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A small molecule that preferentially binds the closed conformation of Hsp90

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ABSTRACT

Described is the synthesis of three different fluorescein-tagged derivatives of a macrocycle, and their binding affinity to heat shock protein 90 (Hsp90). Using fluorescence polarization anisotropy, we report the binding affinity of these fluorescein-labeled compounds to Hsp90 in its open state and ATP-dependent closed state. We show that the compounds demonstrate a conformation-dependent preference for binding to the closed state.

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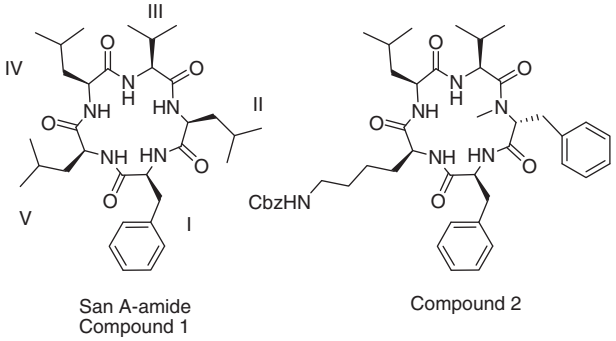
Molecules that bind to and inhibit the function of heat shock protein 90 (Hsp90) have proven to be excellent clinical candidates in oncogenic therapies.[1–6] Hsp90 is an ATP-dependent chaperone protein that helps fold and regulate more than 200 substrate 'client' proteins that are involved in cell growth and signaling.[4,7] Since pathway-specific inhibitors can be problematic in drug-resistant cancers, shutting down multiple pathways at once is a promising approach when developing new therapeutics. Given Hsp90's ability to modulate many growth and cell signaling pathways simultaneously,[8] this protein has become an attractive target in the field of cancer therapeutics.

Recently, we reported the mechanism of action of the cyclic pentapeptide Sansalvamide A-amide (Fig. 1, compound **1**).[9–11] 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.[12] We recently reported that San A-amide binds to and modulates Hsp90[9,11] as does compound **2**.[10] Compound **2** (Fig. 1) exhibited lower IC_{50} values than compound **1** and other San A-amide structures, and has been shown to modulate the binding between numerous client proteins and Hsp90.[10] Thus, it was chosen as a tool for further investigation.

Hsp90 is a constitutive dimer that contains three domains per monomer: the N-terminal domain, which houses an ATP-binding site, the middle domain, and the C-terminal domain (known as N, M, and C, respectively).[13] It functions in conjunction with several co-chaperones, progressing through a complex ATP-dependent conformational cycle (Fig. 2). Without ATP, Hsp90 maintains an open dimerized conformation (Fig. 2a). Upon ATP binding (**2b**), it converts to a stabilized closed and twisted conformation (**2c**), which is favorable for the subsequent hydrolysis of ATP. Upon hydrolysis of ATP Hsp90 forms a more compact ADP-bound state (**2d**). After ADP is released, Hsp90 reverts back to its open state (**2a**).[14–16]

Prior to our work, two types of Hsp90 inhibitors had been reported: those that bind to the ATP-binding pocket in the N-domain, thus inhibiting Hsp90's function,[17] and those that bind to the C-domain.[18,19] All of the compounds currently in clinical trials bind to the N-domain and most are closely related to the natural product



San A-amide
Compound 1

Compound 2

Figure 1. Sansalvamide A-amide and derivative.

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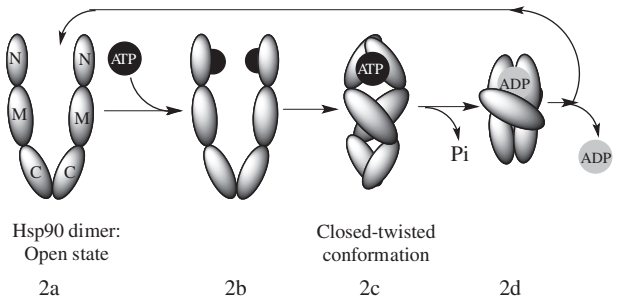


Figure 2. Hsp90 open–close mechanism.

Geldanamycin (K_d ~1 μM).[2] Compounds that bind to the C-domain include the natural product antibiotic novobiocin and its derivatives. The advancement of novobiocin as a chemotherapeutic agent has been hampered by its high micromolar cytotoxic activity (~700 μM) and poor binding affinity for Hsp90 (calculated K_ds in the millimolar range).[20,21] Recent studies have reported novobiocin derivatives with low micromolar affinities for Hsp90,[17] but to date no molecules that bind to the C-terminal site of Hsp90 have been tested in clinical trials.[21] Unlike the current inhibitors, we have established that San A-amide and compound **2** bind to the N–M domain and allosterically modulate the C-domain client protein IP6K2, and co-chaperones FKBP52, and HOP.[9,10]

In an effort to further explore San A-amide's unique mechanism of action we synthesized three fluorescein-tagged derivatives of compound **2** (Fig. 1). Using fluorescence polarization anisotropy, we determined the binding affinities of these tagged derivatives to Hsp90 in both the open (**2a**) and closed state (**2c**). We report that compound **2** demonstrates preferential binding showing a significant preference for the closed state (**2c**) over the open state (**2a**). Compound **2**'s enhanced affinity for the closed state likely contributes to its increased potency over San A-amide (K_d = 100 μM using this approach and 20 μM using biotin-labeled analogs)[11] and suggests a novel mechanism of action. Although current Hsp90 inhibitors disrupt closure by binding to Hsp90's ATP-binding site, this is the first report of a small molecule favoring the closed state.

We placed the tags at positions I, III, and IV of compound **2** (Fig. 3). We have established that the presence of an *N*-methyl-D-phenylalanine is an important motif in our potent molecules, thus we did not place a tag at position II as that would require replacing this residue. In addition, our SAR has indicated that the Cbz-protected lysine at position V is also important for biological activity hence we did not tag that position.

The tagged compounds were synthesized following a solid-phase synthesis and tagging method (Scheme 1). Beginning with pre-loaded 2-chlorotrityl-leucine resin, Fmoc-protected amino acids were subsequently coupled and deprotected until the desired linear pentapeptide was reached. A Boc-protected lysine was used in place of the amino acid at positions I, III, and IV, respectively. The peptide was cleaved from the resin in 50% trifluoroethanol in methylene chloride followed by cyclization using optimized macrocyclization conditions.[11] After purification, the Boc-protecting group of the lysine was removed, and fluorescein was coupled to the macrocycle using activated NHS-fluorescein and 8 equiv of DIPEA. The final fluorescein-tagged compounds were purified by reverse-phase HPLC.

Using fluorescence polarization anisotropy, we determined that all three tagged derivatives of compound **2** bind effectively to Hsc82, the full-length yeast homolog of Hsp90, where their micromolar K_ds were reasonably similar to our cytotoxicity data for the non-tagged compounds (Fig. 4, and Table 1).[22] In addition, we found that the placement of the tag was very important, where 2-T-III had a K_d of 57 μM for the open apo state, 2-T-I and 2-T-IV had K_ds almost 4- and 2-fold higher, respectively (Table 1).

In addition to testing the affinity of compound **2** for Hsc82 in the absence of nucleotide, we also examined compound binding to Hsc82 pre-incubated with the non-hydrolyzable ATP analog, AMPPNP. While AMPPNP only subtly shifts the equilibrium of human Hsp90 towards the closed state, the yeast homolog (Hsc82) is quantitatively shifted towards the closed conformation (Fig. 5c).[14] Thus, using Hsc82 allows us to probe conformational discrimination of our molecules. All three compounds, 2-T-I, 2-T-III, and 2-T-IV prefer the closed conformation (conformation **5c**) of Hsc82 over the open conformation (conformation **5a** and Table 1). Previous data showed that compound **2** binds to a site on the N–M domain of Hsp90. The interface between N and M domains changes

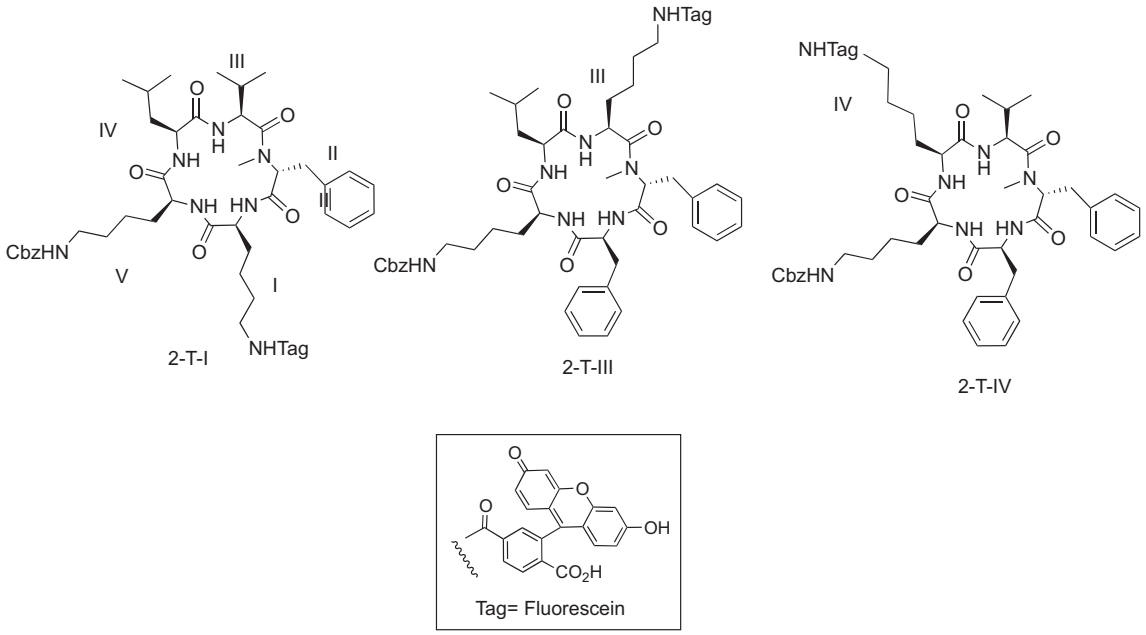
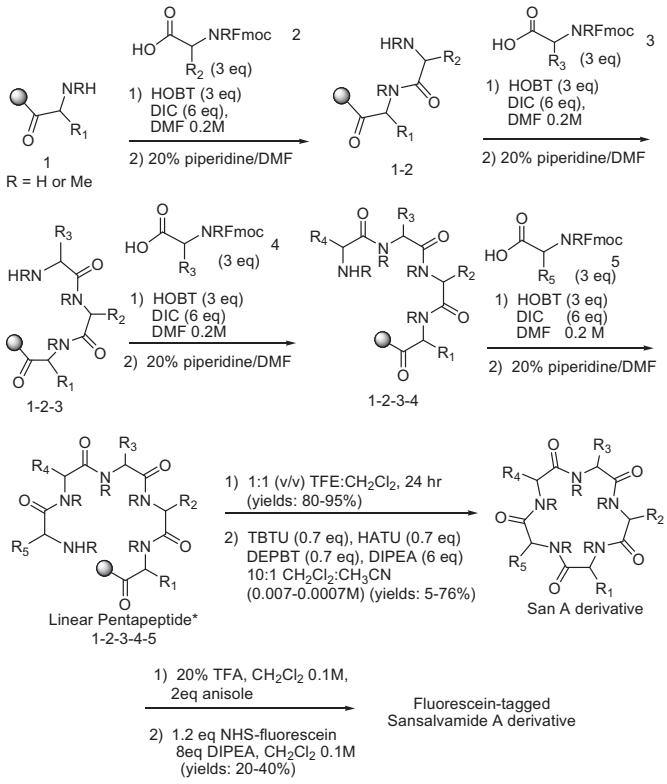


Figure 3. Fluorescein-tagged derivatives of compound **2**.



Scheme 1. Solid-phase synthesis and tagging of San A derivatives.

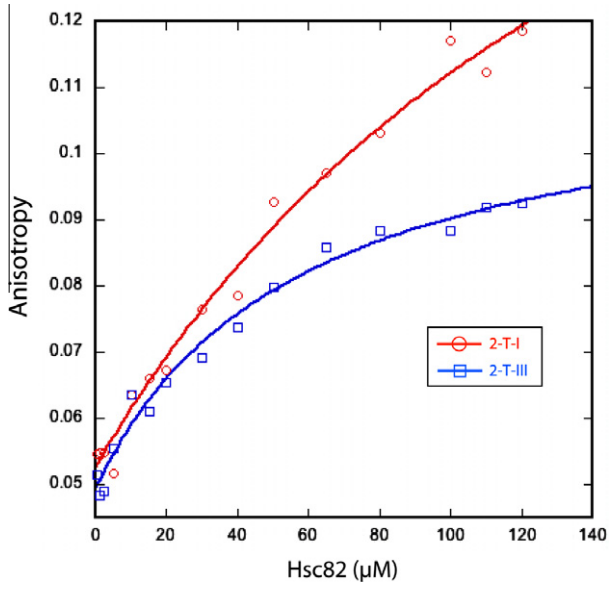


Figure 4. Exemplary anisotropy experiments and corresponding graph with Hsc82 and San A compound **2** tagged with fluorescein at position 1 (red) or at position 3 (blue). Data fitting and analysis is described inside the Supplementary data.

Table 1
Anisotropy data

Protein	Molecule	Measured K_d (μM)	+AMPPNP K_d (μM)
Hsc82	2-T-I	180 ± 60	120 ± 19
Hsc82	2-T-III	57 ± 13	27 ± 4
Hsc82	2-T-IV	103 ± 46	55 ± 21

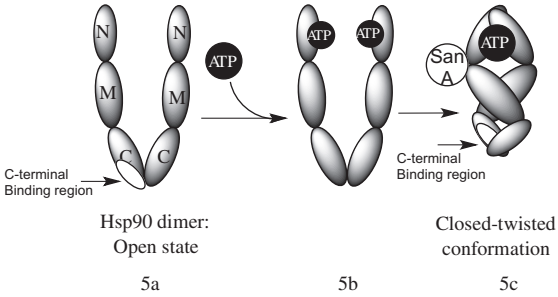


Figure 5. Hsp90 open–close mechanism with San A.

dramatically during the ATPase cycle.[10,11] We had also already reported that allosteric modulation of Hsp90 by San A-amide and compound **2** effectively inhibit the interactions between Hsp90 and C-terminal-interacting co-chaperones and client proteins. The observed preference of compound **2** for the closed conformation is consistent with compound **2**'s binding to the N–M domain, and to its allosteric inhibition of C-terminal client proteins and co-chaperones. That is, binding to a site that is sensitive to conformational rearrangements during the ATPase cycle of Hsp90, and having a preference for a particular Hsp90 conformation, may block client protein access to Hsp90's C-terminus (Fig. 5). Although only 2-fold preference, this modification could be optimized by SAR, constituting an approach that is unique among known Hsp90 inhibitors, and making compound **2** a useful tool in exploring Hsp90 pathways.

In summary, we report the synthesis of fluorescein-tagged derivatives of a San A macrocycle, compound **2**, which binds to Hsp90 at the N–M domain. We used these compounds in fluorescent assays to determine their preference for binding to Hsp90 in its open versus ATP-induced closed state and we show that compound **2** binds preferentially to the closed state. This is the first observation of preference of a non-nucleotide mimetic for the closed state. Based on these findings and our previous reports, compound **2** has an inhibitory profile unlike that of any Hsp90 inhibitor reported to date. It's ability to bind to the N–M domain of Hsp90, allosterically modulate C-terminal client proteins, and a preference for Hsp90's ATP-driven closed state will expand the range of mechanistic tools useful in exploring Hsp90's protein folding cycle. Further studies are currently underway to gain a deeper understanding of this interaction.

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Supplementary data

Supplementary data (spectral data and experimental details for compounds and fluorescence polarization methods) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.09.096.

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22. See Supplementary data for experimental details.