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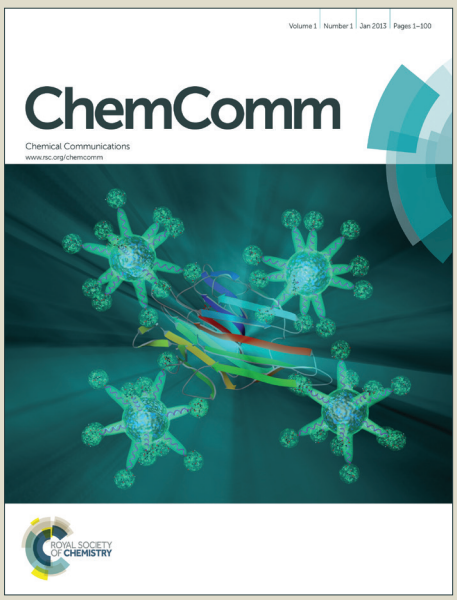

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N-terminal and C-terminal modulation of Hsp90 produce dissimilar phenotypes

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Classic oncogenic heat shock protein 90 (Hsp90) inhibitors target the N-terminus of the protein, triggering a survival mechanism in cancer cells referred to as the heat shock response (HSR). Inhibiting Hsp90 by modulating the C-terminus does not trigger a HSR, making it a highly attractive chemotherapeutic approach.

The heat shock protein 90 (Hsp90) is an ideal therapeutic target for cancer therapy as it plays a key role in controlling over 400 client proteins and co-chaperones, most of which are involved in growth-related signaling events.[1] Up-regulation of Hsp90 is triggered in cancer cells in order to control the abundance of mutated and mis-folded proteins that accumulate, thereby making tumors significantly more dependent upon Hsp90's chaperone activity than normal tissues.[2] The classical Hsp90 inhibitor targets the ATP binding site located at the N-terminus, and there are over 50 clinical trials testing 15 different N-terminal Hsp90 inhibitors (ClinicalTrials.gov database). However, only 4 compounds are still active in the clinic,[3] and only in combination therapies (ClinicalTrials.gov database).[4-6]

The induction of the heat shock response (HSR) is a well-established compensatory mechanism that counteracts the effects of stress on a cell. The HSR is a cellular stress response that initiates the transcription of mRNAs that encode multiple heat shock proteins (HSPs), specifically Hsp70 and Hsp27.[7] These two HSPs facilitate protein folding, support tumor growth, and prevent apoptosis, thereby facilitating cancer cell growth.[8, 9]

All clinical Hsp90 inhibitors target the ATP-binding site of Hsp90, and trigger high levels of Hsp70, which are typically used as a phenotypic marker for identifying Hsp90 inhibitors.[10-13] Indeed, patients treated with inhibitors targeting Hsp90's N-terminus display significantly elevated expression levels of HSPs (predominantly Hsp70).[14-16] This inherent increase of HSPs has driven researchers to find alternative molecules that repress Hsp90 activity without inducing this cytoprotective mechanism.

Herein we evaluated how two different chemical inhibitors of Hsp90, an N-terminal Hsp90 modulator (17-AAG) and a C-terminal Hsp90 modulator (SM122)[17-22] regulated the heat shock process. Evaluation of the compounds' impact on i) Hsp90's ability to fold proteins, ii) the mRNA transcriptional levels of HSPs and the iii) protein expression levels of HSPs allowed us to definitely prove that these two inhibitors produce unique cellular phenotypes (Figure 1).

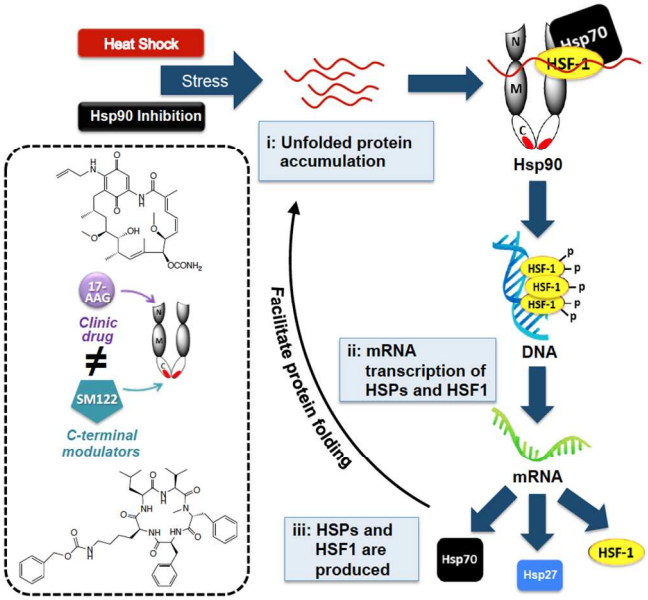

Figure 1. Depiction of the widely proposed model of heat shock- and Hsp90 inhibition-induced HSR. The heat shock-induced HSR is initiated by accumulation of unfolded proteins, which compete with HSF-1 for binding to Hsp90 causing the disassembly of HSF-1-Hsp90 protein complex. Released HSF-1 trimerizes and translocates into the nucleus, binds to DNA, and initiates the transcription of mRNAs encoding for Hsp90, Hsp70, and Hsp27 proteins, which are then synthesized and used to fold the aggregated and stressed proteins.

Although 17-AAG and SM122 have very different growth inhibitory values against HCT116 colon cancer cells (GI_{50} = ~ 50 nM and 8 μM for 17-AAG and SM122 respectively) comparison of 17-AAG treatment versus SM122 treatment on the production of unfolded proteins produced surprising results. HSR induction is well know to produce high levels of unfolded

or aggregated proteins,[23] as is treatment of cells with 17-AAG. Comparison of how effectively 17-AAG and SM122 inhibited protein folding, and produced aggregated proteins was accomplished using a rabbit reticulocyte lysate (RRL)-based luciferase-refolding assay. Using an Hsp90-dependent refolding system in the RRL to re-nature the heat-denatured firefly luciferase protein provides a luciferase-based readout of how effectively Hsp90 is in folding proteins.[24, 25] Renaturation of firefly luciferase is prevented by 17-AAG and SM122 at IC_{50} values of 1.90 and 2.39 μM, respectively (Figure 2). Thus, both inhibitors have similar effects on suppressing the Hsp90-dependent protein folding machinery, thereby inducing the mis-folded or unfolded protein accumulation similar to a HS event.

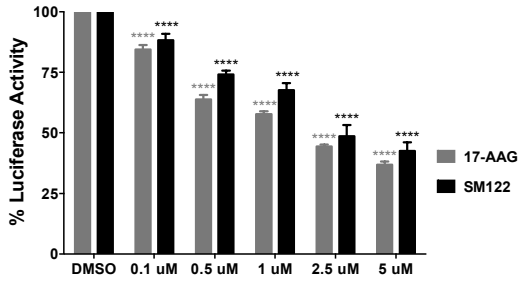

Figure 2. The impact of Hsp90 inhibitors 17-AAG and SM122 on Hsp90 chaperone function leading to unfolded protein accumulation (Figure 1, i). The results of denatured luciferase refolding assay in rabbit reticulocyte lysate (RRL) system were shown. 17-AAG and SM122 showed potent suppressive impact on Hsp90 chaperone function in protein-folding. All experiments were performed in triplicate, and representative results are shown. All values are average ± s.e.m. from three independent experiments. Differences between drug treatments and DMSO treatment are represented with *P* values (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; and ****, $P < 0.001$).

The impact on mRNA transcription of HSPs (including heat shock inducible Hsp70, consitutively expressed Hsc70, and Hsp27) when treating cells with heat shock (HS) versus 17-AAG or SM122 was then evaluated in HCT116 human colon cancer cells. (Note: since Hsp90 behaves similarly in all cancer cells, the specific cancer cell type does not impact the study). Heat-inducible Hsp70, constitutively expressed Hsc70, and Hsp27 HCT116 cells were treated with HS for 60 minutes at 45°C and subsequently incubated at 37°C for responding to the HS treatment.

In parallel, treatment regiments of 17-AAG were administered at 250 nM, 8 fold below 17-AAG's IC_{50} for modulating protein folding (2 μM), which was at the cytotoxicity threshold for the cells (17-AAG GI_{50} = 50 nM). SM122 was administered at 4 fold over its IC_{50} for Hsp90, (but just over its GI_{50} = 8 μM).[19, 26] It is important to note that 17-AAG's IC_{50}, like the other clinical candidates termed "Hsp90 inhibitors" actually modulates Hsp90 with an IC_{50} = 1.9 μM (Figure 2) but kills cells at 50 nM, which suggests it has multiple off target effects. In contrast SM122 has a similar GI_{50} and IC_{50} of binding to Hsp90. Treatments of HS, 17-AAG, and SM122 induced different mRNA transcriptional levels of both inducible Hsp70 (*HSPA1A*) and constitutive expressed Hsc70 (*HSPA8*) mRNA (Figure 3). Specifically for the inducible Hsp70 gene (*HSPA1A)*, HS treatment produced a maximum mRNA increase of 10 fold over the control level (0 time point), whereas treatment with 17-AAG produced ~ 70 fold increase in the mRNA expression over the control (0 time point). Such an increase was time dependent, and dropped back to the control level 24 hours after drug treatment. The C-terminal modulator SM122, however, dramatically decreased the mRNA level of *HSPA1A* in HCT116 cells even at early time points (2 hours after the treatment), and repressed gene expression at low levels throughout the 24 hr-monitoring period. These results show that although SM122 inhibits Hsp90 function, it does not trigger the production of the inducible Hsp70; instead, SM122 effectively suppresses its expression at the mRNA transcriptional level.

The mRNA encoding *HSPA8* was overexpressed during the first 12 hours of 17-AAG treatment, and then dropped back to the control levels after 24 hours. Conversely, *HSPA8* expression was intensively down-regulated by SM122 throughout the 24 h-treatment. These data clearly show that 17-AAG and SM122 have unique phenotypes in regulating both inducible and constitutive Hsp70s at transcriptional levels. Moreover, evaluation of mRNA levels of *HSPB1* (encoding for Hsp27) showed that both HS and 17-AAG up-regulated the Hsp27 expression at mRNA transcriptional level in HCT116 cells. However, similar to *HSPA1A* and *HSPA8*, treatment with SM122 did not induce an increase in *HSPB1* expression, but rather treated cells showed an immediate (starting at the 6 h-time point) and significant decrease (~ 2 fold after the 6 h-time point) relative to the control. Together, these data demonstrate that 17-AAG produces a phenotype similar to the heat shock treatment but distinct from treatment with the C-terminal modulator.

a) mRNA expression levels of *HSPA1A* gene encoding for inducible Hsp70

b) mRNA expression levels of *HSPA8* gene encoding for consitutively expressed Hsp70

c) mRNA expression levels of *HSPB1* gene encoding for Hsp27

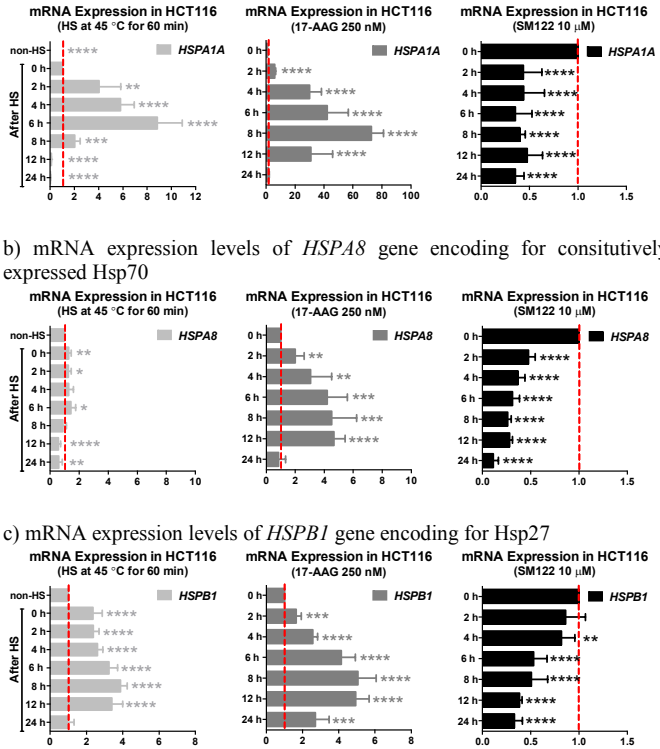

Figure 3. mRNA expression levels of genes encoding for (a) heat-inducible Hsp70 (*HSPA1A*), (b) constitutively expressed Hsp70 (*HSPA8*), and (c) Hsp27 (*HSPB1*) over multiple time points in HCT116 cells treated with DMSO (control), versus HS versus HS at 45 °C for 60 minutes, or 17-AAG, or SM122 for 24 hours. Values are presented as fold change in mRNA expression relative to the indicated controls. Data are average ± s.e.m. from three independent experiments. Differences between treatments and indicated control treatment are represented with *P* values (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; and ****, $P < 0.001$).

A commonly accepted pharmacodynamic marker of Hsp90 inhibition is the induction of Hsp70 protein expression in treated cancer cells.[27] A common assumption is that high levels of Hsp70 protein expression indicate Hsp90 inhibition. Consistent with this expectation, treatment of HCT116 cells with varying concentrations of 17-AAG (100-1000 nM) for 24 hours significantly induced high protein expression levels of HSF-1 and multiple HSPs including Hsp70 and Hsp27 (3-5 fold increase over the untreated control), but

showed no impact on Hsp90 protein expression (Figure 4a). Even at low doses, 17-AAG (250 nM) produced high protein levels of HSPs. In contrast to 17-AAG, treatment with the C-terminal Hsp90 modulator SM122 (5-50 μM) significantly repressed the protein expression of HSF-1, Hsp70, and Hsp27 (up to 4 fold decrease over the untreated control), with a small impact on Hsp90 protein expression (Figure 4b).

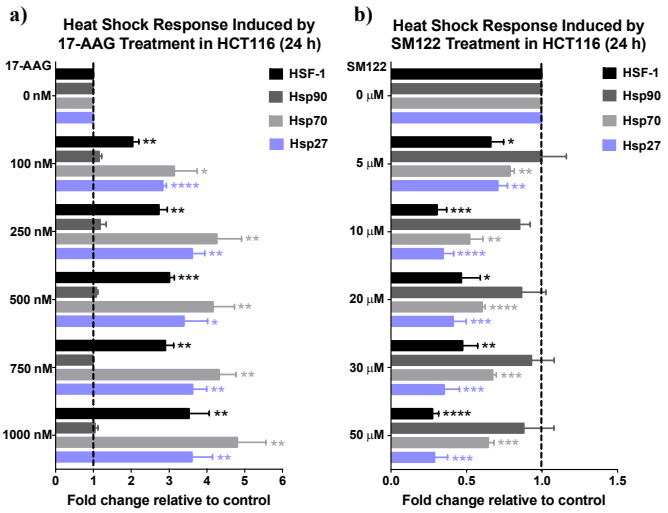

Figure 4. Immunoblot for the proteins of interest in the lysate of HCT116 cells treated over 24 hours with the HS, DMSO (control, shown as 0 μM or 0 nM of indicated inhibitor), 17-AAG, and SM122 at indicated conditions. (a) The quantitative bar graph for immunoblot results in 17-AAG treatments. (b) The quantitative bar graph for immunoblot results in SM122 treatments. Actin was used as the protein loading control. Experiments were performed in triplicate, and representative results are shown in western blots. Values are presented as fold change in specific treatment relative to the controls. Data are represented as average ± s.e.m. from three independent experiments. Differences between drug treatments and indicated control treatment are represented with *P* values (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; and ****, $P < 0.001$).

As seen by others, HS treatment of HCT116 cells (45°C for 60 minutes) strongly elevated the expression of cytoprotective proteins HSF-1 (2-3 fold), Hsp70 (3.5-7.5 fold), and Hsp27 (2-4 fold) during the 24 h-response period after the HS (Supplementary data S2). Treatment with 250 nM of 17-AAG treatment and then examination of HSF-1, Hsp70, and Hsp27 protein expression levels over 24hrs demonstrated that both HS and 17-AAG treatments caused significantly increased levels of all three proteins (~ 4 fold) (Figure 5a). In contrast, treatment of HCT116 cells with 10 μM of SM122 resulted in a significant decrease (~ 4 fold) in protein expression of HSF-1, Hsp70, and Hsp27 over 24 hours (Figure 5b).

In summary, the C-terminal modulator SM122 shows significant repressive effects on the expression of HSP proteins in treated cancer cells, which is distinct from the effects observed with both HS and 17-AAG treatments. Thus, inhibiting or modulating Hsp90 activity does not inevitably lead to an induction of Hsp70 or Hsp27 proteins. Indeed, our data provides solid evidence that the regulatory effect of Hsp90 inhibition on HSF-1 and HSPs (including Hsp70 and Hsp27) at both mRNA expression levels (transcription) and protein expression levels is dependent upon the specific mechanism by which Hsp90 is inhibited (i.e. N versus C-terminal modulation).

Small molecules that modulate the N-terminal site produce distinctly different phenotypes than those that modulate the C-terminus. Although 17-AAG and SM122 have different growth inhibitory values (GI_{50} = ~ 50 nM and 8 μM for 17-AAG and SM122 respectively), both 17-AAG and SM122 effectively inhibit Hsp90's chaperone function in protein folding with approximately the same IC_{50} of ~ 2 μM (Figure 2). However, these two inhibitors exhibit opposite regulatory effects on mRNA transcription and protein expression levels. Where an increase in ~ 70 fold in mRNA expression of the inducible Hsp70 gene (*HSPA1A)* is seen when cells are treated with 250 nM (8 fold below 17-AAG's Hsp90 binding affinity ~ 2 μM); while the same gene expression is decreased by ~ 2 fold when treated with 10 μM, which is 5 fold over above SM122's binding affinity for Hsp90 (~ 2 μM).

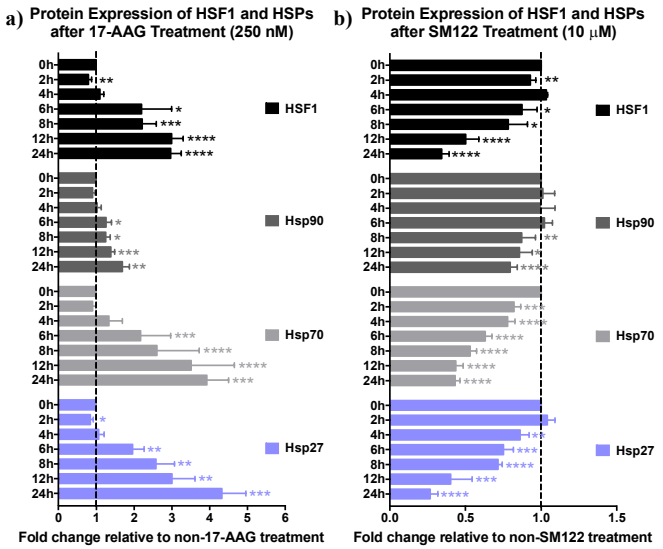

Figure 5 (a) Cells were treated with 250 nM of 17-AAG and the expression levels of indicated proteins were monitored over 24 hours. (b) Cells treated with 10 μM of SM122 and the expression levels of indicated proteins were monitored over 24 hours. Note: The expression levels of indicated proteins in the cells heated at 45°C for 60 minutes as the heat shock control are located in the supplementary data as a comparision. Experiments were performed in triplicate, and representative results are shown in western blots. Values are presented as fold change in specific treatment relative to the controls. Data are represented as average ± s.e.m. from three independent experiments. Actin was used as the protein loading control. Differences between drug treatments and indicated control treatment are represented with *P* values (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; and ****, $P < 0.001$).

Finally, treatment with 8 fold below 17-AAG's IC_{50} for Hsp90 (250 nM) still produces significant increases in HSF-1, Hsp70, and Hsp27 protein levels. In contrast, SM122 (10 μM) is used at 5 fold over its IC_{50} for controlling Hsp90 function and yet it reduces mRNA levels and protein levels of HSF-1, Hsp70, and Hsp27. Thus, it is evident that SM122 has a distinctly different modulation effect on Hsp90 than 17-AAG, and our data support investigation of C-terminal inhibitors of Hsp90 as potential chemotherapeutics. It is likely that this new class of inhibitors will have a very different clinical outcome than the N-terminal inhibitors.

Notes and references

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Electronic Supplementary Information (ESI) available: [details of all experimental methods, and raw data are included in the supplementary]. See DOI: 10.1039/c000000x/

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