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Macrocycles That Inhibit the Binding between Heat Shock Protein 90 and TPR-Containing Proteins

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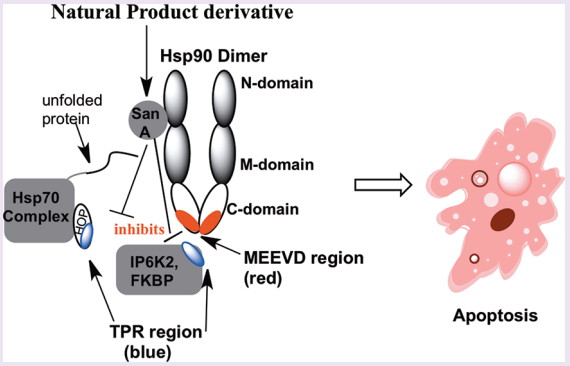
Supporting Information

ABSTRACT: Heat shock protein 90 (Hsp90) accounts for 1−2% of the total proteins in normal cells and functions as a molecular chaperone that folds, assembles, and stabilizes client proteins. Hsp90 is over-expressed (3- to 6-fold increase) in stressed cells, including cancer cells, and regulates over 200 client and co-chaperone proteins. Hsp90 client proteins are involved in a plethora of cellular signaling events including numerous growth and apoptotic pathways. 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. Hsp90's ability to modulate many growth and signaling pathways simultaneously makes this protein an attractive target in the field of cancer therapeutics. Herein we present evidence that a small molecule modulates Hsp90 *via* binding between the N and middle domain and allosterically inhibiting the binding interaction between Hsp90 and four C-terminal binding client proteins: IP6K2, FKBP38, FKBP52, and HOP. These last three clients contain a tetratricopeptide-repeat (TPR) region, which is known to interact with the MEEVD sequence on the C-terminus of Hsp90. Thus, this small molecule modulates the activity between co-chaperones that contain TPR motifs and Hsp90's MEEVD region. This mechanism of action is unique from that of all Hsp90 inhibitors currently in clinical trials where these molecules have no effect on proteins that bind to the C-terminus of Hsp90. Further, our small molecule induces a Caspase-3 dependent apoptotic event. Thus, we describe the mechanism of a novel scaffold that is a useful tool for studying cell-signaling events that result when blocking the MEEVD-TPR interaction between Hsp90 and co-chaperone proteins.



Heat shock proteins (Hsps) are a set of highly conserved proteins that are activated in response to heat, nutrient deprivation, oxidative conditions, and other stresses that may threaten a cell's survival.[1−7] There are five mammalian Hsps, which are identified by their molecular weights: Hsp100, Hsp90, Hsp70, Hsp60, and the small Hsps.[8] Heat shock protein 90 (Hsp90) is known to play an important role in cells, accounting for 1−2% of protein in a normal, unstressed cell.[9] When cells become stressed, the level of Hsp90 increases, with most cancer cells having elevated levels of Hsp90 that account for 3−5% of all protein.[2,10−15] Not surprisingly, Hsp90 plays a critical role in cancer cells because it functions as a molecular chaperone for folding, assembling, and stabilizing proteins and for facilitating intracellular cell signaling.[2,3] There are 6 distinct characteristics displayed by a cancerous cell: (1) growth factor independence, (2) bypassing normal cell cycle checkpoints, (3) unlimited replication potential, (4) tissue invasion and metastasis, (5) avoidance of apoptosis, and (6) sustained angiogenesis.[16] Increased levels of Hsp90 sustain cancer cell growth *via* stabilization and interaction with over 200 client proteins, many of which are responsible for inducing growth, inhibiting apoptosis, and sustaining angiogenesis.[1,17,18,2,4,19] Hsp90 facilitates cell growth by protecting these client proteins from degradation and thereby maintaining the cell rather than directing the cell to the appropriate apoptotic pathway.[20,21] Hsp90 requires a variety of co-chaperones to function, including p23, Aha1, cdc37, Hip, HOP, and Hsp70. These co-chaperones assist in Hsp90's protein folding cycle (Figure 1) and facilitate the maintenance of client proteins. Since Hsp90 is involved in modulating numerous pathways, inhibiting the function of Hsp90 simultaneously affects multiple oncogenic pathways that are involved in cancer cell growth and programmed cell death.[22−25] The redundancy of pathways involved in cancer mean that targeting multiple mechanisms simultaneously is key to developing a successful therapy. Given that the efficacy of target-specific anticancer drugs is often lost over time, blocking a protein that affects numerous cancer-related pathways, such as Hsp90, can be an effective and efficient means of treating drug-resistant cancers.[22−24]

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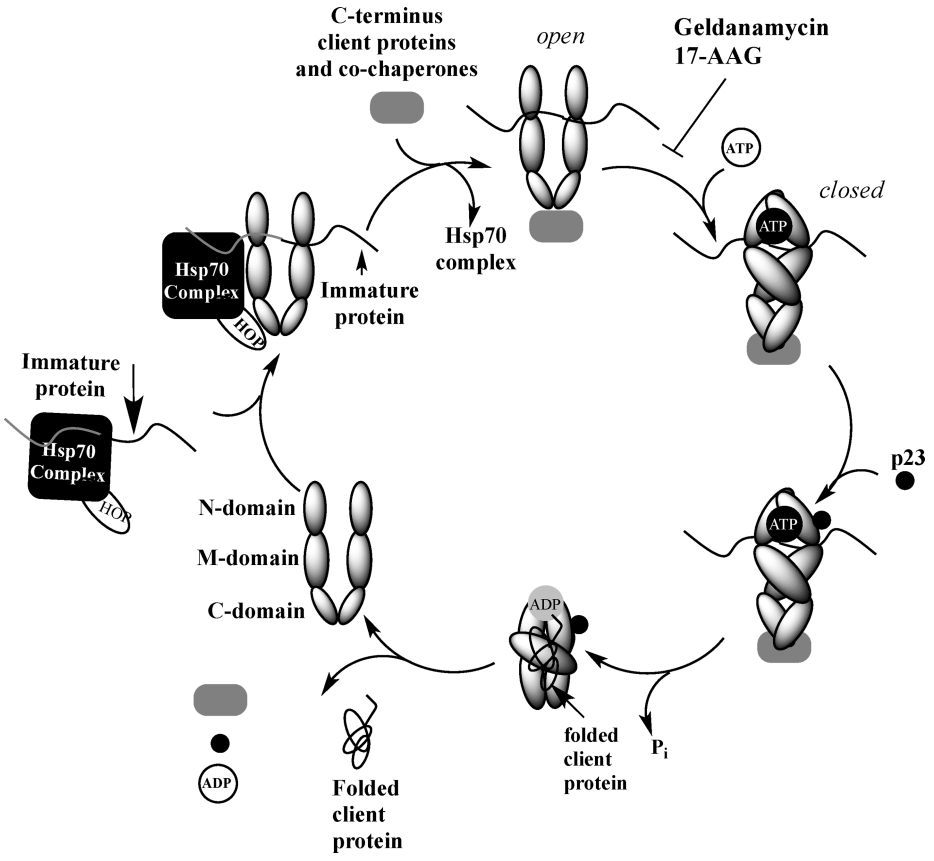

Figure 1. Protein folding mechanistic cycle currently proposed for Hsp90.

Hsp90 has 3 distinct regions: the N-terminal, the middle, and the C-terminal domains (Figure 1). It exists as a dimer in which the subunits interact at the C-terminal domain.[26−28] The N-terminal domain (24−28 kDa) contains an ATP binding site. Hsp90 undergoes a series of ATP-dependent conformational changes including conversion from an open to closed state and reversion to an open state following ATP hydrolysis. Without ATP, Hsp90 maintains an open dimerized conformation. Upon ATP binding, it converts to a closed and twisted conformation that is favorable for the subsequent hydrolysis of ATP to ADP. After ADP is released, Hsp90 reverts back to its open state.[29−31] This hydrolysis and conformational change plays a critical role in Hsp90's ability to regulate the function of many oncogenic client proteins, and it is on the ability of ATP to bind that current inhibitors act as they block the ATP binding event.[32] The middle domain (38−44 kDa) is where most client proteins bind, and this domain plays a key role in stabilizing numerous cell-signaling proteins. By refolding these proteins, Hsp90 protects them from being degraded, thereby allowing them to promote cell growth or to inhibit apoptotic pathways.[33]

The C-terminal domain (11−15 kDa), is the dimerization site for Hsp90.[34] The C-terminus contains a MEEVD sequence (in red, Figure 2). Of the 26 client proteins and co-chaperones known to bind to the C-terminus of Hsp90, 19 contain a tetratricopeptide-repeat (TPR) (blue) region, where this TPR domain binds to the MEEVD region of Hsp90.[35] The TPR domain is a degenerate 34 amino acid sequence that occurs in tandem repeats in the protein. The most common is a three TPR motif that forms a helix-turn-helix three-dimensional structure. FKBP38 and FKBP52 are immunophilins that both bind to the C-domain of Hsp90 *via* the MEEVD-TPR interaction. When free in the cytosol, FKBP38 prevents Bcl-2 from inducing anti-apoptotic effects. Thus, inhibiting FKBP38's interaction with Hsp90 is likely to induce apoptosis *via* the Bcl-2 pathway.[36] FKBP52 is known to be important to immunosuppressant activity within the cell as well as in intracellular trafficking of steroid receptors.[37] It too is regulated *via* a TPR-MEEVD interaction. HOP (Hsp organizing protein) is a co-chaperone that contains 3 TPR binding domains with one binding to the MEEVD region of Hsp90. HOP is also responsible for docking Hsp70 to Hsp90 and allowing for the transfer of nascent polypeptides from Hsp70 to Hsp90 in the protein folding cycle (Figure 1). Finally, inositol hexakisphosphate 2 (IP6K2) is reported to be a C-terminal binding protein, and although it does not appear to not contain a TPR binding domain, it is known to mediate apoptosis *via* a Caspase-dependent pathway.[38] Further, molecules that inhibit the interaction between IP6K2 and Hsp90 are shown to induce apoptosis. Disrupting binding interaction between Hsp90 and TPR-containing proteins, including FKBP38, FKBP52, and HOP, or between Hsp90 and pro-apoptotic C-domain binding proteins such as IP6K2, is one therapeutic approach to inhibiting multiple oncogenic pathways and activating apoptosis simultaneously.

The ATP-binding pocket in the N-terminal domain is the binding site for all Hsp90 inhibitors currently in clinical trials.[39−44] There are currently 15 Hsp90 inhibitors in development, with two of these in phase III clinical trials, and most are structurally related to a single compound: 17-AAG. Of the three non-17-AAG analogues currently in clinical trials, none modulate C-terminal client

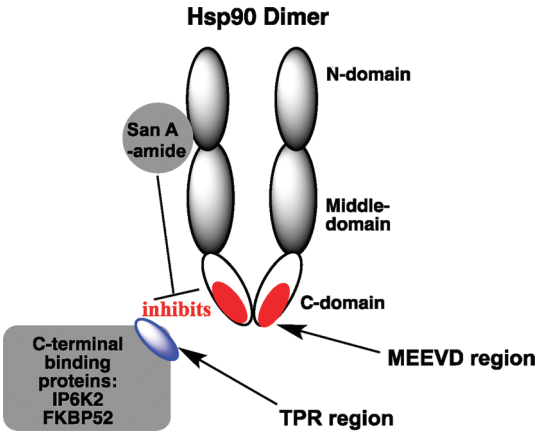

Figure 2. Molecular model for San A-amide analogues' mechanism of action on Hsp90 and its client/co-chaparones.

proteins. Thus, inhibition at a unique site of Hsp90 and modulation of C-terminal binding and/or TPR-containing proteins may represent a new therapeutic approach and as such should be explored.

Unlike current inhibitors, we have shown that a single peptide analogue (compound **1**, Figure 3) of the natural product Sansalvamide A (San A) binds between the N and middle domain and allosterically inhibits IP6K2 and FKBP52 from binding to Hsp90 (Figure 2).[48−50]

Here we present data showing that a new scaffold, a Sansalvamide A derivative (compound **2**, Figure 3), inhibits the binding between Hsp90 and four proteins that bind to the C-terminus, where three of these proteins interact with the MEEVD sequence on Hsp90 *via* their TPR motifs (FKBP38, FKBP52, and HOP). Not only is compound **2** distinct from all current Hsp90 inhibitors, it uniquely modulates the interaction between the MEEVD region of Hsp90 and TPR regions on these co-chaperones. Thus, this small molecule affects a unique subset of Hsp90-client/co-chaperone proteins that are not regulated by any other Hsp90 inhibitors and induces Caspase-3-mediated apoptosis by controlling the pathways associated with these proteins. These data show the promise of small molecule analogues that can be used as tools to block the TPR-MEEVD interaction and access to Hsp90's C-terminus.

RESULTS AND DISCUSSION

Defining the Targeted Hsp90 Domain for Compound 2. Compound **2** was designed to include a phenyl serine residue at position I and three D-amino acids (positions I, IV, and V) in the macrocyclic core (Figure 3).[51] It exhibits good ADME properties and ∼10-fold increase in cytotoxicity over compound **1**.[51] Although we have shown that compound **1** acts *via* an Hsp90-directed mechanism, it is not clear if compound **2** acted *via* the same mechanism. In order to evaluate where compound **2** bound on Hsp90 and to compare its binding site directly to that of analogue **1**, we ran

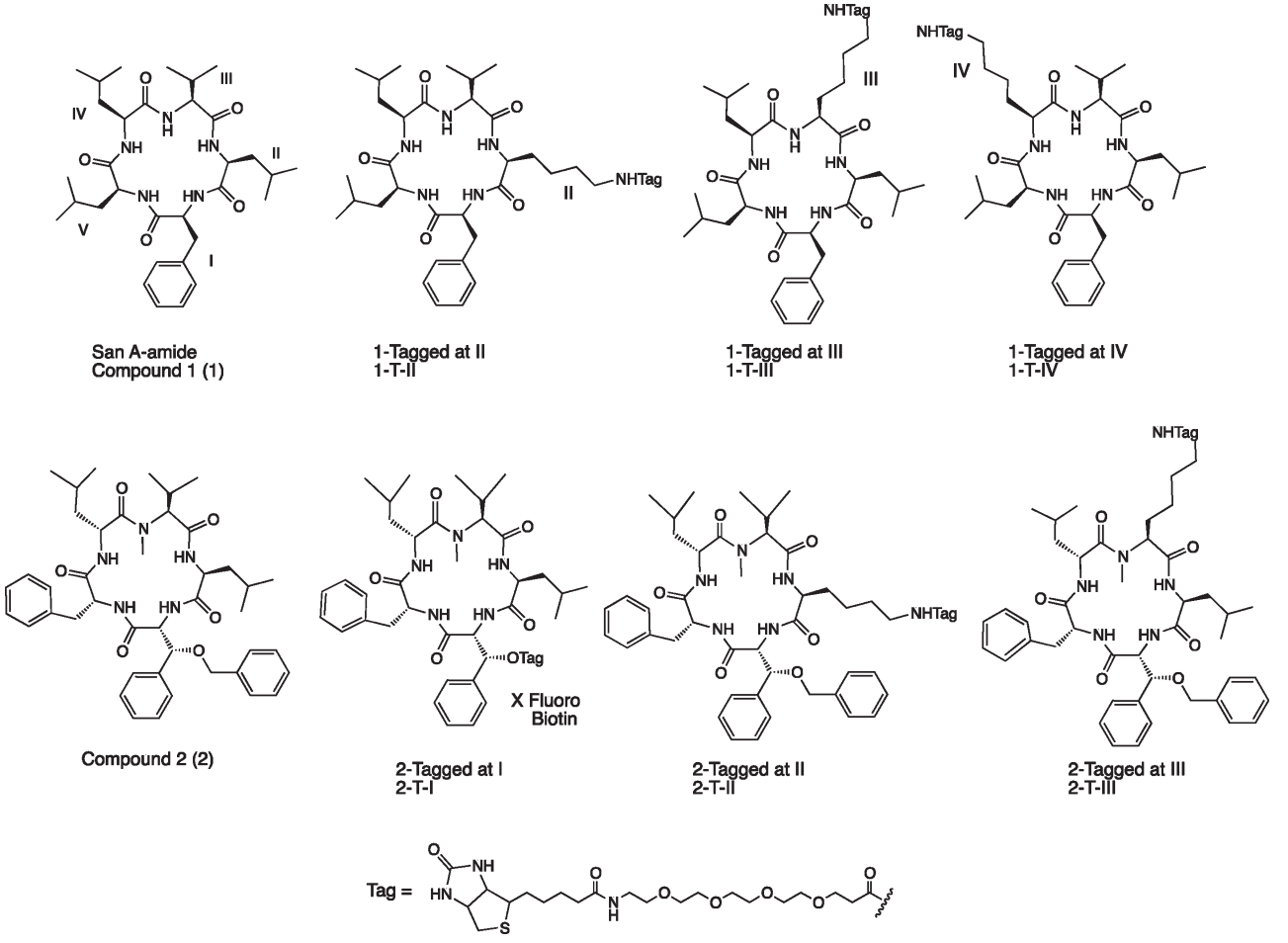

Figure 3. San A-amide structures for compounds **1** and **2** and their PEG-biotinylated analogues. The roman numerals on San A-amide compound **1** represent the location of the PEG-biotin tag for compound **1** tagged at -II, -III, or -IV and **2** tagged at -I, -II, or -III.

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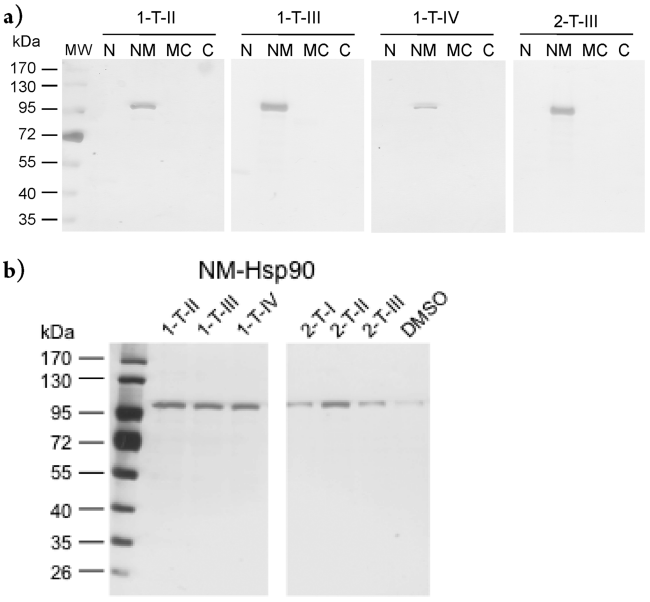

Figure 4. Hsp90 protein pull-downs. (a) Pull-down data for compounds **1** and **2** using mammalian purified Hsp90 domains: N, N-middle (NM), middle-C (MC), and C domains, respectively. Position of molecular weight markers in kDa are indicated on the left. (b) Pull-down data for compounds **1** and **2** using mammalian purified Hsp90 N-middle (NM) domain.

pull-down assays using the binding domains of Hsp90 (N, C, N-middle, and middle-C mammalian domain variants). The pull-down assays require the molecules to be tagged in order to pull out the protein that is bound to them, so three derivatives of both compounds **1** and **2** were made with biotinylated tags. Since we are unsure as to the exact amino acid residues of the compounds involved in the molecule−protein interaction, the tags were rotated around the peptide ring to ensure that the tag would not interfere with the compounds binding to Hsp90. These molecules were made using our solid phase synthesis, where a Boc-protected lysine was incorporated into the designated tagging position.[52]

These tags allowed us to perform pull-down assays with purified Hsp90 domains (Figure 4a). All compounds pulled down the N-middle domain selectively over the other domains, as evidenced by the band in the lane labeled NM for each tagged molecule shown in Figure 3. Data is shown for compound **1** with tags at 3 different positions and then a comparison to compound **2** tagged at III (**2-T-III**). Further, a comparison of all 6 compounds with the N-M domains (Figure 4b) shows that all 6 molecules pull down the N-M domain. These data indicate that the molecules have the same binding site, although it is not surprising they bind with different affinities, which is presumably related to the placement of the tag.

In order to determine the difference in binding affinities to Hsp90, we performed competitive binding assays using the tagged and untagged compounds. We had previously reported a binding affinity of 20 μM for compound **1** using this method.[51] As expected, we found that compound **2** had a lower dissociation constant, and thus higher affinity, of 3.6 μM. The difference in affinities is also consistent with the IC_{50} values of the two compounds (Supplementary Figure 1). In addition, competitive binding assays between compounds **1** and **2** were run and demonstrated that compound **2** inhibits the binding between compound **1** and Hsp90 with an IC_{50} = 5.2 μM (Supplementary Figure 4). These data therefore support the conclusion that both compound **1** and **2** bind to the same binding site on Hsp90, where both require the presence of the N-middle domain for optimal binding. As reported with compound **1**, we suspect that compound **2** binds to the hydrophobic cavity between the N and middle domain.[51]

Hsp90 Inhibition by San A-Amide Leads to Apoptosis. To verify that the cytotoxicity of compounds **1** and **2** is a result of an Hsp90-dependent pathway, siRNA experiments were run in order to evaluate whether cells depleted of Hsp90 were more susceptible to the effects of San A-amide analogues than control cells. HeLa cells were treated with small interfering RNAs (siRNA) against Hsp90 (si-Hsp90), while control cells were treated with random siRNA sequences (where the siRNA construct contained no known homology to mammalian genes). Western blot analyses confirmed that HeLa cells treated with 2 nM si-Hsp90 expressed only 46% of Hsp90 protein relative to si-control treated cells (Supplementary Figure 2a). After 48-h siRNA treatment, compounds **1**, **2**, 17-AAG, or DMSO (control) were added to both si-Hsp90 and si-control treated cells for a further 48-h incubation. The cells depleted of Hsp90 (gray bars Figure 5a) were 21% and 43% more susceptible to the treatment by analogues **1** and **2**, respectively, than control cells (white bars Figure 5a). Interestingly, there was minimal effect on the si-Hsp90 treated cells upon 17-AAG addition (only 14% increased susceptibility).

To validate the siRNA results, we rescued the compound-induced cytotoxicity by overexpressing Hsp90 in HeLa cells. An Hsp90 inhibitor has a reduced effect on cells when Hsp90 is overexpressed compared to control cells (where Hsp90 was at normal levels). This increased ratio of Hsp90 to the small molecule inhibitor allows the constitutive Hsp90 to stabilize, fold, and facilitate cell-signaling events, effectively rescuing cells that are treated with Hsp90 inhibitors. We transfected HeLa cells with pCMV-Hsp90 plasmids to induce overexpression of Hsp90 or with an empty vector (pCMV control). After 24 h of transfection, HeLa cells were harvested and analyzed for Hsp90 overexpression. Hsp90 levels were 2- to 4-fold higher in cells transfected with pCMV-Hsp90 (Supplementary Figure 2b). Overexpressing Hsp90 cells (black bars, Figure 5b) compared to control cells (white bars, Figure 5b) showed less susceptibility to both analogues **1** and **2**. That is, compounds **1** and **2** had diminished cytotoxic effects on cells with increased Hsp90 relative to control cells. This was also true for 17-AAG, a known Hsp90 inhibitor, and is in agreement with the siRNA results. These Hsp90 siRNA and overexpression results coupled with the previous binding domain experiments suggest that compound **2** is acting *via* modulation of Hsp90.

It has become accepted that induction of Hsp70 is an indicator that molecules inhibit Hsp90[45] whereby Hsp70 rescues the cell from apoptotic pathways, allowing for continued cell survival and conferring resistance to 17-AAG.[47] It is believed that Hsp70 takes on the protein folding and stabilization role usually performed by Hsp90.[46,47] It is known that 17-AAG induces overexpression of Hsp70 at mid-nanomolar concentrations (Figure 5c). We also found that compounds **1** and **2** induce the production of Hsp70 at concentrations consistent with their GI_{50} values (Figure 5c), which indicates that our molecules are going through an Hsp90 inhibition pathway, that is, the fact that compound **2** induces the production of Hsp70 at significantly lower concentrations and at a faster rate is consistent with compound **2** being a more effective Hsp90 inhibitor than compound **1**.

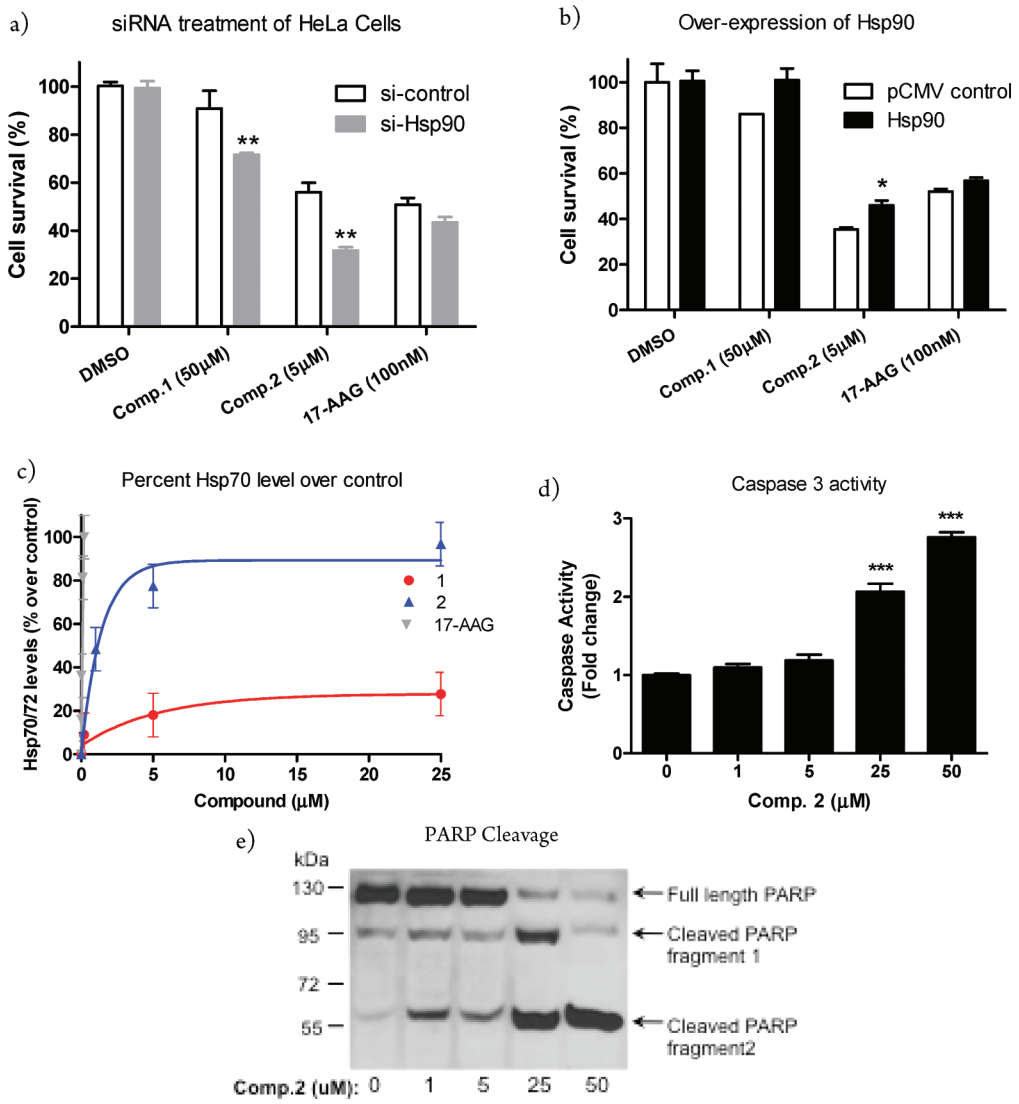

Figure 5. Cell-based analysis of compounds acting *via* an Hsp90 pathway and inducing apoptosis. (a) siRNA experiments in HeLa cells treated with control siRNA (white bars) or siRNA against Hsp90 (gray bars). Cells depleted of Hsp90 were more sensitive to treatment by compounds **1** and **2** than control cells. Statistical analysis was performed by a two-way ANOVA. $^{**}p < 0.01$. (b) Hsp90 overexpression in HeLa cells. Compounds **1** and **2** had a decreased effect in overexpressing cells (black bars) compared to control cells (white bars). Statistical analysis was performed by a two-way ANOVA. $^{*}p < 0.05$. (c) Hsp70 up-regulation analysis in compound-treated cells. Compound **1** up-regulated Hsp70 by 25% compound **2** by 85%, and 17-AAG induced overexpression 100% over control. (d) Analysis of Caspase-3 activity in cells treated with compound **2**. Statistical analysis was performed by an unpaired *t* test. $^{***}p < 0.001$. (e) Cell lysates from panel d were analyzed for full length and cleaved PARP by Western blot. Position of molecular weight markers in kDa are indicated on the left.

Given that compounds **1** and **2** modulate Hsp90, they were likely to induce apoptosis. To verify this, we incubated HCT-116 cells with compound **2** (0−50 μM) for 24 h and then examined whether Caspase-3 activity was increased in treated cells compared to nontreated cells. Not surprisingly, Caspase-3 activity increased in a dose-dependent manner with treatment of compound **2** (Figure 5d). Significantly, a 2- and 3-fold increase in activity over nontreated cells was observed at 25 and 50 μM, respectively.

In addition, a well-known method for detecting apoptosis is the evidence of cleaved poly(ADP-ribose) polymerase (PARP) fragments, as PARP is involved in programmed cell death. Examination of whether an analogue triggers Caspase-dependent apoptosis can be measured by evaluating the levels of full-length PARP and its corresponding apoptotic enzyme-cleaved fragments.[53] An increase in the degradation of full length PARP upon the addition of increasing amounts of small molecule verifies that the compound is inducing Caspase-dependent apoptosis.[53,54] Cell lysates from HCT-116 treated for 24 h with increasing concentrations of compound **2** (0−50 μM) 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 **2** (Figure 5e). Consistent with these results is the appearance of cleaved PARP, fragment 1 (~89 kDa) and fragment 2 (~54 kDa), which increased in a dose-dependent fashion upon the addition of compound **2**. Thus, the elevated levels of Caspase-3 and the cleavage of full-length PARP support that compound **2** induces apoptosis in a Caspase-dependent manner.

Inhibition of Hsp90−Client Protein Interactions. Anticipating the cytotoxic effect of San A might be a direct result of its

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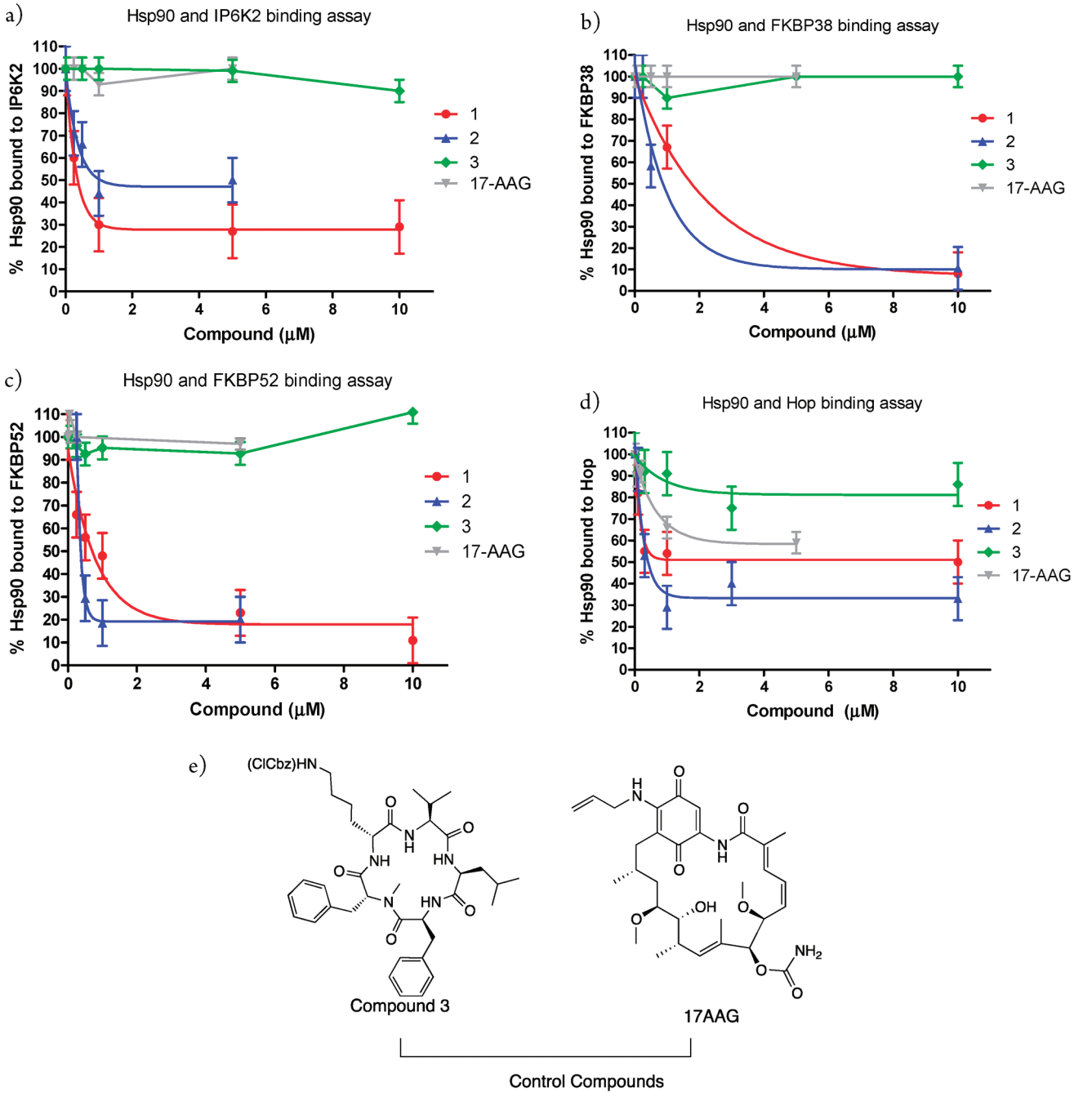

Figure 6. Pure protein binding assays. (a) IP6K2 and Hsp90 binding assay. Compounds **1** (red line) and **2** (blue line) inhibited the binding interaction between IP6K2 and Hsp90, whereas compound **3** (green line) and 17-AAG (gray line) did not. (b) FKBP38 and Hsp90 binding assay where compounds **1** and **2** inhibited the binding interaction between FKBP38 and Hsp90, whereas compound **3** and 17-AAG did not. (c) FKBP52 and Hsp90 binding assay showed that compounds **1** and **2** inhibited the binding interaction between FKBP52 and Hsp90, whereas compound **3** and 17-AAG did not. (d) Hop and Hsp90 binding assay showed that compounds **1** and **2** inhibited the binding interaction between Hop and Hsp90, whereas compound **3** and 17-AAG did not. (e) Structures of both control compounds, where compound **3** does not appear to inhibit any binding between Hsp90 and C-terminal clients but is structurally similar to both compound **1** and **2**. 17-AAG is a known Hsp90 inhibitor.

ability to inhibit the interaction between Hsp90 and client proteins integral to cell signaling events, we probed the effect of both compounds **1** and **2** on the binding interaction between Hsp90 and IP6K2, FKBP38, FKBP52, and Hop, four proteins that bind to the C-terminus of Hsp90. Since FKBP38, FBBP52, and HOP are co-chaperones that are intricately involved in Hsp90's protein folding mechanism and bind to Hsp90 *via* the MEEVD-TPR interaction, understanding how these two San A analogues affect these co-chaperones' interaction with Hsp90 will potentially elucidate their role in Hsp90's protein folding mechanism and their ability to modulate access to the MEEVD region on Hsp90's C-terminus.

We recently showed that compound 1 inhibited IP6K2 from binding to Hsp90 (red line Figure 6a),[48] and we show here that

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compound **2** also inhibits this binding event (blue line Figure 6a), whereas both of our negative controls, 17-AAG and compound **3** (structures shown Figure 6e), do not (green and gray, respectively in Figure 6a). Compound **3** is a cytotoxic macrocycle that differs in the amino acids used to form the cyclic backbone, and it does not appear to interact with Hsp90 (data not shown). We found that both compounds **1** and **2** inhibit the binding between Hsp90 and FKBP38 or FKBP52 (Figure 6b and c, respectively), whereas both 17-AAG and analogue **3** do not affect either co-chaperone's interaction with Hsp90. Although FKBP38 has been shown to play a role in preventing Bcl-2's anti-apoptotic effects, modulation of the FKBP38-Bcl-2 pathway *via* Hsp90 is not well understood, and therefore compounds **1** and **2** will be useful tools for investigating the mechanism of the Hsp90/FKBP38/Bcl-2 interaction. Compounds **1** and **2** also modulate FKBP52, and therefore these molecules will be useful in deducing what role Hsp90 plays in FKBP52's immunosuppressive pathway. Finally, both compound **1** and compound **2** effectively block the interaction between HOP and Hsp90 (Figure 6d), whereas compound **3** and 17-AAG do not. As observed with the binding event between IP6K2 and Hsp90, compound **2** is more effect at inhibiting the binding event between HOP and Hsp90 than compound **1**. We believe this is due to compound **2**'s better binding affinity for Hsp90 compared to that of compound **1** (*i.e.*, 3.6 versus 20 μM). Given HOP's vital role in the transfer of the nascent polypeptides from Hsp70 to Hsp90 (Figure 1), compound **2** can be used to further investigate HOP's role in modulating the interaction between these two important heat shock proteins and what affects inhibiting access to the MEEVD region will have on the transfer of unfolded client proteins. Evidence supporting the use of a small molecule to modulate a large protein's conformation is not unprecedented.[55,56] Further, a recent publication by Agard *et al.*[57] supports the idea that the N-M domain is a hinge-like region where our molecules affect Hsp90's ability to fold and appropriately expose its MEEVD region to client or co-chaperones. An alternative explanation, also supported by Agard *et al.*,[57] is that the middle domain of Hsp90 contains a site that interacts with TPR domains. Thus, our molecule could be directly inhibiting binding between Hsp90 and the three proteins containing a TPR domain (FKBP38, FKBP52, and HOP). This explanation would be consistent with the binding data, where compound **2** only inhibits the Hsp90-HOP interaction at 35% and at an IC_{50} = 400 nM, that is, a reasonable explanation for only inhibiting 65% of this protein binding event is that another HOP binding site exists on Hsp90 and it is not affected by the binding of compound **2**. However, this would not explain our ability to inhibit IP6K2 from binding to the C-terminus of Hsp90. Thus, we believe the most reasonable hypothesis based on current knowledge is that our compounds act *via* an allosteric modulation.

Mechanism of Action of San A-Amide Analogues. The ATP pocket in the N-terminal domain is the binding site for all Hsp90 inhibitors currently in clinical trials.[39−44] Unlike current inhibitors, which target proteins that interact only with the N or middle domain of Hsp90, we have shown that analogue **2** pulls down a truncated version of Hsp90 that contains the N-middle domain (Figure 4a and b), binding most effectively when both domains are present. These pull-down results suggest that our compound binds to the interface between the two protein domains.

The treatment of cells with siRNA against Hsp90 leads to decrease in cell survival upon the addition of compound **1** or **2** compared to treatment with DMSO. More importantly, the cells treated with compound and siRNA controls were significantly less affected than those treated with siRNA against Hsp90 and compound (Figure 5a). Verifying that these two analogues act *via* an Hsp90-controlled mechanism, the addition of compound **1** or **2** to cells where Hsp90 was overexpressed rescued the cells from the compound's cytotoxic effects (Figure 5b). The induction of Hsp70 overexpression upon treatment with both compounds **1** and **2** are accepted phenotypes for Hsp90 inhibitors (Figure 5c), and Caspase-3 assays along with PARP analyses from cells treated with increasing concentrations of analogue **2** (Figure 5d and e) showed an increase in Caspase-3 levels and a corresponding fragmentation of full-length PARP in cells. These data all support that compound **2** induces an apoptotic event *via* an Hsp90-dependent pathway.

Unique to the San A-amide scaffold, data in Figure 6 shows that compound **2** allosterically inhibits four different C-terminal client/co-chaperone proteins from binding to Hsp90. In contrast to 17-AAG, which binds to the N domain and inhibits Hsp90's catalytic cycle by blocking ATP−Hsp90 interactions, compound **2** is potentially inhibiting Hsp90's catalytic cycle earlier than 17-AAG, blocking co-chaperones from binding during or prior to transferring the unfolded protein between Hsp70 and Hsp90 (Figure 7, step 1 or step 2). Three of the C-terminal binding proteins inhibited from interacting with Hsp90 contain a TPR binding domain, and thus our data support the mechanism that analogue **2** inhibits the TPR region from binding to the MEEVD sequence on Hsp90 (Figure 7, step 1). Further, compound **2** inhibits HOP from docking, perhaps preventing Hsp70 from transferring the unfolded protein to Hsp90 (Figure 7, step 2).

In summary, compound **2** is a novel structure that inhibits four C-terminal binding proteins from interacting to Hsp90. It has a unique mechanism of action, one that is distinct from other Hsp90 inhibitors published to date, as it appears to modulate the binding of the MEEVD−TPR interaction that occurs between Hsp90 and co-chaperone, respectively, *via* an allosteric effect. Compound **2** is likely impacting access to Hsp90's MEEVD region on the C-terminus by inducing or stabilizing an Hsp90 conformation that obstructs access to the region where TPR binding domains interact, thus modulating TPR-containing proteins and their ability to bind Hsp90. This is the first example of such a remarkable mechanism. As such, it provides the opportunity for compound **2** to be utilized as a novel tool that can modulate Hsp90's TPR-MEEVD interaction, making it a perfect instrument for delineating many unexplored Hsp90-protein pathways including the Hsp90/FKBP38/Bcl-2 pathway. Studies on the subset of protein pathways involving Hsp90's MEEVD region are currently underway and will be reported in due course.

METHODS

Binding Assay. The binding affinity between Hsp90 and its co-chaperone (*i.e.*, FKBPs and Hop) or client proteins (*i.e.*, IP6K2) were completed using 100 nM (final concentration) of human native protein Hsp90 (Stressgen, cat. no. SPP-770D) and 50 nM (final concentration) of recombinant co-chaperone or client proteins (GST-FKBP38, Abnova, cat. no. H00023770-P01; GST-FKBP52, Abnova, cat. no. H00002288-P01; GST-IP6K2, Abnova, cat. no. H00051447-P01). Three independent experiments ($n = 3$) were conducted using five different concentrations of San A (0−10 μM). 17-AAG was used as the control compound. Protein pull-down was completed using Immobilized Glutathione agarose (Pierce, cat. no. 20211) or Talon-Metal Affinity Resin (Clontech, cat. no. 635501),

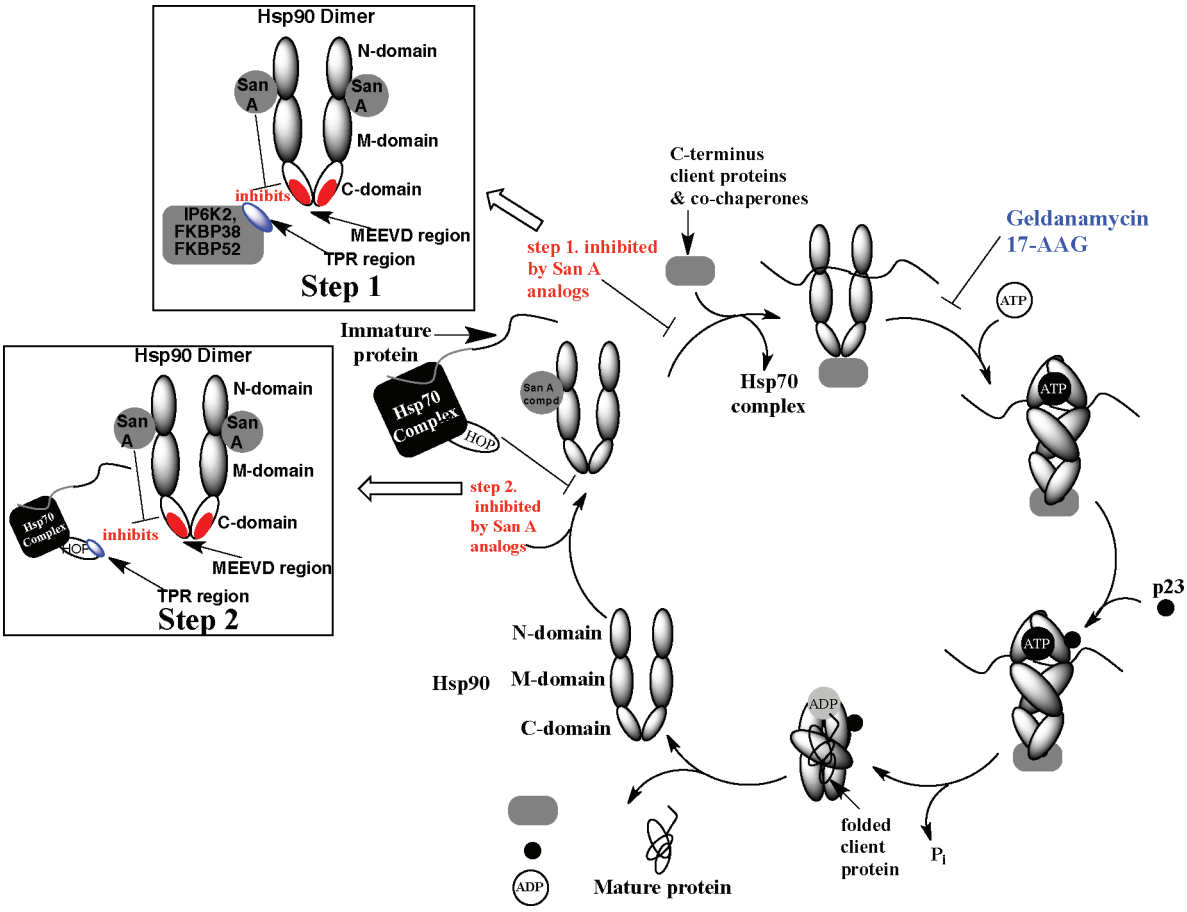

Figure 7. Schematic of Hsp90 protein folding cycle and the proposed mechanism of action of compounds **1** and **2**.

followed by three washes of the bead (20 mM Tris-HCl, 300 mM NaCl, 1% (v/v) triton X-100), and finally boiling the beads with 4x Laemmli sample buffer. Samples were analyzed using 4−20% SDS-PAGE gel, followed by standard Western blot protocol to detect Hsp90 (Stressgen rabbit polyclonal anti-Hsp90 antibody, cat. no. SPA-836) and its co-chaperone or client proteins (R&D Systems rat monoclonal anti-FKBP38 antibody, cat. no. MAB3580; Abcam rabbit polyclonal anti-FKBP52 antibody, cat. no. ab2926; Santa Cruz Biotechnology goat polyclonal anti-Hop antibody, cat. no. sc-27962; Santa Cruz Biotechnology goat anti-IP6K2 antibody, cat. no. SC-10425). The respective ratio of Hsp90 to its co-chaperone or client proteins were analyzed *via* Image J and transformed to a percent of Hsp90 bound to co-chaperone or client proteins.

Domain Pull-Down Assay. Gst-tagged Hsp90 proteins containing N-, NM-, MC-, or C-domain were expressed and purified from BL21(DE3) *Escherichia coli*. A batch purification was completed according to manufacturer's protocol (Thermo Scientific, Immobilized Glutathione cat. no. 15160). To verify that San A-amide derivatives are binding to a specific domain of Hsp90, we performed a domain pull-down using 15−25 μM of purified Hsp90 proteins (*i.e.*, N-, NM-, MC-, or C-domain). San A-amide-biotin or DMSO (control) was added to each reaction and incubated for 2 h at RT. Then, 50 μL of NeutrAvidin agarose resin was prepared according to manufacturer's protocol (Thermo Scientific, cat. no. 209200), added to each reaction, and incubated for an additional 30 min at RT. After 4 washes (20 mM Tris-HCl, pH7.5, 100 mM NaCl, 10% (v/v) glycerol, and 1.5% (v/v) NP-40), proteins were eluted from NeutrAvidin resin with 30 μL of sample buffer and boiling. Samples were ran on a SDS-PAGE gel and visualized by silver staining using standard manufacturer's protocol (Pierce, cat. no. 24621).

Co-immunoprecipitation of Hsp90. coIP assay of Hsp90 was performed according to Hernandez *et al.* (2002). A slight modification included using 1.5×10^6 HCT-116 cells treated with San A (0−50 μM) or with 17-AAG (0−1 μM). Control cells were treated with 1% DMSO (final concentration). After 72 h of compound treatment, cells were harvested and lysed (according to Hernandez *et al.*, 2002), and total protein was quantitated. Next 500 μg of total protein lysate was incubated with 1 μg of Hsp90 antibody (StressMarq mouse monoclonal anti-Hsp90 antibody, cat. no. SMC-109A/B) for 24 h at 4 °C. Mouse IgM antibody (Rockland, cat. no. 010-0107) was used a control for a set of treated HCT-116 cells. After addition of IgM agarose (Abcam, cat. no. 65867) for an additional 2-h incubation at 4 °C, the beads were washed according to protocol (Hernandez *et al.*, 2002) and analyzed by standard Western blot protocol to detect Hop and Hsp90. Each experiment was repeated ($n = 4$).

siRNA Experiments. HeLa cells (~30% confluent) were transfected with 2 nM of si-control (Qiagen Ctl_AllStars_1) or 1 nM of si-Hsp90α (AACCCTGACCATTCCATTATT) and 1 nM of si-Hsp90β (CAAGAATGATAAGGCAGTTAA) for 48 h using standard Qiagen HiPerFect protocol (cat. no. 301704). DMSO (1% final, control), compound **1** (50 μM), compound **2** (5 μM), or 17-AAG (100 nM) was added to transfected cells for 48 h before cell survival was measured using standard CCK-8 protocol (Dojindo, cat. no. CK04-11). Down-regulation of Hsp90 was confirmed by Western blot (Supporting Information, Figure 2a).

Hsp70 Up-regulation in Compound-Treated Cells. HCT-116 cells were treated with increasing concentrations of compound **1** (0−25 μM) or 17-AAG (0−200 nM) for 24 h after which cells were harvested and lysed. Lysates were analyzed for Hsp70 *via* Western blot, using GAPDH as a control.

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Hsp90 Overexpression Experiments. HeLa cells (~30% confluent) were transfected with HSP90α and HSP90β together (0.5 μg each) or control PCMV (1 μg) using standard Qiagen PolyFect protocol (cat. no. 301107). After 24-h transfection, HeLa cells were then treated with DMSO (control), compound **1** (50 μM), compound **2** (5 μM), or 17-AAG (100 nM). Cell survival was determined using standard CCK-8 protocol (Dojindo, cat. no. CK04-11). Overexpression of Hsp90 was confirmed by Western blot (Supporting Information, Figure 2b).

Caspase-3 Assay. Caspase-3 analyses were completed according to manufacturer's protocol (Promega, cat. no. G7351). Briefly, 1×10^6 HCT-116 cells was treated with San A (0−50 μM) for 24 h. Cells were harvest and lysed according to manufacturer's protocol; 30 μg of total protein lysate was used in the Caspase-3 assay.

Statistical Analysis. All experiments were repeated three times. Means ± SEM was calculated using GraphPad Prism 5 Software and Microsoft Excel. Statistical analysis was performed by unpaired Student's *t* test for *, $P < 0.05$.

■ ASSOCIATED CONTENT

Supporting Information. General experimental procedures, pull-down assay, binding assay, and NMR and mass spectral data for compounds. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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