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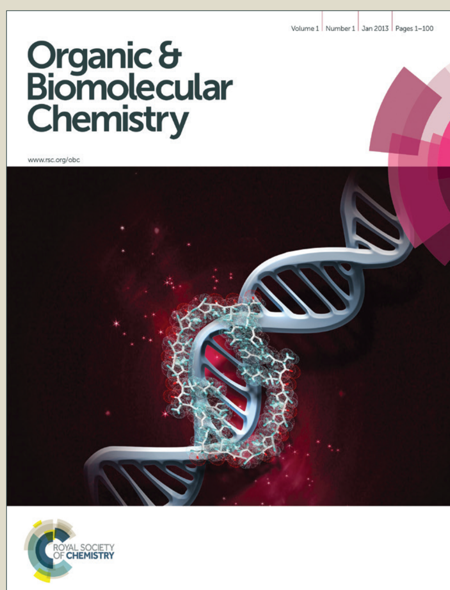

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PERSPECTIVE

C-terminal heat shock protein 90 modulators produce desirable oncogenic properties

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The cellular protection mechanism, the heat shock response, is only activated by classical heat shock 90 inhibitors (Hsp90) that "target" the N-terminus of the protein, but not by those that modulate the C-terminus. Significant differences in cytotoxicity (nanomolar) for classical inhibitors versus their ability to modulate Hsp90 (low micomolar) are discussed. Incontrast, molecules that modulate Hsp90's C-terminus show similar IC_{50} values for cytotoxicity and hsp90 inhibition. A comparison between the two types of Hsp90 inhibitors suggests that classical inhibitors may be modulating an alternative biological target that stresses the cell rather directly inhibiting Hsp90, whereas C-terminal modulators are most likely acting by directly inhibiting Hsp90.

Introduction

Exposure to environmental and temperature challenges has produced an evolutionary reaction referred to as the heat shock response (HSR).[1] The HSR is usually activated when the cell has prolonged exposure to temperature or rapid growth stress. Stress produces unfolded, misfolded, and aggregated proteins, which trigger high expression levels of protein-folding chaperones, or heat shock proteins (HSPs, Figure 1, A). Thus, detection of high levels of HSPs is often referred to as a HSR regardless of whether temperature, chemical treatments or prolonged cellular trauma caused the HSP increases.[2] Both in temperature and chemically induced stress, the high levels of HSPs are proposed to refold the aggregated and misfolded proteins that accumulate in the stressed cell, and aid in protein degradation, thereby rescuing the cell from apoptosis.[3]

The HSR mechanism is proposed to involve release of HSF-1 from Hsp90 (Figure 1, B) under cellular stress.[4-8] Transport of HSF-1 from the nucleus into the cytoplasm is inhibited, leading to a build-up of HSF-1 in the nucleus.[9] The HSF-1 trimer is phosphorylated form of HSF-1,[10] whereupon it binds to specific sequence of DNA and induces transcription of genes encoding for itself and multiple cellular chaperones, including heat shock protein 70 (Hsp70) and heat shock protein 27 (Hsp27). In the absence of stress, promoters for these genes are occupied and unavailable.[11, 12] However, under stressed conditions, the promoters for Hsp27 and Hsp70 become available (Figure 1, C).[13, 14] The mRNAs encoding for inducible and constitutive Hsp70 are produced (*HSPA1A* and *HSPA8*, respectively), as well as mRNA that encodes for HSF-1 and Hsp27 (Figure 1, C). These mRNAs are then translated into proteins (HSPs, Figure 1, D). Together these induced proteins attempt to rescue the cell from the accumulation of unfolded protein by either facilitating their degradation, or refolding them as salvageable proteins.

Over the past 10 years, Hsp90 has been investigated as an oncogenic target for treating cancer. There have been over 50 clinical trials (ClinicalTrials.gov database)[15-17] during this time, and 15 structurally distinct Hsp90 inhibitors were tested as clinical candidates against numerous types of cancer. All the clinical candidates, termed "classical inhibitors", act by the same mechanism of action, and bind to Hsp90's ATP-binding site at its N-terminus, thereby blocking ATP from interacting with Hsp90 (Figure 1). One significant issue for all classical Hsp90 inhibitors is that they induce high levels of HSPs. Induction of these HSPs using chemical moieties are assumed to protect the cell in a manner similar to when they are produced during environmental and prolonged stress conditions. Indeed, the high levels of HSPs produced when inhibiting the ATP-Hsp90 binding interaction is a possible reason that the clinical trials involving Hsp90 inhibitors have produced disappointing results.

The cytoprotective response triggered by treating tumours with classical Hsp90 inhibitors has been termed a "HSR" because treatment of numerous cancer types these inhibitors induce a large increase in Hsp70 and Hsp27 protein levels (5-10 fold over background).

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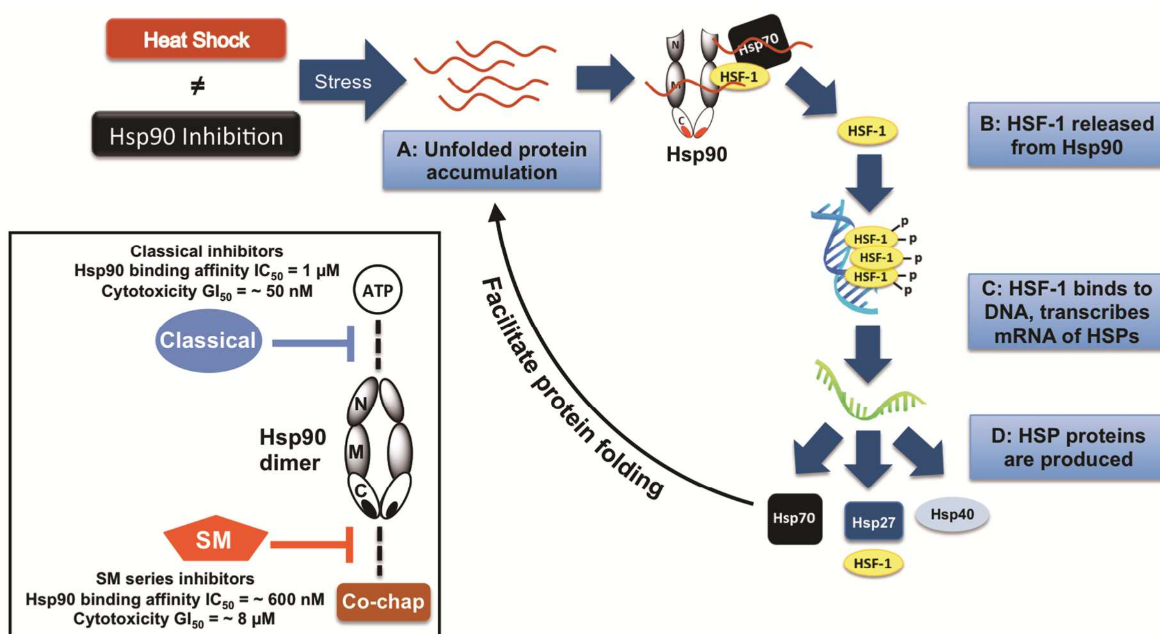

Figure 1: Depiction of the widely accepted model of the induction of the heat shock response (HSR), and the mechanisms of Hsp90 inhibition by different classes of inhibitors.

However, in this perspective, we highlight our recent work that shows modulating Hsp90 using classical inhibitors produces a phenotype that is unique from one induced under high temperature or prolonged stress conditions. [18] Furthermore, we summarize our recent developments towards modulating the C-terminus of Hsp90, which we have demonstrated does not produce an increase in HSPs at both translational and transcriptional levels.[18-23]

Impact of classical Hsp90 inhibitor-induced versus temperature-induced stress on HSPs' mRNA production

It is unclear what role classical inhibitors play in activating such high levels of HSPs, and thus we set out to explore the mechanism by which this occurred. Our recent work demonstrated that both a classical inhibitor, 17-AAG, and a C-terminal modulator, SM122 (Figure 2), have identical impacts on the ability of Hsp90 to fold protein with an inhibitory concentration required to block 50 % of the protein folding effect (IC_{50} = 1.9 μM and 2.4 μM respectively).[18] Yet, as noted in Figure 1, the growth inhibitory concentration required to inhibit 50 % of cancer cells to grow (GI_{50}) of most classical inhibitors are at low nanomolar range, while SM inhibitors are at low micromolar levels. These data clearly indicated that the classical inhibitors are not acting via modulation of only Hsp90, because their IC_{50} of direct Hsp90 inhibition (regardless of the assay i.e. cellular or biochemical) ranges from 1-3 μM, while these classical inhibitors inhibit cancer cell growth at low nanomolar level. Specifically pure protein binding assays showed that 17-AAG binds to Hsp90 with IC_{50} = ~ 1 μM, and surface plasma resonance (SPR) indicated that it binds to Hsp90 with an IC_{50} = 10 μM.[24] Clearly these classical inhibitors although appearing to target Hsp90, must act primarily via another mechanism. Comparison of the impacts caused by classical inhibitors, SM compounds, and the heat shock (HS) treatment on multiple mechanistic steps in the HSR induction process provides more evidence supporting this hypothesis (Figure 1).

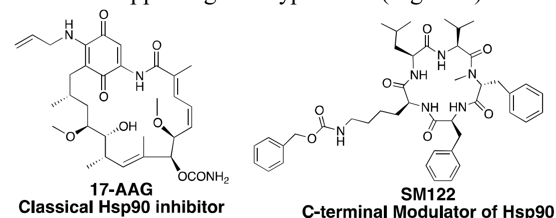

Figure 2: Chemical structures of 17-AAG and SM122.

Comparing how HSPs' mRNA production (Figure 1, C) is impacted in the cancer cells treated with the classical inhibitor17-AAG versus HS provides evidence that these two treatments induce two phenotypes that are distinct from each other. We showed that when HCT116 cells were incubated under HS conditions (45°C) for 60 min, they intensively increased *HSPA1A* (encodes for the inducible form of Hsp70) mRNA level by more than 20000 fold over the non-HS control (Figure 3, A) after a 6 h-recovery period. However, there was essentially no change in *HSPA8* (encodes for constitutively expressed Hsp70) mRNA expression. In contrast, 17-AAG with high concentration (250 nM) only caused a maximum ~ 70 fold increase in *HSPA1A* mRNA level over 24 hrs (Figure 3, A) compared with non-treated control. In addition, very different from HS, 250 nM of 17-AAG raised the mRNA expression level of *HSPA8* gene up to 6 fold. Thus, despite the ineffectiveness of 17-AAG to inhibit Hsp90's function at 250 nM, the mRNA levels of both two Hsp70 isoforms are being escalated significantly, which is distinct from the impact of HS treatment. These data strongly support the hypothesis that 17-AAG is not triggering a heat

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shock response, as defined by Morimoto and co-workers.[1] Rather, 17-AAG is producing a cytotoxic effect that is unrelated to inhibiting Hsp90, but is activating protection mechanisms in cancer cells.

Impact of classical inhibitors versus C-terminal modulators (SM series) on HSPs' mRNA production

Comparison between the phenotypes observed when treating HCT116 cells with 17-AAG versus SM122 shows distinct differences. Treatment with 10 µM of SM122 (5 fold over the concentration required to inhibit Hsp90) produced a decrease in mRNA expression levels of both inducible and constitutive Hsp70 (a decrease by 2.5 fold and 10 fold relative to controls respectively, Figure 3, A). These data support the hypothesis that the SM class of molecules, and perhaps all C-terminal inhibitors of Hsp90 produce a phenotype that does not activate a HSR. Despite inhibiting Hsp90 using high concentrations of SM122, there was no cytoprotective HSR induced. SM inhibitors' effect on the HSPs' mRNA expression was consistent with a molecule that inhibits Hsp90's function without stressing cancer cells or inducing a HSR as a rescue response. Furthermore, in contrast to 17-AAG, the SM molecules produced this effect at concentrations that significantly impact Hsp90's function.

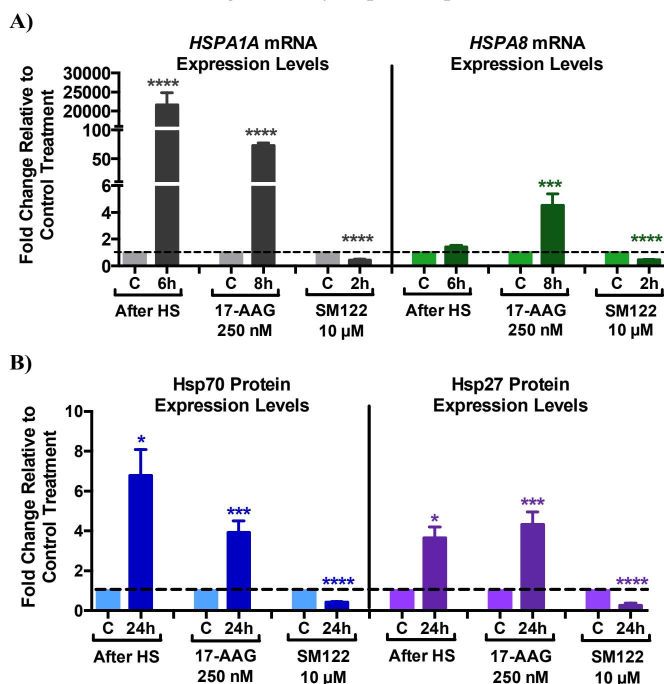

Figure 3: A) mRNA expression levels of inducible and constitutive Hsp70 in HCT116 cells treated by heat shock (60 min at 45 °C), 17-AAG (250 nM), or SM122 (10 µM). B) Protein expression levels of Hsp70 and Hsp27 in HCT116 cells treated by heat shock (60 min at 45 °C), 17-AAG (250 nM), or SM122 (10 µM). "C" represents non-treated control.

Impact of classical Hsp90 inhibitor-induced versus temperature-induced stress on HSPs' protein production

The disappointing clinical results observed when using classical inhibitors has led to most companies dropping their Hsp90 programs, and no inhibitors have yet been approved by the FDA. Our work supports the hypothesis that failure in the clinical may be related to the high levels of Hsp70 and Hsp27 proteins induced in cancer cells upon treatments. Discovery that Hsp70 and Hsp27 proteins are reverse prognostic prediction factor in patients is a critical fact that has not yet been assimilated into the Hsp90 community. Critically, when these chaperones have been induced, tumor cells maintain their growth patterns and remain healthy and robust.[25-27] Supporting these facts, we demonstrated that treatment of HCT116 cells with heat shock produced an increase in Hsp70 and Hsp27 protein expression up to ~ 7.5 fold over non-treated cells (Figure 3, B).[18] Similarly, treatment with 250 nM of 17-AAG produced a 4-5 fold increase in both Hsp70 and Hsp27 protein levels. Given that 17-AAG is not modulating Hsp90 at this concentration, it is likely that 17-AAG is inducing a cytotoxic stress response unrelated to inhibiting Hsp90 function. Thus, the classical drug-induced phenotype appears to be similar to the cytoprotective HSR, and proposes a reason for these molecules' failure in the clinic. However, in contrast to others opinion, these molecules are not producing a cytoprotective response that is connected with Hsp90, but rather through an as yet unidentified stress-inducing mechanism.

Impact of classical inhibitors versus C-terminal modulators (SM series) on HSPs' protein production

Indeed, the current theory that all Hsp90 inhibitors produce the cytoprotective HSPs is contradicted by the data gathered by using our SM C-terminal modulators. Specifically, we evaluated Hsp70 and Hsp27 protein levels in HCT116 cells treated by SM122 with high concentrations (up to 50 µM). We observed that treatment with 50 µM of SM122, which is 25 fold over its binding affinity to Hsp90, produced a significant decrease in Hsp70 and Hsp27 protein levels. Indeed, Hsp70 and Hsp27 protein levels dropped by ~ 2 fold and 3 fold respectively relative to non-treated controls. Furthermore, monitoring the HSP protein expression over time showed that after 24 hrs, 10 µM of SM122 produced a drop of Hsp70 and Hsp27 levels to 2 fold and 3 fold over controls. In contrast, 250 nM of 17-AAG produced a progressive increase in Hsp70 and Hsp27 expression with ~ 4 fold over controls. Thus, despite using low concentrations of 17-AAG, which do not inhibit Hsp90 function, 17-AAG still induces a large HSP induction. Since the 250 nM concentration does not reach the amount required to inhibit Hsp90, classical inhibitors are likely acting via a yet undiscovered mechanism to kill cancer cells.

Several recent reports support our hypothesis, where Vielhaurer shows that another class of compounds that modulate the C-terminus also decrease Hsp70 protein levels.[28] A recent study by Butler[29] found that 17-AAG behaves very differently *ex vivo* than two other classic Hsp90 inhibitors, specifically AUY922 and HSP990, despite both compounds targeting the ATP-binding site of Hsp90. Similar to 17-AAG, both of these molecules induced high levels of the cell-protecting protein, Hsp70. However, Butler also found that despite all three molecules inducing high expression levels of Hsp70 and Hsp27, AUY992 and HSP990 both triggered a biologic response in *ex*

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vivo-cultured tumors, whereas 17-AAG did not. Butler's conclusion was that the HSP accumulation was not a reasonable indicator of successful Hsp90 inhibition. Importantly, Butler concluded that the enhanced potency of AUY922 and HSP990 is likely a result of these compounds inhibiting other targets. Thus, markers other than high HSP protein expression levels should be considered when determining whether molecules are targeting Hsp90. Furthermore, these data indicated that as expected, the effects observed when targeting Hsp90 are dependent on the molecule used to modulate it's function, where even the two classical inhibitors function via distinct mechanisms.

Conclusions

Although the evaluation of heat shock on the production of HSPs, specifically Hsp70 and Hsp27, has already been extensively investigated,[1, 25-27, 30-33] the unique mechanism by which a C-terminal inhibitor acts, and the distinct difference between heat shock, classical Hsp90 inhibitors, and C-terminal modulators of Hsp90 was only recently documented in cells.[18] Indeed, in earlier reports several issues have been overlooked regarding classical inhibitors. First, they all maintain GI_{50} values of 10-100 nM depending on the cancer cell line or tumour source.[34] However, numerous data has proven that their binding affinity for Hsp90 protein ranges from 1-2 μM depending on the assay used.[19, 20, 24, 28, 35, 36] Classic inhibitors' potential promiscuity is also seen by its highly potent effect on cancer cells despite inhibiting the binding of only a few client proteins (e.g. HER-2 and Akt) to Hsp90 at high concentrations (IC_{50} = ~ 500 nM for binding assays).[20, 21, 35] That is, classical inhibitors do not block access to a large number of Hsp90 clients, but only a few, and at concentrations 10 fold over their ability to kill cancer cells. These data clearly indicate that classical inhibitors are killing cancer cells via alternative mechanisms. Finally, there is evidence showing that classical inhibitors block Hsp90's protein-folding function with an IC_{50} = ~ 2 μM.[18, 20, 21] Thus, clearly these classic inhibitors are killing cancer cells via other mechanisms rather via their modulation of Hsp90.

Our conclusion is that heat shock produces a distinct phenotype from that observed when treating cancer cells with a classical inhibitor, or when treating with a C-terminal modulator of Hsp90. Furthermore, "classical Hsp90 inhibitors" trigger a cytoprotective response, likely due to inducing a toxic effect on cancer cells via an unknown mechanism, rather than modulating Hsp90. Finally, the cytoprotective response induced by classical inhibitors may be responsible for their disappointing clinical trials. Targeting the C-terminus of Hsp90 appears to provide an alternative route for modulating this critical chaperone without inducing such protection mechanism in caner cells. Based on these conclusions, it would be optimal if strategies for modulating the C-terminus of Hsp90 were investigated in in-vivo studies.

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