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A heat shock protein 90 inhibitor that modulates the immunophilins and regulates hormone receptors without inducing the heat shock response

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Abstract

When a cell encounters external stressors, such as lack of nutrients, elevated temperatures, changes in pH or other stressful environments, a key set of evolutionarily conserved proteins, the heat shock proteins (hsps), become overexpressed. Hsps are classified into six major families with the hsp90 family being the best understood; an increase in cell stress leads to increased levels of hsp90, which leads to cellular protection. A hallmark of hsp90 inhibitors is that they induce a cell rescue mechanism, the heat shock response. We define the unique molecular profile of a compound (SM145) that regulates hormone receptor protein levels through hsp90 inhibition without inducing the heat shock response. Modulation of the binding event between heat shock protein 90 and the immunophilins/homologs using SM145, leads to a decrease in hormone receptor protein levels. Unlike N-terminal hsp90 inhibitors, this hsp90 inhibitor does not induce a heat shock response. This work is proof of principle that controlling hormone receptor expression can occur by inhibiting hsp90 without inducing pro-survival protein heat shock protein 70 (hsp70) or other proteins associated with the heat shock response. Innovatively, we show that blocking the heat shock response, in addition to hsp90, is key to regulating hsp90-associated pathways.

Keywords

Heat shock protein 90 (hsp90); Tetratricopeptide-repeat (TPR); FKBP51; FKBP52; Heat shock response; Heat Shock Factor 1 (HSF1)

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Supporting information

Supporting information includes experimental details for biological assays and imaging experiments. This material is available free of charge at <http://pubs.bmcl.org>.

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When a cell encounters external stressors, such as lack of nutrients, elevated temperatures, changes in pH or other stressful environments, a key set of evolutionarily conserved proteins, the heat shock proteins (hsps), become overexpressed.^{1–7} The hsps are molecular chaperones broken down into six distinct families based on their molecular size (hsp100, hsp90, hsp70, hsp60, hsp40 and the small hsps).⁸ While all of these hsps are important in normal cells and become overexpressed in stressed cells, hsp90 is the most prominent. In an unstressed cell, hsp90 makes up 1–2% of the total protein load, and upon external stressors this is increased to 3–5%.⁹ One major stressor known to induce this up-regulation of hsp90 is malignancy.^{2, 10–15} The large amount of mutated and mis-folded proteins in cancer cells cause them to become dependent upon the molecular chaperone activity of hsp90; because hsp90 protects the function of more than 200 client proteins, many of which are associated with oncogenesis (Figure 1).^{1, 2, 4, 16–18} Thus, cancer cells are significantly more dependent on hsp90 than normal cells.¹⁹

Hsp90 is ATP dependent, functional only when dimerized and broken down into 3 domains, the amino (N), middle (M), and carboxy (C) domains (Figure 1). The C-terminal domain is known to interact with a specific subset of proteins that contain a tetratricopeptide-repeat (TPR) domain.²⁰ The TPR domain is a protein scaffold consisting of a semi-conserved sequence of 34 amino acids that occur in repeats throughout the protein.²¹ Within the group of sixteen TPR proteins that interact with hsp90, four are immunophilins: FK506 binding protein 52 (FKBP52), FKBP51, cyclophilin 40 (Cyp40), and FKBP38. There are also several are homologs including: C-terminus of Hsc70 interacting protein (CHIP), Unc45, and mitochondrial import receptor of 70kDa (Tom70).²² In addition, a key co-chaperone that regulates hsp90's function is the TPR-containing heat shock organizing protein (HOP). These TPR-containing proteins are all regulated via their interaction with hsp90's MEEVD region (M= methionine, E= glutamic acid, V= valine, D= aspartic acid), located at the C-terminus (Figure 1).

Three of the four immunophilins (FKBP51, FKBP52 and Cyp40) are well established to regulate cell growth through controlling hormone receptor (HR) interactions with hsp90.²³ In addition, the homologs CHIP, Unc45 and Tom70 facilitate hormone receptor-regulated cell growth via hsp90.²³ These co-chaperones regulate the maturation of hormone receptors by forming a multi-chaperone complex with hsp90 and the co-chaperone p23. This complex induces a signaling cascade leading to cell growth.²³ Since the interaction of hsp90 with the immunophilins regulates HR development, blocking the interaction between the immunophilins' TPR domain and hsp90's MEEVD region will likely affect HR protein levels. Since hsp90 its co-chaperones regulate HR maturation, targeting this pathway may avoid existing cell rescue mechanisms.

There are two general classes of hsp90 inhibitors that have been extensively investigated: those that bind to the N-terminus and those that bind to the C-terminus of hsp90. There are 4 inhibitors in clinical development and they all bind to the ATP-binding pocket at the N-terminal domain. Of these four drugs, three are structurally related and contain a resorcinolic acid motif (similar to NVP-AUY992) and the fourth is an analogue of 17-AAG, which has been previously clinically tested and removed due to toxicity (Figure 2). These four drugs all impact the same signaling pathways and have no impact on the binding between

immunophilins and hsp90. Thus, they are ineffective tools for delineating the relationship between the immunophilins, hsp90, and HR production. Furthermore, the hsp90 inhibitors currently in clinical trials are combating drug resistance, which is caused by the activation of the heat shock response (HSR).^{24, 25} The HSR is a cell survival mechanism that induces the over-expression of hsp70 and other heat shock proteins that rescue the oncogenic pathways usually controlled by hsp90.⁶

The second class of molecules that bind to hsp90 are compounds that target its C-terminus. The most effective are coumermycin A1 (CA1) and its analogs (Figure 2).^{20, 26, 27} There is currently no data on how the C-terminal hsp90 inhibitor CA1 controls the immunophilins. However, work done by Ratajczak and co-workers showed that a millimolar concentration of a structurally similar C-terminal inhibitor, novabiocin, disrupts immunophilins from binding to hsp90.²⁸ Despite extensive research on hsp90 as a therapeutic target, there is a large and important knowledge gap within the hsp90 field: how do we inhibit hsp90 pathways without inducing a heat shock response?

Herein we describe the first small molecule, SM145, which binds at a novel site (between the N and middle domain of hsp90) and modulates binding between hsp90 and multiple immunophilins/homologs via blocking the interaction between MEEVD and TPR binding sites. We show that disrupting the association between hsp90 and the immunophilins leads to a decrease in hormone receptor protein levels without inducing the heat shock response (HSR). This work is proof of principle and the first example showing that directly inhibiting hsp90 can modulate the hormone receptor levels without inducing the pro-survival protein heat shock protein 70 (hsp70) or causing the HSR.

We have previously reported that SM145 binds to the NM-domain of hsp90, induces apoptosis and inhibits its binding to specific client proteins and co-chaperones.^{29–31} To prove that SM145 inhibits the molecular chaperone function of hsp90, we performed two types of luciferase refolding assays. The first was a pure protein assay, where hsp90 and necessary co-chaperones were incubated with heat-denatured luciferase. The second assay was a rabbit reticulocyte lysate (RRL, Promega) based luciferase refolding assay, where denatured luciferase was incubated in the complete system of the lysate. In the pure protein assay inhibition of luciferase activity can be tied directly to inhibiting hsp90. The second, lysate-based assay, shows that hsp90 inhibition also occurs in the more complex system of proteins that are normally involved in protein refolding. 17-AAG was used as a control.^{32, 33} Addition of SM145 to the pure protein system or to the RRL and subsequent measurement of luciferase activity showed that SM145 decreased luciferase activity over time, indicating a decrease in hsp90 function. In the pure protein assay refolding of luciferase was decreased to 55% of control after 2 hours, where as in the RRL luciferase refolding was reduced to 60% compared to control. (Figure 3). 17-AAG and CA1 also reduce the luciferase activity over time (Figure 3, Supplemental Figure S3). These data support our previously published evidence and show that SM145 inhibits hsp90's ability to fold proteins.^{29–31}

The disruption of hsp90 function has had clinical success, however, a hallmark of all current hsp90 inhibitors in clinical trials is that they bind to the N-terminal ATP-binding site and produce a rapid HSR. By inhibiting hsp90, these molecules promote the over-expression of

all the hsp related proteins including pro-survival protein hsp70, hsp90, and HSF1 which facilitate cell rescue.^{34, 35} The effect of C-terminal hsp90 modulators on inducing the HSR is not well established. In order to understand the impact of all 3 inhibitor classes (N, N-Middle, and C) on the HSR, the protein levels of these HSR elements were evaluated in HeLa (cervical carcinoma cell line) cell lysate after treatment with the IC₅₀ and ten-fold over the IC₅₀ values of 17-AAG, CA1 or SM145.³⁶

In HeLa cells, we observed that treatment with 17-AAG (100nM and 1μM, bars 2 and 3 respectively, Figure 4) increased protein levels of hsp70, hsp90, and HSF1 (~2.5, 3 and 4-fold increase respectively). These results are consistent with data generated by others and they show that 17-AAG is triggering the heat shock response.^{24, 25} When HeLa cells were treated with the C-terminal inhibitor CA1, (5μM and 50μM, bars 4 and 5 respectively, Figure 4) in contrast to 17-AAG, the HSR was not triggered. These data show the maintenance of hsp70 protein levels, and a decrease in the protein levels of hsp90 and HSF1 (~50% and 50% compared to DMSO respectively). Our data are consistent with previous reports of other C-terminal inhibitors that do not cause the heat shock response.³⁶ Treatment with SM145 (5μM and 50μM, bars 6 and 7 respectively, Figure 4) yields a decrease in hsp90 and HSF1 levels to ~50% of control, and causes almost no change in the hsp70 levels. Our data show that SM145 does not trigger the HSR. This is exciting because SM145 avoids the heat shock response, which is associated with cell survival and drug resistance. This indicates that it can overcome the main resistance mechanism associated with the current clinical hsp90 inhibitors. The lack of induction of the heat shock response indicates that SM145 is acting via a unique mechanism from that of 17-AAG.

To investigate this mechanism of action we explored the binding event between hsp90 and its co-chaperones. Previously we reported that SM145 inhibits the binding of three carboxy-terminal interacting co-chaperones while 17-AAG does not.³¹ Two of these (HOP and FKBP52) contain a TPR motif. In this report, we show that SM145 inhibits the ability of hsp90 to bind to all the TPR-containing co-chaperones tested (Figure 5a). These TPR containing co-chaperones are essential members of the hsp90 chaperone complex, and each plays an important role in protein folding and maturation. Briefly: HOP is an organizing protein responsible for bringing hsp70 and hsp90 together to facilitate protein transfer;²³ Unc45 is a molecular chaperone for myosin, and also regulates the progesterone receptor pathway;^{37, 38} CHIP is an E3 ligase that causes the selective ubiquitination of proteins including the hormone receptors;³⁹ TOM70 is a mitochondrial import receptor essential for transferring pre-proteins to hsp90;^{39, 40} Cyp40, FKBP51 and FKBP52 are immunophilins that bind cyclosporine and FK506 respectively and are essential players in the hsp90 multi-protein complex leading to mature hormone receptors.²³ Disrupting the interaction between these proteins and hsp90 will halt the proper folding and maturation of many proteins, including the hormone receptors.

The binding of these co-chaperones with hsp90 was evaluated by combining pure native hsp90 protein with pure co-chaperones, and adding increasing amounts of compound (detailed in materials and methods section).^{31, 41} Indeed, six of seven TPR-containing proteins are inhibited by 0.5–1 μM of SM145, which is below SM145's IC₅₀ value. By comparison, 17-AAG only partially inhibits FKBP51 and TOM70 at 5μM (Figure 5b),

despite 17-AAG's IC₅₀ being ~100nM. This lack of inhibition is likely because 17-AAG binds at the N-terminus and has no impact on the structure of the C-domain. CA1 is more effective than 17-AAG, inhibiting CHIP, TOM70 and Cyp40 at 10μM (Figure 5c) but not as effective as SM145.³¹ Furthermore, CA1 has no impact on the binding between hsp90 and FKBP51, FKBP52, Unc45 and HOP. The TPR domain of each co-chaperones is different and requires interactions with sites on hsp90 in addition to the MEEVD region.²³⁴² By binding to the C-terminus CA1 likely blocks some of these regions, but leaves some available. This may account for the variable binding inhibition. Thus, SM145 is the first hsp90 inhibitor that controls binding between hsp90 and all TPR-containing proteins, likely by altering the C-domain in a way that it becomes less accessible to all the TPR domains.

The cellular effects of the *in vitro* inhibition of these TPR proteins by SM145 were evaluated by examining associated co-chaperone protein levels in treated cell lysates. We examined the protein levels of two immunophilins that are closely associated with hormone receptor expression FKBP51 and FKBP52.⁴³⁻⁴⁵ We found decreased protein levels of both FKBP52 and FKBP51 (60% and 20% of control levels respectively) occurred upon treatment with SM145 (bars 6 and 7, Figure 6). This correlates with the inhibition of hsp90 binding to these proteins in the *in vitro* binding assay (Figure 5). However, treatment of HeLa cells with 17-AAG (lane 2 and 3, Figure 6) showed ~4-fold and ~2-fold increase of FKBP52 and FKBP51 protein levels respectively. Although these data may appear contradictory to the binding assay data in Figure 5, this increase is likely due to the dramatic induction of the HSR. Although there is a decrease in binding affinity between hsp90 and FKBP51 when 17-AAG is present, the HSR causes large levels of hsp90 induction. The large quantities of hsp90 protein now available are able to interact with FKBP51 and prevent its degradation.⁶ Treatment of cells with CA1 (lanes 4 and 5, Figure 6) caused little change in the protein levels of FKBP51 and slightly decreases in FKBP52 at high concentrations relative to control, consistent with the binding assay data. These protein level data support the binding assay data (Figure 5), whereby SM145 inhibits binding of FKBP52 and FKBP51 to hsp90, which then leads to the degradation of these co-chaperones.

HeLa cells were also evaluated for their GR levels upon treatment with 17-AAG, CA1, and SM145. Treatment of cells with 17-AAG resulted in no change to the GR consistent with previous reports⁴⁶ (bars 2 and 3, Figure 6). Treatment with CA1 resulted in a slight increase in GR protein level ~1.3-fold only at high concentrations (bar 5, Figure 6). In contrast, treatment with SM145 resulted in a dramatic reduction of GR to only 10% of control levels. These data are consistent with the depletion of both FKBP52 and FKBP51 protein levels after treatment with SM145. Further, these data strongly support the hypothesis that SM145 regulates HR protein levels through an hsp90 inhibition event, via the immunophilins.

In conclusion, we present evidence that SM145 inhibits hsp90's ability to re-fold proteins, inhibits hsp90's interaction with TPR-containing immunophilins and homologs, and causes depletion of these immunophilins and associated HRs. Through protein level quantification of treated cell lysates we demonstrate that SM145, unlike 17-AAG, induces an anti-proliferative effect without inducing the drug resistance-associated heat shock response. Through pure protein assays we prove that SM145 inhibits the direct binding event between the TPR-domain and MEEVD region of the immunophilins/ homologs and hsp90

respectively. Finally, we establish that via hsp90 regulation, SM145 controls protein levels of FKBP51, FKBP52, the GR, and the AR through a proteasome independent pathway. This is the first molecule to inhibit hsp90, not induce the heat shock response, and yet subsequently impact the immunophilins and hormone receptors. Overall, the described mechanistic characteristics of SM145 make this compound a unique hsp90 inhibitor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DMSO	dimethyl sulfoxide
IC₅₀	inhibitory concentration (50%)

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Figure 1. Hsp90 cartoon depiction

The hsp90 dimer indicating where 17-AAG, coumermycin A1, SM145, and various co-chaperones important in hormone receptor development bind. (M= methionine, E= glutamic acid, V= valine, D= aspartic acid)

Figure 2. Structure of hsp90 inhibitors

SM145, 17-AAG (amino-terminal hsp90 inhibitor), NVP-AUY922 (resocinoic acid containing amino-terminal hsp90 inhibitor) and coumermycin A1 (carboxy-terminal hsp90 inhibitor).

Figure 3. Firefly luciferase refolding assay

Treatments are 17-AAG 1 μ M and SM145 50 μ M. a) Refolding in rabbit reticulocyte lysate b) Refolding in pure protein system containing 1 μ M hsp70, 0.2 μ M hsp40, 0.1 μ M HOP and 0.5 μ M hsp90. (n=3, graph mean $\pm$ SEM, compound controls appear Supplemental Figure S1 & S2)

Figure 4. HSR protein level analysis

HeLa cell lysates were analyzed for levels of HSR proteins. a) hsp70 b) hsp90 and c) HSF1. All western blots appear in Supplemental Figure S4. Bands were quantitated with image j software, normalized to GAPDH levels and to DMSO control (n = 3, graph mean±SEM).

Figure 5. TPR co-chaperone and hsp90 binding assay

The binding affinity of TPR containing proteins (Unc45, CHIP, TOM70, HOP, Cyp40, FKBP52 and FKBP51) for hsp90 was evaluated in the presence of increasing amounts of: a) 17-AAG (0–5μM) b) coumermycin A1 (0–10μM) and c) SM145 (0–10μM). (note: * previously published³¹)

Figure 6. Immunophilins and HR protein level analysis

HeLa cell lysates were analyzed for protein levels of HR complex proteins after treatment with 17-AAG (100nM and 1μM), CA1 (5μM and 50μM) and SM145 (5μM and 50μM): a) FKBP51, b) FKBP52, c) glucocorticoid receptor (GR). All western blots appear in Supplemental Figure S5. Bands were quantitated with image j software, normalized to GAPDH levels and compared to the DMSO control. (n = 3, graph of mean ± SEM)