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Synthesis and Evaluation of Biotinylated Sansalvamide A Analogs and their Modulation of Hsp90

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Abstract

Described are the syntheses of 3 Sansalvamide A derivatives that contain biotinylated tags at individual positions around the macrocycle. The tagged derivatives indicated in protein pull-down assays that they bind to Hsp90 at the same binding site (N-middle domain) as the San A-amide peptide. Further, these compounds inhibit binding between Hsp90 and multiple C-terminal client proteins. This interaction is unique to the San A analogs indicating they can be tuned for selectivity against Hsp90 client/co-chaperone proteins.

Keywords

Sansalvamide A; Hsp90; N-middle domain

Recent work describing the synthesis and biological activity of pentapeptide derivatives, based on the natural product sansalvamide A (San A), has brought attention to this compound class.^{1,2,3,4,5} San A is a pentadepsipeptide that was discovered by Fenical and coworkers from a marine fungus of the genus *Fusarium* and exhibits anti-tumor activity.¹ The pentapeptide structure (San A-amide, figure 1, compound 1) has recently been reported to bind to and inhibit Heat shock protein 90 (Hsp90).^{4, 5} Hsp90 is a well-established oncogenic target that modulates client proteins involved in cellular growth, angiogenesis, and apoptosis.⁶ The redundancy of pathways involved in cancer growth mean that targeting multiple mechanisms simultaneously is key to developing a successful therapy. Recent evidence shows that Hsp90 controls ~100 client proteins and co-chaperones, many of which are involved in multiple cancer-related cell signaling proteins, this makes it an excellent oncogenic target.⁷ Further, Hsp90 is up-regulated in most cancers, and cancer cells are more susceptible to Hsp90 inhibitors than normal cells because Hsp90 plays a vital role in maintaining the functionality of these pathways during cancer cell growth.⁶ There are currently 15 Hsp90 inhibitors in development, with 2 of these in phase III clinical trials.⁸ Hsp90 interacts with client proteins at one or more of its three domains: N-, Middle, or C

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(N, M, C (respectively). All compounds currently in clinical development bind to the ATP binding pocket in the N-domain, and most are structurally related to a single compound, Geldanamycin, including 17-AAG, which is currently in Phase II and III clinical trials. Of the 3 non-Geldanamycin analogs in clinical trials, none modulate C-terminal client proteins.⁸ We have reported that San A-amide (1, Figure 1) is a cytotoxic molecule that modulates the activity of multiple client proteins and co-chaperones, acting via an allosteric effect.⁵ Indeed, we have published data showing that San A-amide (1) allosterically modulates C-terminal client proteins FKBP52 and IP6K2, unlike other Hsp90 inhibitors. We have also recently published the synthesis of analog 2 (Figure 1), where 2 exhibits greater cytotoxicity than compound 1 against HCT-116 colon cancer cells.⁴

In order to evaluate compound 2's mechanism of action, we report here the synthesis of biotinylated analogs of compound 2. In addition we describe Hsp90 pull-down assay results using these biotinylated analogs and binding assay data using compound 2. We show that compound 2 has an enhanced ability to inhibit binding between Hsp90 and four of its client/co-chaperone proteins (Her-2, HOP, FKBP52, and IP6K2) over compound 1 and 17-AAG. These proof of principle experiments show that structural variations of San A allow us to selectively "tune" client/co-chaperone contacts with Hsp90, thereby controlling unique subsets of Hsp90- protein interactions and subsequent pathways with each structural variant.

Our previous SAR data indicated that placing a tag at positions I–IV are all reasonable possibilities and it was not clear if any single position would be an ideal choice (although position V had been ruled out).^{4, 9} Data from compound 1-tagged at position IV (1-T-IV) pulled down Hsp90 N-middle domain, and we were interested in evaluating how the placement of the tag might alter compound 2's ability to bind to its protein target. Thus, we designed and synthesized compounds 2-T-I, 2-T-III, and 2-T-IV (Figure 2).¹⁰

These compounds were synthesized using the same solid-phase, protocol previously published (Scheme 1).^{4, 11} 2-T-I and 2-T-III were synthesized using a pre-loaded 2-chlorotrityl-leucine resin while 2-T-IV utilized preloaded 2-chlorotrityl-phenylalanine resin. Subsequent coupling of Fmoc-protected amino acids and deprotection was 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 our standard macrocyclization conditions.⁹ 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.

Compound 2 tagged at all 3 positions was then run in protein pull-down assays using purified N, Middle, C, N-Middle, and Middle-C domains of mammalian Hsp90 (Figure 3). Although all three tagged compound 2 molecules pulled down the anticipated N-Middle domain, it is noted that compounds 2-T-I and 2-T-III are most effective at pulling down this domain. These data indicate that the tag placement is important and affects the ability of the molecule to bind to its target. Further, it suggests that the preferred binding mode of compound 2 to Hsp90 does not involve residues I or III and most likely involves residues IV and V.

Given our previous data on compound 1, we anticipated that the cytotoxic effect of San A might be a direct result of its ability to inhibit the interaction between Hsp90 and client proteins that are integral to cell signaling events. Thus, we probed the effect of 1 and 2 on the binding interaction between Hsp90 and four client/co-chaperone proteins: Her2, HOP, FKBP52, and Inositol hexakisphosphate kinase-2 (IP6K2) (Figure 4).^{5, 12} Her2 is a well-established client protein that binds to the middle domain, and is inhibited from binding to Hsp90 by 17-AAG.¹³ Binding between Her2 and Hsp90 was inhibited by both 2 and 17-

AAG (Figure 4). HOP (Hsp organizing protein) binds to Hsp90's C-domain and facilitates the transfer of unfolded protein from Hsp70 to Hsp90. (Figure 5). Both 1 and 2 block this interaction, however, 17-AAG does not. Since HOP is vital to the Hsp70-Hsp90 interaction, compounds 1 and 2 will be excellent tools for investigating the mechanism of this complex. FKBP52 is a co-chaperone that binds to the C-domain of Hsp90 and assists with protein folding and trafficking.¹⁴ Both compound 1 and 2 inhibit the binding between Hsp90 and FKBP52. As expected, 17-AAG does not inhibit this binding event. Finally, IP6K2, which binds to the C-terminus of Hsp90, and is known to be involved in an apoptotic pathway, is also modulated by 1 and 2, while 17-AAG does not affect this binding event. Thus, these data show that 17-AAG gave traditional N-terminal inhibition effects, blocking Her2 interactions with Hsp90, but not inhibiting Hsp90's interaction with 3 C-terminal binders: HOP, FKBP52, and IP6K2. Compounds 1 and 2 inhibited binding between Hsp90 and all 3 C-terminal binding proteins. Further, compound 2 inhibited binding between Hsp90 and Her-2, suggesting that the compounds can be "tuned" via modification to San A side chains to allosterically modulate interactions between Hsp90 and its proteins. These compounds can be used as tools to investigate the cell signaling pathways by effectively inhibiting C-terminal binders to Hsp90.

Our proposed model for how analogs 1 and 2 play a role in the binding of C-terminal client proteins to Hsp90 is outlined in Figure 5. Based on the pull-down results, our molecules bind between the N-middle domain and inhibit the binding of C-terminal client protein IP6K2 and co-chaperones FKBP52 and HOP. San A analogs do this via induction of conformational change when binding to Hsp90 that is translated from the N-middle domain to the C-terminal domain of Hsp90. This induced conformational change makes the C-terminus of Hsp90 inaccessible FKBP52, HOP, and IP6K2. Interestingly, inhibiting the binding between HOP and Hsp90 is likely to inhibit the ability of Hsp70 to dock to Hsp90 via HOP and transfer unfolded proteins. Thus, it appears that San A derivatives may control the ability of protein transfer between these two oncogenic targets: Hsp70 and Hsp90.

In summary, we have described the synthesis of three new biotinylated Sansalvamide A analogs. These tags were placed at multiple positions around the macrocycle in order to determine which position would be optimal for binding to the protein target: Hsp90. Although all 3 tagged compound 2 molecules pulled down the expected N-middle domain, it was discovered that a tag at positions I or III were optimal for compound 2 to bind to Hsp90. Further, we ran client-binding assays with compounds 1, 2, and 17-AAG and found that both San A amide compounds modulate, via an allosteric interaction, binding between all 3 C-terminal binding client proteins and Hsp90. Further, the ability to block HOP from binding to Hsp90 would likely affect the key interaction between Hsp70 and Hsp90. Data on additional mechanistic aspects of these compounds is ongoing and will be published in the near future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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10. Compound 1-T-I was not made because it was known that the phenylalanine residue at position I in San A-amide was important for binding.
11. It should be noted that 2-T-II was not synthesized as we have shown the D-N-methyl-phenylalanine is critical for biological activity.
12. See supplemental data pS32 for experimental binding methods.
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Figure 1.
Sansalvamide A molecules 1 and 2⁴

Figure 2.
Tagged Compound 2 with tags at positions I–IV

Figure 3. Pull-down data for compounds 2-T-I, 2-T-III, and 2-T-IV using mammalian Hsp90 domains: N, Middle, C, N-middle, and Middle-C domains respectively.

Figure 4.
Compound 2 and client protein binding data

Figure 5.
Model of how San A analogs interrupt binding between Hsp90 and key proteins

Scheme 1.
 General solid-phase synthesis of tagged derivatives