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**Discovery of novel Hsp90 C-terminal domain inhibitors that disrupt co-chaperone binding**

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| ARTICLE INFO | ABSTRACT |
| Article history:  Received  Revised  Accepted  Available online | Heat shock protein 90 (Hsp90) is an essential molecular chaperone that performs vital stress-related and housekeeping functions in cells and is a current therapeutic target for diseases such as cancers. Particularly, the development of Hsp90 C-terminal domain (CTD) inhibitors is highly desirable as inhibitors that target the N-terminal nucleotide-binding domain may cause unwanted biological effects. Herein, we report on the discovery of two drug-like novel Hsp90 CTD inhibitors by using virtual screening and intrinsic protein fluorescence quenching binding assays, paving the way for future development of new therapies that employ molecular chaperone inhibitors.  2009 Elsevier Ltd. All rights reserved. |
| Keywords:  Hsp90  Molecular chaperone  Virtual screening  Inhibitor  Cancer |

Heat shock protein 90 (Hsp90) is a molecular chaperone that is essential for maintaining homeostasis in the cells during stress as well as under normal conditions by promoting and regulating the folding, stabilisation and activity of its client proteins (for recent reviews, see references 1–5). Hsp90 is as a dimer requiring the consumption of adenosine triphosphate (ATP) and the binding of other co-chaperones to perform its function.6–9 The Hsp90 monomer can be divided into three domains, each playing different structural and functional roles.1–5 The N-terminal domain (NTD) consists an ATP-binding site that is responsible for the ATPase activity of the catalytic cycle, the middle domain (MD) mediates ATP hydrolysis and client proteins binding, and the C-terminal domain (CTD) contains the dimerisation interface, a nucleotide binding site, as well as binding site(s) for the binding of co-chaperone proteins.

To date, over 500 client proteins of Hsp90 have been discovered.10–13 These include oncoproteins such as kinases and transcription factors that are involved in cancer initiation or growth. As such, Hsp90 may promote tumorigenesis and metastasis.14–16 Not surprisingly, Hsp90 is a current inhibition target for the development of new treatments against cancers.17–20 Nineteen Hsp90 inhibitors have entered clinic trials although so far none has yet been approved by the United States Food and Drug Administration (FDA).21–24

The vast majority of Hsp90 inhibitors that have entered clinical trials target the NTD. However, Hsp90 NTD inhibitors may stimulate heat shock response (HSR),25 which is a cellular adaptive mechanism in response to the accumulation of misfolded proteins.26–28 HSR encourages the transcription of genes encoding for the expression of heat shock proteins, thus rendering the treatment ineffective and limiting the clinical potential of Hsp90 NTD inhibitors. In addition, the specificity of Hsp90 NTD inhibitors is also a concern for potential off-target effects.29

Due to the drawbacks of Hsp90 NTD inhibitors, there are potential advantages to design alternative Hsp90 inhibitors that do not target the NTD.30–37 With that in mind, a number of CTD inhibitors were identified and developed over the last decade.38 The coumarin antibiotic novobiocin and its derivatives are some of the best known examples inhibitors that bind to the nucleotide binding pocket of the Hsp90 CTD.39,40 Hsp90 CTD inhibitors have also been identified from natural products, which include the green tea catechin (−)-epigallocatechin-3-gallate,41,42 flavonolignans such as silibinin,43,44 and the flavonoid deguelin and its synthetic structural analogues such as L18.45,46 Peptide-based inhibitors have also been developed to disrupt the dimerisation between the CTD and its co-chaperones.47–50 These alternative approaches have shown to be fruitful. For example, RTA 901, entered phase 1 clinical trial in January 2017.51

Nonetheless, the development of Hsp90 CTD inhibitors is not trivial. This, in part, is due to the lack of complete structural information and the complex conformation dynamics of the CTD. For example, the nucleotide-binding site on CTD is attainable only when the N-terminal domain active site is bound.52 Multiple strategies, including computational methods such as molecular docking and molecular dynamics simulations, and experimental method such as photocrosslinking followed by mass spectrometry have been attempted to identify potential ligand binding pockets at Hsp90’s CTD.53–58 However, despite these efforts, the exact binding sites for nucleotides and inhibitors like novobiocin are not known. To date, no crystal structure of Hsp90 CTD in complex with its ligands or inhibitors has been reported.



**Figure 1.** Structures of the 24 virtual hits. Compounds that bind to Hsp90 CTD and disrupt the interactions between Hsp90 CTD and its co-chaperone experimentally are highlighted in blue.

We are interested in the discovery of novel hit matter that bind the CTD of Hsp90, with the aim to develop drug candidates. Herein, we describe our work in using a structural-based approach that combines virtual screening and binding assays, a workflow that we have successfully applied to discover Hsp90 NTD and MD binders,59,60 to identify novel natural product ligands of the Hsp90 CTD. Modelling studies were also conducted to gauge the potential of our Hsp90 CTD ligands for chemical modification to enhance affinity and specificity. Our work provides novel scaffolds for future development of Hsp90 CTD inhibitors.

Since there is no well-defined binding site(s) that can be targeted by small molecules reported in the literature, we first performed heuristic docking to determine potential small molecule binding pocket(s) at the Hsp90 CTD. A promiscuous drug-like compound was used for the blind docking against the crystal structure of Hsp90α CTD (PDB id: 3Q6M,61 resolution 3.0 Å). A thieno[2,3-*b*]pyridine-based compound was chosen (Supplementary Figure S1) because they are known to modulate the activities of many molecular targets, including (but is not limited to) G protein-coupled receptors,62 the platelet P2Y12 receptor,63 the DNA repair enzyme tyrosyl DNA phosphodiesterase 1,64 the colchicine binding site of tubulin,65 phospholipase C-δ1,66 kinases such as PIM-167 and eukaryotic elongation factor-2 kinase,68 and cyclooxygenases.69 The scoring functions GoldScore,70 ChemScore,71,72 Piecewise Linear Potential (ChemPLP)73 and Astex Statistical Potential (ASP)74 were used to evaluate the relative binding energies of the thienopyridine-containing compound to the different potential binding sites. Twelve potential binding sites were evaluated (Supplementary Table S1), which upon visual inspection had the attributes of a probable binding pocket, i.e. a lipophilic cavity containing hydrogen bond donors and/or acceptors. Consensus scoring between the four functions revealed a site within a 10 Å radius around the oxygen atom (from the backbone carbonyl group) from Leu-696 residue (x = -67.161, y = -81.781, z = 22.414) as the most tractable pocket to be targeted by small molecules.

The virtual screen was then performed against the identified binding site. 9049 molecular entities were downloaded from the natural product collection from InterBioScreen Ltd. Using the GOLD software,70 ten docking runs were carried out for each ligand with 30 % search efficiency. The search efficiency, when set at 100%, GOLD will attempt to apply optimal settings for each ligand. At 30%, fewer operations are performed thereby speeding up the docking; however, the search space is less well explored. From the consensus scoring, all ligands with low ChemScore (< 8.0), GoldScore (< 30.0), ChemPLP (< 30.0) and ASP (< 15.0) and those with limited number of hydrogen bonding (HB < 0.1) were eliminated in the initial screen (roughly top 20% scoring ligands were brought forward for each scoring function). 921 candidates remained and were screened again with 100 % default search efficiency and 50 genetic algorithm (GA) runs. The candidates with low scores ChemScore (< 16.0), GoldScore (< 40.0), ChemPLP (< 43.0), ASP (< 20.0) and number of HB (< 1.0) were filtered out. This resulted in 172 compounds. Visual inspection was then conducted for consensus of the best predicted configuration of the ligands between each scoring function. Finally, we eliminated compounds that do not show plausible configurations (e.g. structurally strained ligands and those that have lipophilic moieties pointing into the aqueous environment) and those that contain undesirable moieties that are linked to cell toxicity and chemical reactivity.75 As a result, 24 compounds were selected for further experimental testing (Figure 1 and Supplementary Table S2).

Hsp90 CTD regulates the chaperoning activity through dimerisation as well as the binding of co-chaperones. Hence, Hsp90 CTD inhibitors may target the CTD dimerisation interface or the binding site between the CTD and its co-chaperone proteins.32 The tetratricopeptide-containing repeats (TPR)-domain proteins belong to a class of Hsp90 co-chaperones that recognise and bind to the MEEVD motif at its C-terminus of the CTD.33 As the identified ligand binding site in this study is located towards the C-terminus of the CTD, we reasoned that our compounds may interfere with the interactions between the Hsp90 CTD and its co-chaperones. One of the TPR-domain co-chaperones of Hsp90 CTD is peptidylprolyl isomerase D (PPID, also known as cyclophilin 40).34 We therefore first tested the ability of our virtual hits to disrupt the interactions between Hsp90 CTD and PPID by using an Alpha (amplified luminescence proximity homogeneous assay)-based assay that employs biotin-labelled Hsp90 CTD (Hsp90α535-732) and glutathione S-transferase (GST)-tagged PPID.35,36 Initial screening was carried out at 500 μM. Excitation was applied at 680 nm and fluorescence was measured at 615 nm. Our results showed that, seven (out of the twenty-four) compounds (**4**, **8**, **10**, **15**, **16**, **18** and **21**) were able to reduce the ability of Hsp90 CTD to bind with PPID by >60% (Supplementary Figure S2). Control experiments were also carried out in absence of inhibitor as the positive control and absence of PPID as the negative control. Our results thus showed that these compounds may inhibit the binding between Hsp90 CTD and its TPR-domain co-chaperones although the mechanism is not clear.

To investigate the hits’ mode of inhibition, e.g. whether they disrupt the interactions between Hsp90 CTD and PPID through binding to the CTD, binding experiments with Hsp90 CTD were conducted. Experiments were carried out using recombinant human Hsp90 CTD (Hsp90α626-732) with an N-terminal GST fusion tag, without it the protein denatures. Intrinsic protein quenching fluorescence spectroscopy was applied.36 As Hsp90 CTD contains multiple tyrosine residues and GST contains both tryptophan and tyrosine residues, irradiation was done at 280 nm to excite both the tryptophan and tyrosine residues. Control binding experiments were also conducted with GST on its own. All twenty-four virtual hits were tested.

Initial binding experiments were carried out using 20 µM of the GST-tagged Hsp90 CTD protein and 1 mM of compounds. The results showed that six compounds (**10**, **13**, **14**, **15**, **22** and **23**) induced more than 30% fluorescence quenching (Supplementary Figure S3), indicating that they bind. Control experiments were conducted with these six compounds against GST. The results showed that all six induced fluorescence quenching of GST although the magnitude of the quenching were less than those of GST-tagged Hsp90 CTD (Supplementary Figure S3). Our results thus showed that the active hits are binders of Hsp90 CTD. However, they may also bind to GST (presumably non-specifically), which is not surprising given the promiscuous nature of this protein.76

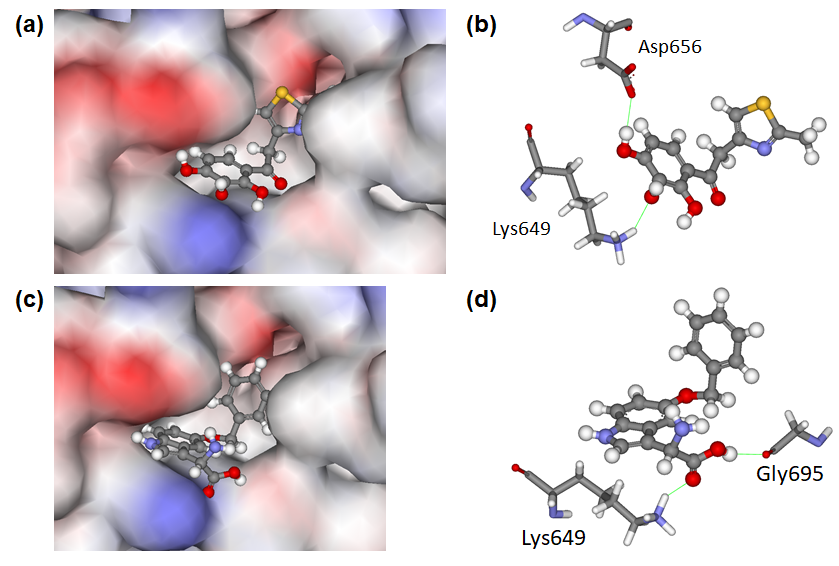
Combining the data from the two assays, it is clear that **10** and **15** bind to GST-tagged Hsp90 and disrupt the interactions between Hsp90 CTD and PPID. Both compounds **10** and **15** are derivatives of natural products. Compound **10** is a 2-methylthiazol-4-yl-acetyl substituted analogue of pyrogallol and compound **15** is 5-benzyloxy-substituted tryptophan. These two compounds were then subjected to further analyses. First, by using the Alpha assay, the inhibition potency (IC50) values were measured. **10** was found to be the more potent with an IC50 value of 35 ± 1 μM followed by **15** at 220 ± 9 μM (Table 1 and Supplementary Figures S4 and S5). Furthermore, titration experiments were conducted to obtain the binding affinity of **10** and **15** between GST-tagged Hsp90 and GST. Both compounds showed a lower dissociation constant (*K*D) values with GST-tagged Hsp90 CTD than with GST, indicating that they have a stronger affinity to Hsp90 CTD than to GST (Table 1 and Supplementary Figures S6 and S7). The differences in *K*D values between GST-tagged Hsp90 and GST for both **10** and **15** are roughly threefold, indicating that they have some selectivity for Hsp90 CTD over GST.

**Figure 2.** Predicted binding modes and interactions of **10** and **15** at the Hsp90 CTD binding site using the ChemPLP scoring function (hot spot at residue Leu-696). (a) The docked configuration of **10**. Red depicts a negative partial charge on the surface, blue depicts positive partial charge and grey shows neutral/lipophilic regions; (b) Hydrogen bond interactions (depicted as green lines) between **10** with Lys-649 and Asp-656. (c) The docked configuration of **15**; (d) Hydrogen bond interactions are predicted with Lys-649 and Gly-695 for **15**.

**Table 1.** Table summarising the *K*D values of **10** and **15** against GST-tagged Hsp90 CTD and GST, and Hsp90 CTD and PPID.

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| --- | --- | --- | --- |
| Compound# | *K*D (GST-tagged Hsp90 CTD) / μM | *K*D (to GST) / μM | IC50 (Hsp90 CTD-PPID binding interactions) / μM |
| **10** | 102 ± 1 | 310 ± 3 | 35 ± 1 |
| **15** | 28 ± 3 | 83 ± 5 | 220 ± 9 |

Molecular modelling studies indicate that both **10** and **15** have plausible binding modes and reasonable scores to Hsp90 CTD at the identified binding site (see Supplementary Table S2), which is distinct from the proposed nucleotide binding site at the CTD.53–58 Our docking results predict that **10** and **15** bind in a pocket that is formed by the dimerisation of the C-terminal domain (i.e. across two monomers) (Figure 2). In particular, **15** can form a hydrogen bond with the sidechain of Lys649 from one monomer, and the backbone carbonyl of Gly695 from another monomer. The pyrogallol moiety of **10** and the indole of **15** sit on a hydrophobic plane created by a number of lipophilic amino acid residues at the dimerisation interface. Furthermore, the methylthiazole moiety (**10**) and phenyl ring (**15**) sit in a lipophilic pocket formed by the dimerisation of the C-terminal domain, which is formed by two α-helices (His640-Asp653 and Asp656-Ser674) from one monomer and one α-helix (Asp680-Leu696) from the other monomer. This pocket creates favourable binding conditions as affinity is often associated with hydrophobicity, i.e. small lipophilic molecules are pushed into the binding pocket resulting in entropy gain, whereas hydrogen bonding gives specificity.77

In addition to molecular docking, the physicochemical properties of compounds **10** and **15** were derived in order to gauge their compatibility with biological systems. This is important because most Hsp90 CTD inhibitors to date are peptides and or structurally complex small molecules.38 Six molecular descriptors; molecular weight (MW), lipophilicity (log P), number of hydrogen donor (HD), number of hydrogen acceptor (HA), polar surface area (PSA) and number of rotatable bonds (RB) were calculated for the ligands (Table 2). Both **10** and **15** occupy lead-like and drug-like space (see Supplementary Table S3 for definitions).78–80 We also evaluated our hits against the Known Drug Indexes 2a and 2b (KDI2a/2b),81 which describe the overall balance of the six molecular descriptors. Compound **10** has a KDI2a value of 5.6 and compound **15** has a KDI2a value of 5.17. The theoretical maximum of is KDI2a 6 and the average is 4.08 for known drugs. The KDI2b values of compounds **10** and **15** are 0.65 and 0.39, respectively, with a theoretical maximum of 1.00, and an average of 0.18 for drugs in clinical use. Also, the one-electron oxidation (ionisation potentials, IP) and reduction (electron affinity, EA) potentials were calculated using the Density Functional Theory (DFT) as described in Matuszek and Reynisson (Supplementary Table S4).82 The IPs for **10** and **15** are 7.6 and 6.7 eV respectively, which is in the 6.0–9.0 eV range as known drugs. As for the EAs they are -0.1eV (**10**) and 0.4 eV (**15**), both are well within the limits of known drug space (-1.5–2.0 eV). It can therefore be concluded that **10** and **15** are redox stable in biological systems. Together with the high KDI2a/2b values, our results indicate that these compounds possess favourable drug-like properties, and hence they can be considered excellent starting points for chemical transformation and further development.

**Table 2.** Molecular descriptors of compounds **10** and **15**.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Cmp# | MW | HD | HA | Log P | PSA | RB | KDI2a/2b |
| **10** | 265.3 | 2 | 4.75 | 1.4 | 95.7 | 6 | 5.60/ 0.65 |
| **15** | 310.4 | 4 | 3.75 | 0.8 | 91.9 | 7 | 5.17/ 0.39 |

Overall, by using virtual screening and intrinsic protein fluorescence quenching binding studies, we have successfully identified two analogues of natural products (compounds **10** and **15**) that bind human Hsp90 CTD and disrupt the binding interactions between Hsp90 CTD and PPID, a co-chaperone of Hsp90. The IC50 values and apparent *K*D values to Hsp90 CTD are in the μM region, indicating that they are reasonable inhibitors/binders to the protein. For example, they are comparable or better than the IC50 values for novobiocin and silibinin to disrupt Hsp90 CTD-PPID binding, which were ~500 μM and 1 mM respectively.44 Molecular modelling was used to predict the most probable binding modes of the hits to Hsp90 CTD suggesting that our compounds can bind to hydrophobic pockets that are created through the dimerisation of the C-terminal domains. Cheminformatics studies showed that both compounds are in lead-like and drug-like spaces, favourable for cell permeability and biocompatibility in general. Our work therefore adds to a number of recent studies that use computer modelling to identify, design and develop small molecule-based CTD inhibitors.44,83–87

The development of Hsp90 CTD inhibitors is not straightforward. There are two ways that an inhibitor could target the Hsp90 CTD. First is to disrupt the dimerisation of the CTDs, and second to target the interactions between the Hsp90 CTD and its co-chaperones.32 It is worth noting that disruption of Hsp90 dimerisation may also affect co-chaperone binding. Numerous Hsp90 CTD inhibitors have been reported to disrupt the dimerisation. This can be achieved either through binding at the C-terminal nucleotide binding pocket (e.g. novobiocin and coumarin derivatives)39,40 or by directly interfering the dimerisation interface (e.g. through the use of peptides such as aminoxyrone (AX)).49 Examples that cause disruption between Hsp90 CTD and its TPR-domain co-chaperones through allosteric modulation have also been reported.88 However, to date, there are no examples of small molecule inhibitors that directly target the Hsp90 CTD-TPR-domain co-chaperone interface although there are peptide-based inhibitors that target the MEEVD motif to stop co-chaperone binding to Hsp90 CTD such as those that were reported by Rahimi and co-workers.47,48 As our docking results showed that our compounds likely bind to the Hsp90 CTD dimer, our work may represent the first example of using small molecules to directly disrupt Hsp90 CTD-TRP-domain co-chaperone interactions. The goal of our studies was to discover novel chemical matter that bind to Hsp90 CTD that can be used for further developments. As the two hit compounds are small and drug-like, we therefore believe that they are excellent starting points for further development of Hsp90 CTD inhibitors that interfere with co-chaperone binding interactions.

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Supplementary Material

Supplementary material that may be helpful in the review process should be prepared and provided as a separate electronic file. That file can then be transformed into PDF format and submitted along with the manuscript and graphic files to the appropriate editorial office.

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