme 1), where the synthesis of one analog, **1**, is described. Phenylalanine (Phe) was loaded onto 2-chlorotrityl chloride resin. The resin-bound peptide underwent sequential coupling and Fmoc removal with Fmoc-protected tyrosine(*t*-Bu), serine(*t*-Bu), alanine, and lysine(Boc) amino acids, respectively. Once couplings were complete, the pentapeptide was cleaved from the resin under mildly acidic conditions. Crude linear pentapeptide was cyclized using a combination of three coupling agents (HATU, TBTU, and DMTMM). Subsequent deprotection of the side-chains was accomplished using trifluoracetic acid (TFA) with anisole as the carbocation scavenger. HPLC purification produced pure compound, and LC−MS, ^{1}H NMR, and 2D NMR were used to confirm the final structure.

All compounds were evaluated for their ability to inhibit the Hsp90−Cyp40 interaction using a commercially available Hsp90β (C-terminal) Inhibitor Screening Kit (BPS Bioscien-

Scheme 1. Synthesis of 1[a]



[a]A similar strategy was used to produce the library of analogs.

ces, cat. # 50314). Each compound was incubated at the designated concentration, with the C-terminal fragment of Hsp90β (UniProt P08238, a.a. 527−724) and Cyp40 protein following the commercial protocol. DMSO (1%) was used as a control, representing 100% binding between the C-terminus of Hsp90β and Cyp40.

Based on the concentration-dependent binding data (Figure 2b), substitutions of alanine and lysine effectively eliminate the binding activity of the compound when replacing the tyrosine residue (Series 3) or the lysine residue (Series 5). These data indicate that both tyrosine and lysine play an important role in the binding event with Hsp90 and are required to block the interaction between Cyp40 and Hsp90. Similarly, substituting the natural L-stereochemistry with the unnatural D-amino acid diminishes activity at both the Tyr and Lys positions.

In contrast, replacement of the asparagine (series 1), serine (series 2), or phenylalanine (series 4) with alanine or lysine

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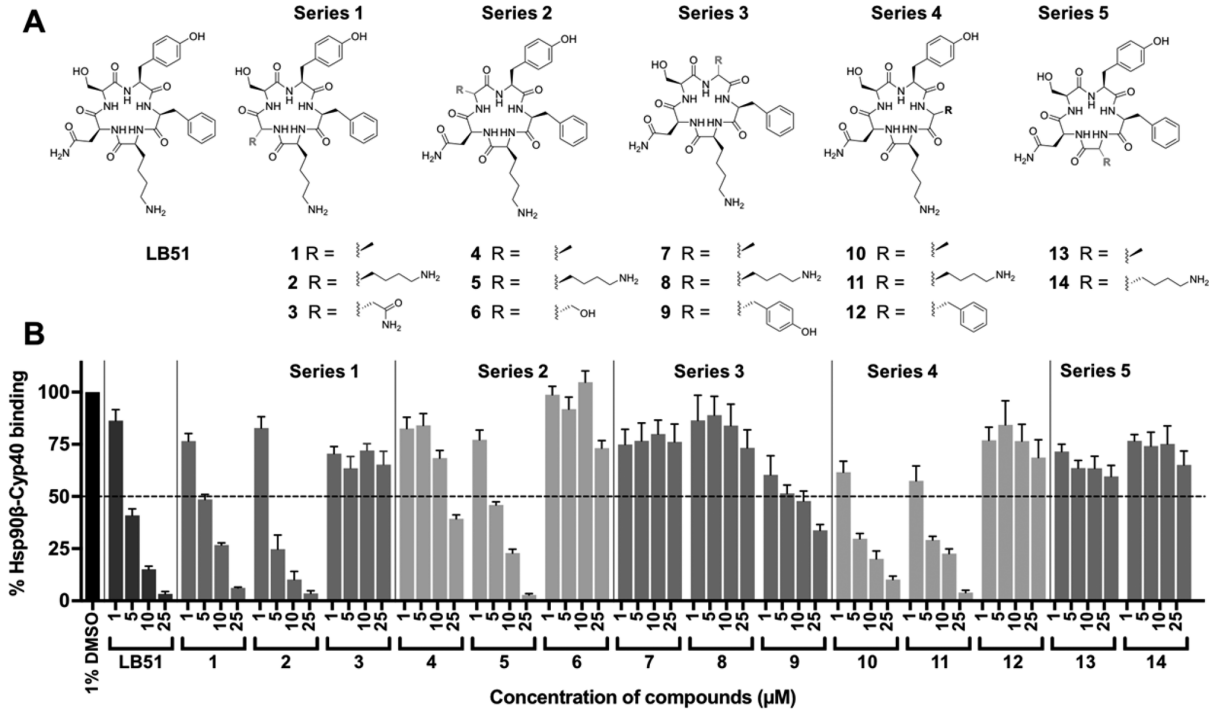

Figure 2. (a) Structures of **LB51** analogs, where each position is substituted with alanine, lysine, or the D-isomer. (b) Impact of analogs on Hsp90−Cyp40 interaction.

produces compounds that maintain their ability to block the binding between Hsp90 and Cyp40. The compounds in series 1 and 4 (originally the location of asparagine and phenylalanine, respectively) maintain binding affinity when substituted with alanine or a lysine residue. However, for series 2, replacing serine with lysine (**5**) produced a more effective molecule than the alanine substitution (**4**). Inverting the L-stereochemistry to D at the asparagine (**3**), serine (**6**), and phenylalanine (**12**) positions results in a loss of inhibitory activity in all three of these series, indicating that the L-stereochemistry provides an effective conformation control that is required for tight binding affinity to Hsp90. Comparing the most active molecules **1**, **2**, **5**, **10**, and **11**, using IC_{50} values (Table 1), showed that replacing the asparagine (series 1) or phenylalanine (series 4) with alanine (**1** and **10**) or lysine (**2** and **11**) produced the most effective compounds.

Table 1. IC_{50} Values of the Most Active Molecules

compd	IC_{50} (μM)
LB51	3.72 ± 0.26
1	3.90 ± 0.27
2	2.58 ± 0.30
5	3.57 ± 0.31
10	1.84 ± 0.24
11	1.85 ± 0.26

In order to evaluate whether alternative residues would improve binding affinity to Hsp90, we modified a single series. Choosing series 1, we replaced the asparagine with multiple amino acids. These modifications included introducing hydrophobic moieties leucine (**15**) or phenyl alanine (**16**), changing the side chain charge from positive to negative with an aspartic acid (**17**), and examining how stereochemistry impacted binding affinity by incorporating a D-alanine (**18**) (Figure 3).

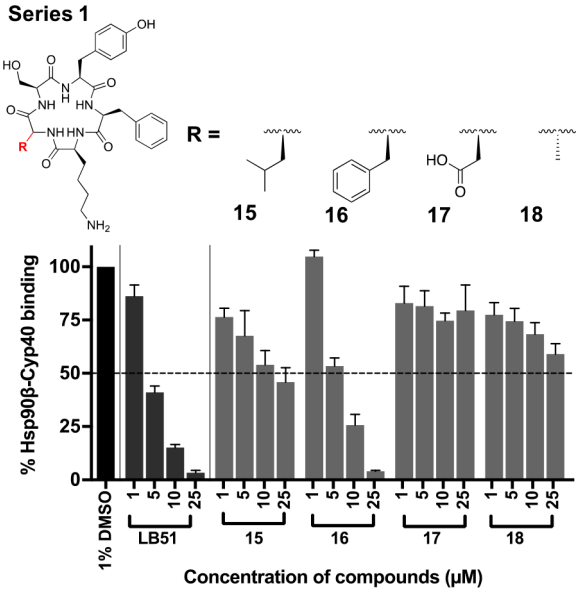

Figure 3. Structures with modifications made to the asparagine in **LB51** (series 1) and the binding affinity of those molecules.

Biological evaluation revealed that molecules of phenylalanine (**16**) were an effective inhibitor and had similar activity to the alanine (**1**) and lysine (**2**) derivatives. In contrast, substituting a leucine (**15**), aspartic acid (**17**), or D-alanine (**18**) resulted in molecules that were significantly less effective at blocking the binding event.

Two conclusions can be drawn from these data. First, electrostatic interactions are critical for binding between Hsp90 and these molecules. Specifically, the acidic residues on the CTD of Hsp90 in the MEEVD region must be interacting with the basic residues on the compounds. Thus, the inclusion of

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two lysine residues at three different positions (**2**, **11**, **5**) produced compounds that were more active than the lead structure **LB51**. Supporting this hypothesis is the compound that incorporated an acidic residue (**17**), producing an inactive compound. The second conclusion that can be drawn is that a specific conformation of the molecule is critical in order to produce a successful binding event with Hsp90. Inversion of the alanine from L (**1**) to D (**18**) produced an inactive molecule, indicating that an explicit organization of the backbone is critical and that the natural L-stereochemistry is optimal for binding.

A comparison between the activity of the L-lysine, **LB51**, and D-lysine analog, **14**, showed that **LB51** was significantly more active then **14** (Figure 2). This data suggests that both conformation and electrostatic interactions are critical for binding to Hsp90. In order to confirm this hypothesis, we evaluated a second instance of conversion between L-lysine and D-lysine. Comparing the activity where the L-lysine was inverted to D (**1** and **19** respectively, Figure 4) showed that inverting the

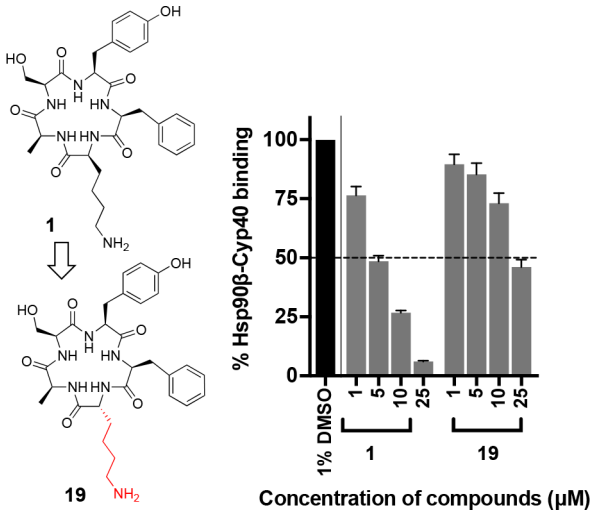

Figure 4. Structure of the molecules with L-lysine (**1**) to D-lysine (**19**) and the binding affinity for both compounds.

lysine dramatically reduced the compound's ability to bind, changing the IC_{50} from ~3.9 to ~25 μM. Thus, both electrostatic interaction(s) of the side chain and the orientation of the amino acids are critical for binding.

In conclusion, this SAR study established that lysine and tyrosine were critical for binding to Hsp90, and replacing these residues eliminated all binding affinity. Increasing electrostatic interactions with Hsp90 produced compounds that were more effective than the lead structure. Maintaining either of these two residues, but substituting the natural L-stereochemistry to D also eliminated binding activity. Specific modifications could be made to the asparagine without eliminating the binding affinity. However, molecules where D-amino acids were substituted into the lead all had decreased binding affinity compared to the L-diastereomer. Thus, a specific conformation and electrostatic interactions are required in order for our molecules to bind to Hsp90. These data have implications for the next generation of compounds and for future production of cyclic peptides designed to bind to protein targets.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.7b00310.

Experimental procedures and all spectra collected during the synthesis (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. M.N.R. synthesized nine molecules and drafted the paper. L.K.B. synthesized a molecule, performed the biological assays, and collated the supplemental data. S.Z. contributed to the synthesis of six molecules and reviewed the paper. J.K. contributed to the synthesis of three molecules and reviewed the paper. V.C., A.G., J.K., S.H.K., L.K.L., M.F.L., M.L., G.M., D.N., and G.T. each contributed to the synthesis of one molecule. S.R.M. reviewed the paper and contributed intellectual direction to the project.

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

This manuscript is dedicated in loving memory of Michael Finbarr Lawler.

■ ABBREVIATIONS

Hsp90, heat shock protein 90; Cyp40, cyclophilin 40; CTD, C-terminal domain; HOBt, 1-hydroxybenzotriazole hydrate; DIC, *N*,*N*-diisopropylcarbodiimide; TFE, 2,2,2-trifluorethanol; HATU, (1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo-[4,5-*b*]pyridinium 3-oxide hexafluorophosphate; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate; DMTMM, 4-(4,6-dimethoxy-[1,3,5]triazin-2-yl)-4-methylmorpholin-4-ium chloride; DIPEA, *N*,*N*-diisopropylethylamine

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