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An Hsp90 modulator that exhibits a unique mechanistic profile

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Abstract

Described is the synthesis of two biotinylated derivatives of a cytotoxic macrocycle. Pull-down assays indicate that this macrocycle targets the N-middle domain of Hsp90. Untagged compound can effectively compete away tagged compound-Hsp90 protein complexes, confirming the binding specificity of the macrocycle for Hsp90. The macrocycle is similar in potency to other structurally-related analogs of Sansalvamide A (San A) and induces apoptosis via a caspase 3 mechanism. Unlike other San A derivatives, we show that the macrocycle does not inhibit binding between C-terminal client proteins and co-chaperones and Hsp90, suggesting that it has a unique mechanism of action.

Keywords

Hsp90; Sansalvamide A; Pull-down; Caspase 3; Heat shock proteins

Recently, we reported the mechanism of action of the cyclic pentapeptide Sansalvamide A-amide and two analogs (Figure 1).^{1–7} The peptide structure is based on the natural product depsipeptide Sansalvamide A (San A) that was isolated by the Fenical group from a marine fungus of the genus *Fusarium sp* and found to exhibit anticancer activity.⁸ Our reports showed that these 3 molecules bind to the N-middle domain of heat shock protein 90 (Hsp90) and modulate its function. Hsp90 is a ubiquitous molecular chaperone that regulates over 200 client proteins involved in multiple growth and signaling pathways.^{3, 4, 6, 7} The roles of Hsp90 in these pathways include: regulating the conformational folding of numerous signal transduction molecules, and refolding denatured proteins under stress conditions.^{9, 10} Because many hormone receptors, kinases and signaling molecules involved in these pathways are targets for chemotherapeutic strategies, Hsp90's role as a master regulator has made it an attractive candidate for drug development.^{11, 12}

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Supplementary Material

Supporting information includes spectral data and experimental details for compounds and biological assay conditions.

Hsp90 exists as a homodimer in the cytosol, with three highly conserved domains.¹³ The 25-kDa N-terminal domain contains an ATP-binding site and is the target of the natural product geldanamycin and its derivative 17-AAG [17-(allylamino)-17-demethoxygeldanamycin]^{14, 15} as well as other drugs under clinical development that modulate Hsp90.¹⁶ The 35-kDa middle domain binds to multiple client proteins and co-chaperones, including human growth factor-2 (HER2) and the anti-apoptotic factor Akt.^{17, 18} Three Sansalvamide A analogs (Figure 1, compounds 1–3) have been shown to bind to the N- middle region, in a pocket between the two domains.^{4–6} Using their tetratricopeptide repeat (TPR) motif¹⁹ several cofactors interact with the MEEVD region of the 12-kDa C-terminal domain of Hsp90. These C-terminal binding proteins are unaffected by all drug candidates that are currently in clinical trials that exploit this pathway.²⁰ And while the C-terminal binder novobiocin was reported to inhibit the binding between Hsp90 and four TPR-containing co-chaperones with an IC₅₀ in the millimolar range,²¹ all three of the previously reported San A-amide compounds (compounds 1–3, Figure 1) were recently shown to allosterically interfere with the binding interactions of four co-chaperones that interact with Hsp90 via their TPR domains with IC₅₀s in the low micromolar range.^{4–7}

During our investigation of San A-amide and its mechanism of action, we came across a San A-amide derivative, compound 4, which exhibits the same GI₅₀ values as those that target Hsp90, but displays a mechanism that is unique from the other San A-amide analogs.^{3, 4, 6, 7} Specifically, compound 4 (Figure 1) has a GI₅₀ of 2.9 μ M in HCT-116 cells (colon cancer) but does not inhibit the interaction between Hsp90 and the client/co-chaperone proteins that bind via their TPR domains to the C-terminus of Hsp90.^{2, 3, 6} Because compound 4 does not interfere with Hsp90-TPR protein complexes, this raised a question as to whether compound 4 binds directly to Hsp90 or exerts its cytopathic effect by another mechanism. To better understand how the conformation of the macrocycle contributed to cell death, we investigated the cytotoxicity of compound 4 analogs.³ These analogs included a) replacing the chlorobenzoyloxycarbonyl (2-Cl-Z) group on the lysine residue (IV) with a carbobenzyloxy (Cbz) group, b) replacing the D-Lys(2-Cl-Z) with L-Lys(2-Cl-Z) (IV), c) moving the N-methyl from the D-Phe residue (V) to the D-Lys(2-Cl-Z) (IV). All modifications resulted in over 100-fold decrease in GI₅₀ values. Interestingly removing the N-methyl from the D-phe residue (V) and adding it to the valine residue (III) generated an active compound with a GI₅₀ value of ~8 μ M (2.6 fold greater than compound 4). Thus, compound 4 has a very distinct conformation and 3-D structure that induces a cytotoxic effect, yet its binding specificity for Hsp90 was unknown.

Herein we report the synthesis of two biotinylated analogs of compound 4 (Figure 2), and the results from the pull-down assay performed using these derivatives. Similar to other derivatives of San A-amide, compound 4 binds to Hsp90 and forms stable interactions within the N-middle domain. As previously published, compound 4 does not interfere with binding interactions between client proteins and the C-terminal domain of Hsp90.⁶ Further, we show that: a) compound 4 binds to the same site as compounds 1, 2, and 3 and competitively inhibits the binding of compound 3 to Hsp90's N-middle domain, b) it displays a distinct phenotype, which indicates it goes through an Hsp90-mediated mechanism, and c) it induces apoptosis via cleavage of poly (ADP-ribose) polymerase (PARP), which suggests a caspase 3-mediated pathway. These results indicate that the mechanism of action of compound 4 is unique from other San A-amide derivatives or 17-AAG, in that its interaction with Hsp90 leads to a pro-apoptotic effect but not through the inhibition of the same co-chaperone or client protein binding interactions that are observed with other Hsp90 inhibitors.

In an effort to explore compound 4's unique mechanism of action we synthesized two biotinylated derivatives (Figure 2): **4**-Tagged at position II (**4**-T-II) and **4**-tagged at position

III (4-TIII). We placed the tags at positions II and III as position I, IV, and V all had phenyl moieties, which are important for activity.^{14, 15} Specifically, through structure activity relationships (SAR) we had found that the phenyl at position I was critical,² the Chloro-Cbz-protected *D*-lysine at position IV was important for biological activity,¹ and the presence of an *N*-methyl-*D*-phenylalanine at V was an important motif.^{1, 15} Thus we chose not to tag these positions. These compounds were synthesized using a solid-phase protocol. (Scheme S1 and S2)^{1, 3} Both 4-T-II and 4-T-III were synthesized using a commercially available chlorotrityl resin pre-loaded with L-phenylalanine or L-leucine, respectively. Subsequent coupling of Fmoc-protected amino acids and amine deprotection were performed until the desired linear pentapeptide was reached. A Boc-protected lysine was incorporated at the tagged position. After cleaving the peptide from the resin with 50% trifluoroethanol in dichloromethane, the linear pentapeptide was cyclized using macrocyclization conditions. The macrocycle was then subjected to 20% TFA to remove the Boc from the lysine, and biotinylated using NHS-peg-biotin and 8 equivalents of DIPEA.

The biotinylated compounds were incubated with HCT116 cell lysate whereupon neutravidin bound agarose beads were added to immobilize the compound along with the bound target protein(s) (Figure S18). The beads were washed ten times to remove non-specifically bound proteins, followed by elution of proteins using sample buffer. The eluted proteins were run on an SDS-PAGE gel and visualized with Coomassie blue staining (Figure S18). Protein targets were isolated from the binding assay containing compound 4-T-III, and five major proteins were visible. The most prominent protein bound to compound 4-T-III was visualized as a band at approximately 90–95 kDa. Four other major proteins appeared and protein sequencing of these five bands was performed using a nano-LC/MS/MS followed by peptide identification using the NCBI Eukaryotic database and fingerprinting software. The bands were identified as: myosin-9, Hsp90, beta tubulin, actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). No protein targets were isolated using compound 4-T-II (Figure 3).

It is well documented that myosin, β -tubulin, and actin are commonly pulled down during these assays due to their hydrophobicity.¹⁶ Comparison of the band isolated from 4-T-III at 90–95 kDa to that region in the gel for 4-Tag-II, the negative control (PEGylated biotin linker alone) or DMSO (Figure 3) indicates that the interaction between Hsp90 and compound 4-T-III is specific to the molecule. Given that Hsp90 is the only protein pulled down that is associated with an oncogenic pathway, we verified the targets by Western blot analysis (Figure 3). In support of the LC/MS/MS data, western blot analysis shows that compound 4-T-III pulls down Hsp90, whereas compound 4-T-II and control reactions do not.

Activation of *hsp90* gene expression can occur through a variety of stress signals, including temperature, heavy metals, cytokines and mitogenic agents.¹⁷ Once expressed, Hsp90 localizes mainly to the cytoplasm due to a C-terminal cytosolic localization signal.¹⁸ To determine if treatment with compound 4 alters localization of Hsp90 in colon cancer cells, immunofluorescence staining was performed using a polyclonal antibody to Hsp90. Small, punctate areas of Hsp90 staining intensify in brightness when the cells are treated with the Hsp90 inhibitor 17-AAG, yet staining remains confined to the cytosol (Figure 4). Treatment with compounds 3 and 4 increased the staining density of Hsp90 in the cytosol, and there was no significant difference in the brightness intensity between cells treated with either compound (Figure 4). From these data, we see that compound 4 affects Hsp90 localization in a similar manner to compound 3, and the change in staining intensity over the DMSO-treated control is likely due to an increase in treatment-induced cellular stress.

The three domains of Hsp90 form a unique scaffold that dynamically responds to client/co-chaperone protein binding and ATP hydrolysis. The N-terminal domain of Hsp90 is the frequent target of drug development because of its ATP-binding pocket¹³ The middle domain contributes significantly to the maintenance and stability of Hsp90's conformation. Recent studies suggest that the middle domain modulates the position of the γ -phosphate group of ATP prior to hydrolysis and directly affects the reaction rate.¹⁹ In addition, the middle domain may also play a key role in client protein recognition and binding.²⁰ Previous data showed that derivatives of San A-amide bound to the N-middle domains of Hsp90.⁴ In order to evaluate where compound 4 bound to Hsp90, we used compound 4-T-III in a pull-down assay with purified domains of Hsp90 (Figure 5a). Similar to other derivatives of San A-amide, compound 4-T-III bound preferentially to the N-middle domain of Hsp90. Significantly, compound 4 competes for the same binding site as compound 3, our most potent analog to date (Figure 5b). That is, as you add increasing concentrations of compound 4 to Hsp90 bound to 4-T-III, you are able to displace 50% of the bound 4-T-III molecules using 6.2 μ M of compound 4. Further, consistent with its binding affinity (3.6 μ M) is the fact that compound 3, outcompetes 4-T-III with a significantly lower IC₅₀ than compound 4. Thus, both compounds bind to the same binding pocket of the N-M domain of Hsp90.

Given that compounds 1, 2, and 3 all induce apoptosis⁴ and that compounds 3 and 4 bind to the same site, it was likely that compound 4 induces apoptosis. A well-known and reliable method for detecting apoptosis is the evidence of cleaved poly(ADP-ribose) polymerase (PARP) fragments. PARP is an enzyme activated in the presence of DNA strand breaks, and it catalyzes the synthesis of poly(ADP-ribose) groups, which it covalently attaches to itself and to several nuclear proteins.²² Caspase 3 is largely responsible for the cleavage of PARP during programmed cell death. Examination of whether a molecule triggers caspase-dependent apoptosis can be measured by evaluating the levels of full-length PARP and its corresponding apoptotic enzyme-cleaved fragments. An increase in the degradation of full length PARP upon the addition of increasing amounts of compound verifies that the compound is inducing caspase-dependent apoptosis.²³ Cell lysates from HCT-116 treated for 24 hrs with increasing concentrations of compound 4 (0–50 μ M) were analyzed by Western blot. We observed that full length PARP (~113 kDa) decreased in a dose-dependent fashion upon the addition of increasing concentrations of compound 4. For cells treated with 50 μ M of compound 4, there was a 48% decrease in full-length PARP compared to non-treated cells, (Figure S22). These data indicate that compound 4 causes apoptosis in a caspase dependent manner.

In addition to the above reported data, we investigated the ability of our compounds to inhibit clients and co-chaperones from binding to Hsp90. Previously, we had found that unlike compounds 1,² 2,³ and 3,⁴ compound 4 did not inhibit the interaction between four client proteins and co-chaperones that bound to the C-terminus of Hsp90, including: inositol hexakisphosphate kinase-2 (IP6K2), FKBP38, FKBP52, and Hsp organizing protein (HOP).⁶ Two other proteins of interest, which bind to the middle-domain of Hsp90 are HER2 and Akt1. The protein kinase B (Akt) and phosphatidylinositol-3-kinase (PI3K) pathways are activated by growth factors such as HER2, which leads to anti-apoptosis or cellular proliferation of cancer cells.²⁴ Hsp90 inhibitor 17-AAG prevents the association of Akt1 with Hsp90, leading to ubiquitination and degradation of Akt1 in the cell.²⁵ To determine if compound 4's cytotoxic effect is due to the binding inhibition of these client proteins to Hsp90, we compared the binding effects of compound 4 to 17-AAG or to San A-amide (17-AAG reduced the binding of Hsp90 to Akt by 80% and to HER2 by 47% (Figure S23). Similar to our earlier results with proteins that bound to the C-terminus of Hsp90, compound 4 had little to no effect on the binding of either client protein to **Hsp90** (Figure

S23). These data indicate that the cytotoxic effect of compound 4 is not due to the disruption of HER2- or Akt-binding interactions to Hsp90.

In summary, compound 4 is a cytotoxic macrocycle that induces apoptosis in colon cancer cells. Through binding to the N-middle domain of Hsp90, compound 4 forms stable interactions and successfully competes for binding with one of the more potent Sansalvamide A analogs (compound 3), thus indicating that it has the same binding site as compound 3. Similar to cells treated with compound 3, cytosolic Hsp90 accumulates in cells treated with compound 4 and is indicative of cellular stress. This accumulation of Hsp90 in the cytosol, seen in cells treated with all three compounds (Figure 4), is indicative of the cells inability to break down misfolded proteins, and suggests that both compound 3 and 4 inhibit the proteasome degradation pathway. This accumulation of Hsp90 and potentially other misfolded proteins due to treatment with compound 4 eventually results in PARP cleavage and apoptosis at 72 hours post-treatment.

Unlike other published analogs of Sansalvamide A, or other Hsp90 inhibitors, compound 4 does not disrupt interactions between Hsp90 and several client proteins and co-chaperones that play key roles in apoptosis. Yet compound 4's potency and ability to compete for the same binding site on Hsp90 with compounds that do inhibit key client proteins and co-chaperones from binding to Hsp90, is intriguing. Further, the specific structural requirements to obtain potency and binding to Hsp90 clearly indicate that compound 4 acts via a unique mechanism. One hypothesis is that compound 4 may disrupt Hsp90's protein folding ability, leaving client proteins bound to the scaffold but inhibiting the correct folding, activation and release of these client proteins into the cytosol. If compound 4 locks Hsp90 into a conformation that prevents folding and release of client proteins, these misfolded proteins would accumulate over time in the cell and eventually trigger cell death. This hypothesis is supported by our binding data that shows compound 4 did not disrupt Hsp90 binding interactions between HER2, Akt1 or other client proteins (Figure S23 and Ardi, et al), yet compound 4 induces apoptosis by PARP cleavage (Figure S22). Conformational changes in the domains of Hsp90 are thought to contribute to co-chaperone and client protein binding and release, thereby promoting cell growth (Wandinger SK; Richter K; Buchner J. (2008) The Hsp90 chaperone machinery. *J Biol Chem*, **283**, 18473-7). Compound 4 represents a unique tool that binds specifically to Hsp90's N-middle domain and allows cochaperone and client binding interactions with Hsp90, yet it inhibits cell growth by potentially altering Hsp90's conformational dynamics and preventing release of client proteins from the Hsp90 scaffold.. Further studies are currently underway to gain a deeper understanding of these interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. San A-amide (compound 1)⁴ Hsp90 inhibitors (compounds 2⁵ and 3⁶, and compound of interest (compound 4).

Figure 2.
Biotin-tagged derivatives of Compound 4.

Figure 3.
Western blot of proteins isolated in pull down assay.

Figure 4.

Visualization of Hsp90 in HCT 116 cells treated with DMSO (72 hours; 1%), 17-AAG (24 hours; 200 nM), compound 3 (72 hours; 5 μ M), or compound 4 (72 hours; 5 μ M).

Figure 5.

a) Compound 4-T-III selectively pulls down the N-middle domain of Hsp90. [11.5 fold above background] b) Compound 4 and 3 compete off the tagged analog 4-T-III (IC_{50} 6.2 μ M and IC_{50} 1.9 μ M respectively).