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Hsp90, an unlikely ally in the war on cancer

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Abstract

On the surface heat shock protein 90 (Hsp90) is an unlikely drug target for the treatment of any disease, let alone cancer. Hsp90 is highly conserved and ubiquitously expressed in all cells. There are two major isoforms and encoded by distinct genes and together they may constitute 1%–3% of the cellular protein. Deletion of the protein is embryonic lethal and there are no recognized polymorphisms suggesting an association or causal relationship with any human disease. With respect to cancer, the proteins absence from two recent high profile articles, ‘Hallmarks of cancer: the next generation’ [Hanahan & Weinberg (2011) *Cell* **144**, 646–674] and ‘Comprehensive molecular portraits of human breast tumours’ [Koboldt *et al.* (2012) *Nature*] underlines the perception that it is an unlikely *bona fide* target to treat this disease. Yet, to date, there are 17 distinct Hsp90 inhibitors in clinical trials for multiple indications in cancer. The protein has been championed for over 20 years by the National Cancer Institute (Bethesda, MD, USA) as a cancer target since the discovery of the antitumor activity of the natural product geldanamycin. This review aims to look at the conundrum of why Hsp90 can even be considered a druggable target for the treatment of cancer. We propose that in contrast to the majority of chemotherapeutics our growing armamentarium of investigational Hsp90 drugs represents an elegant choice that offers real hope in the long-term treatment of certain cancers.

Keywords

cancer; chaperone; combination therapy; drug discovery; extracellular Hsp90; Hsp90; protein activation

Introduction

Undoubtedly, any protein or gene of interest that has relevance to cancer will receive superfluous recommendations to pursue it pharmacologically. Heat shock protein 90 (Hsp90) is no exception. With each new interaction and publication about Hsp90 there is augmenting enthusiasm to pursue clinical applications. The fact is that Hsp90 inhibitors are being pursued and advancing through clinical trials and their applications toward multiple cancers are expanding. Despite these successes, a few recent high profile articles on the mechanisms of cancer [1,2] have overlooked this already established therapeutic target, and it does not appear to be an anomaly with the authors of these cancer reviews. Cancer pharmacologists within our own institution are often blindsided by the clinical trial data or even the notion that one could target an abundant chaperone protein, such as Hsp90, that has no known disease-linked polymorphisms [3,4]. On the surface this seems reasonable: how could one pharmacologically target a protein that is essential for normal cell viability and

has a documented interactome that involves over 400 putative clients and over a dozen cellular pathways and processes?

Because it was hard to envision any clinical success at first, the application of Hsp90 inhibitors was relinquished to purely academic exercises as pharmaceutical companies considered it inconsequential. However, despite the high abundance and pleiotropic effects of Hsp90 protein–protein interactions, Hsp90 inhibitors have advanced into clinical trials. Counterintuitively, the aspects that at first made Hsp90 inhibitors seem inappropriate are now demonstrating advantages over single target therapies, and the potential of combination therapies that include Hsp90 inhibitors should effectively prevent or prolong the development of cancer drug resistance that is commonly seen with tyrosine kinase inhibitors and other target-based therapies [5–7].

To underline the improved successes of Hsp90 inhibitors, we highlight results published for ganetespiib, an Hsp90 inhibitor developed by Synta Pharmaceuticals (Lexington, MA, USA). In *in vitro* tumor cytotoxicity studies, it was shown that a 5-min exposure to ganetespiib at 1 μM (a readily achievable plasma level *in vivo*) was sufficient to reduce cell death within 72 h. This suggests that a brief exposure to Hsp90 inhibitors results in a permanent and lasting effect on the cell. These observations extend to animal studies. A single weekly dose of 100–150 $\text{mg}\cdot\text{kg}^{-1}$ for 3 weeks resulted in 50–90% tumor reduction in solid and hematological xenograft models [8]. Most importantly, in recent clinical studies it was also shown that once-weekly ganetespiib treatment in patients with non-small-cell lung carcinoma resulted in a 50% overall response rate [9,10]. Development of ganetespiib in many ways is therefore a good example of how second generation synthetic Hsp90 inhibitors are surpassing their first generation geldanamycin based counterparts. Ganetespiib not only shows increased efficacy and improved formulation over the geldanamycins, but also significantly reduces cardiovascular and hepatotoxicity side effects [8]. In our view such consistent advances are a testament to the validity of Hsp90 as a druggable target. While safety for off target effects is being improved and desired tumor effects continue to progress, the mechanisms for Hsp90 targeting in tumors over Hsp90 found in normal tissue is less understood.

Hsp90, the basics

To frame the review we offer a brief overview of the basics of Hsp90 and its known biology and then address the distinguishing characteristics of Hsp90 between cancer cells and normal cells. Hsp90 plays an essential role in maintaining cellular protein homeostasis by acting as a molecular chaperone to aid in folding as well as in intracellular trafficking of its protein clients [11,12]. It is expressed as a 90 kDa protein (Hsp90 732 amino acids; Hsp90 724 amino acids) in the cytosol and the nucleus and contains an N-terminal ATP-binding domain that is essential for most of its cellular functions [13]. ATP hydrolysis is thought to drive various conformational changes within Hsp90 and this process is highly regulated by interactions with co-chaperones and possibly a variety of posttranslational modifications (Table 1) [12,14]. Through these mechanisms the apparently ubiquitous cellular chaperone functions of Hsp90 are thought to be acutely regulated.

Much effort has been seen in the past two decades to characterize the specific protein interactions with other proteins termed co-chaperones. Co-chaperones assist Hsp90 throughout its conformational cycling that is required for its normal function, act as substrate recognition proteins and even provide additional enzymatic activity. The predominant class of co-chaperones are the tetratricopeptide repeat (TPR) domain containing proteins, which bind the MEEVD motif found in the C-terminus of Hsp90 [15–18]. Among the co-chaperones with a TPR domain are C-terminus of Hsp70-interacting

protein, Hsp70–Hsp90 organizing protein (Hop), cyclophilin 40, FK506-binding protein and protein phosphatase 5 (PP5). While most co-chaperones facilitate the recruitment of other substrate proteins, some of these co-chaperones add enzymatic functionality to the chaperone complex as is the case for the isomerases, phosphatases and ligases [19–21]. Other co-chaperones that interact with Hsp90 via alternative domains are activator of Hsp90 ATPase homolog 1 (Aha1), which enhances the function of Hsp90 by stimulating its ATPase activity [22,23]. The co-chaperone that is most implicated in facilitating tumorigenesis is cell division cycle 37 (Cdc37) because it associates with mutant kinases that drive cancer progression [24]. Recently it was shown that the Hsp90–Cdc37 complex binds two-thirds of the kinome to varying degrees, while at the same time demonstrating negligible interactions with ligases and transcription factors [25]. Another co-chaperone that is responsible for the complexing of nuclear hormone receptors and Hsp90 is p23. However, its interactions are not as limited as Cdc37, as p23 has been found in a broad range of Hsp90–client complexes [26–29]. Unlike the activating co-chaperone Aha1, the co-chaperones Cdc37, p23 and Hop inhibit ATPase activity. These co-chaperones therefore add another layer of regulation of this multifaceted master chaperone, underlining the complexity of the Hsp90 chaperone cycle and its folding functions. Hsp90 is also regulated transcriptionally, primarily through direct interactions with the transcription factor HSF [30]. Despite its high level of expression in normal cells, cellular stresses such as heat shock (37–42 °C) have been reported to induce Hsp90 levels by as much as twofold [31].

Two other immediate family members, glucose regulated protein 94 (Grp94) and tumor necrosis factor receptor associated protein 1 (Trap1), share sequence similarity in the ATP-binding domain and are also thought to act as cellular chaperones to promote protein stability and folding either in the mitochondria (Trap1) or endoplasmic reticulum (Grp94), although they do not exemplify the same complexity and vast interactions that Hsp90 exhibits [32]. Like Hsp90, Grp94 and Trap1 contain an N-terminal ATP-binding domain and ATPase activity that is also necessary for cellular function [33–36]. Unlike Hsp90, the list of client proteins interacting with either Grp94 or Trap1 is much more limited and less well defined. Several groups have proposed that Grp94 and Trap1 are additional potential targets that can be exploited as cancer chemotherapies, although selective inhibitors of these proteins have yet to be defined.

A surprising and common finding with most Hsp90 inhibitors is their selectivity for certain tumor cells and not other cells. With few exceptions *in vivo*, most cancer cells are more sensitive to Hsp90 inhibition than non-transformed cells and non-toxic doses demonstrate anti-cancer activity. In animals and humans, Hsp90 inhibitors consistently accumulate in tumors whereas they are rapidly cleared from plasma and do not appear to enter most tissues [37–43]. One notable exception may be the retina. Although idiosyncratic in nature, a reversible ocular toxicity has been observed in some patients with some but not all Hsp90 inhibitors [44]. Generally this occurs at higher dosing or after prolonged exposure and manifests as a loss of night vision. Drug withdrawal or dose reduction usually reverses the symptoms. However, the idiosyncratic nature of the phenomenon suggests that the mechanisms of drug accumulation in the retina may be different from tumor cells and perhaps related to some underlying eye pathology in patients that are most susceptible. Alternatively, the structural variations of different Hsp90 inhibitors permit varying degrees of cellular uptake, with the retina being more susceptible than most other tissues. The fact that certain tumor cells preferentially absorb Hsp90 inhibitors strongly suggests that specific mechanisms exist within these cells that contribute to druggability of Hsp90. One early hypothesis suggested that in tumor cells Hsp90 preferentially exists in chaperone complexes and these multiprotein complexes were reported to exhibit a higher affinity for Hsp90 inhibitors as well as higher ATPase activity [45]. Experiments with affinity resins based on immobilized Hsp90 inhibitors suggest a large imbalance of Hsp90 to its co-chaperones in

both normal and transformed cells [46,47]. A simple mechanism that could explain the drug accumulation may be that tumor cells simply express more Hsp90 than normal cells. Certainly, transformation has been shown to induce expression of Hsp90 at the protein level and this can also be measured in tumors isolated from patients. However, even in the most extreme case the fold inductions are 2–3-fold at best at the protein level. As discussed, in normal cells Hsp90 is thought to constitute 1–2% of the cellular protein and many believe that high expression of Hsp90 is evolutionarily conserved. Indeed, normal Hsp90 expression levels beg the question as to why tumor cells would need to induce more expression of the protein. We align our theory with others that the abundance of Hsp90 is evolutionarily conserved for a purpose and not wastefully guarded to bewilder scientists. If we can come to an understanding of the mechanisms that accentuate the tumor differences, Hsp90 inhibitors could be universally employed to fight the constantly evolving battle on cancer.

In this review we discuss the mechanisms that differentiate tumor Hsp90 from the Hsp90 that is abundantly expressed in normal tissues, thereby making it a druggable cancer target. The three mechanisms, not mutually exclusive, that are the most important in distinguishing Hsp90 in cancer cells from Hsp90 in non-transformed cells are (a) the induction of Hsp90 mRNA and protein, (b) the activation of the protein through either client association or post-translational modifications and (c) the localization to ectopic cellular compartments (Fig. 1). As we understand more clearly the mechanistic differences in tumor biology and normal cellular biology regarding Hsp90, the remaining hurdles for Hsp90 inhibitors to enter routine clinical practice will be overcome. For reviews on Hsp90 inhibitors see [37,48–53].

Induction

First we look at the differential expression of Hsp90. Few will argue that Hsp90 is overexpressed in tumors 2–3-fold higher than corresponding non-tumorigenic tissue, but even under basal conditions Hsp90 is abundant, comprising 1–3% of the total cellular protein [46,51,54,55]. Hsp90 is upregulated in response to cellular stress imposed by heat, hypoxia and nutrient deprivation, which are commonly associated with the tumor microenvironment. Thus it has been proposed that Hsp90 upregulation in tumors is essential to surviving the harsh microenvironment by allowing unstable permutations to persist that drive tumor malignancy [56].

In one of the strongest cases for clinical applications, hormone and protein kinase dependent breast cancer, Hsp90 expression levels have been well characterized and correlated to patient outcome of survival. First, Hsp90 expression in the breast has been recently evaluated immunohistochemically for various types of tissue: normal, pre-malignant and malignant. Hsp90 was shown to be expressed at higher levels in cancer tissues compared with non-cancer tissues [57]. Also Pick and colleagues performed immunohistochemical analysis of breast cancer cell lines and 655 primary breast cancers, including 331 estrogen receptor positive (ER+) and 324 ER– tumors, and found detectable Hsp90 expression in all of the breast cancer cell lines and in 90% of primary breast cancers. In addition, they reported that high expression of Hsp90 is associated with poor prognosis [58]. Because of the recognition of specific molecular subtypes of breast cancer, biostatisticians evaluated Hsp90 gene expression from profiles of over 4000 breast cancer patients from 23 publicly available gene expression databases, annotated with overall survival data from over 1000 patients. They found a normal distribution of Hsp90 expression, and confirmed that high expression of Hsp90 was associated with a poor overall survival [59]. It has also been shown in melanomas, leukemia and human colonic carcinoma that Hsp90 is elevated in the transformed and even more malignant tumors [60–62]. Elevated levels are also being detected in the serum of patients with non-small-cell lung cancer and prostate cancer [63,64].

Based on these studies it is believed that high expression of Hsp90 represents an important oncogenic signaling node for malignant behavior in cancer and detecting upregulation or activation of Hsp90 in cancer cells could be an early indicator of malignant behavior. However, increased Hsp90 expression is not sufficient to explain the mechanisms that drive tumorigenicity. One discrepancy is that even though Hsp90 is elevated in breast tumors compared with normal breast tissue, there are other normal tissues such as the bladder, spleen and brain that express higher Hsp90 in the ratio of total Hsp90 : total protein (Fig. 2) [55,65]. This leads to the argument that there is something unique about the protein in cancer cells that distinguishes it from Hsp90 found in normal tissue. With hundreds of putative client proteins and over 30 sites where post-translational modifications can occur, one can easily envision a scenario where tumor Hsp90 proteins are poised to behave differently from normal Hsp90.

Activation

Hsp90 is implicated in cancer cell survival and growth through its transient interactions with client proteins. Over 400 clients have been thought to be identified thus far and many of these are involved in mediating signal transduction pathways that govern cellular growth, apoptotic evasion, differentiation and metastasis [25,66–68]. For a current and comprehensive list of reported Hsp90 protein interactions visit the Picard laboratory's website at <http://www.picard.ch/Hsp90Int/index.php>.

Due to the fact that Hsp90 inhibitors preferentially target tumors, one may infer that these drugs target a subset pool of Hsp90, and this subset exhibits a high affinity conformation of Hsp90 for the inhibitors. One possibility is that Hsp90 exists in a multi-chaperone complex with transformation-specific oncoproteins, such as classical mutant kinases, human epidermal growth factor receptor 2 (Her2) and Bcr-Abl. These proteins may represent a small fraction of the client proteins regulating the transformed phenotype. The latent Hsp90 complexes regulating normal misfolding processes and comprising at any time the majority of total cellular Hsp90 may not be effectively inhibited by these drugs at the relatively non-toxic doses used. Such an interpretation leads to the hypothesis that, under normal conditions, Hsp90 interacts with client proteins in a dynamic, low affinity manner regulated by low affinity binding and release of ATP and ADP. Upon mutation or deregulation, which is characteristic of the cancer phenotype, many of these client proteins may display unusually stable association with Hsp90, representing the active and druggable state.

A 2003 *Nature* paper by Kamal *et al.* [45] claimed that Hsp90 in tumors exists entirely in multi-chaperone complexes and that when Hsp90 is in these specific complexes it has higher ATPase activity and a 100-fold higher affinity for the inhibitor 17-AAG. However, one incorrect assumption was that all Hsp90 has an equal opportunity of binding ATP or its mimetics that are immobilized to a bead. We and others have shown that only a fraction (20–30%) of Hsp90 binds to ATP or its ligands. Radiolabeled PU-H71 also only labeled 30% of the Hsp90 in MDA-MB-468 cells and only half that in CML cells [46]. As far as co-chaperone involvement, Kamal *et al.* demonstrated that when Hsp90 was reconstituted *in vitro* with Hsp70, Hsp40, Hop and p23, the highest ATPase activity was observed. Moulick *et al.* also showed that Hsp90 recognized by immobilized ligand precipitated the co-chaperones Hsp70, Hsp40, Hop and Hip and that these co-chaperones were not found in the fraction of the antibody-isolated Hsp90, but they were found in the flow-through [45,46]. It is thus hypothesized that the population of Hsp90 that binds to the ligand also exists in complex with several co-chaperones, but the 'inactive' pool does not exist with co-chaperones. In their *in vivo* analysis they found that mouse tumors compared with non-corresponding normal tissue do not differ that much in total Hsp90 levels as determined by western blotting. However, their ATPase activity was higher and their affinity for Hsp90

inhibitors was more [45], thus supporting that transformation and malignancy cannot be explained solely by the elevated expression of Hsp90. On the other hand, efforts to replicate this work have failed to show the exclusive complex of Hsp90 found in cancer. With regard to the complex having a higher affinity for Hsp90 inhibitors is believed to be an artifact of non-specific binding to the affinity resin. Our laboratory has shown that non-specific binding to an Hsp90 affinity resin decreases upon extending the ligand away from the immobilized bead. Hsp90 was cleanly and competitively eluted from the affinity resin [47], suggesting an alternative hypothesis that when Hsp90 is in complex with an inhibitor that targets the ATP-binding domain, co-chaperones that should be in stoichiometric abundance are displaced and not recovered.

The studies to elucidate the client–chaperone interactions for Hsp90 are incomplete and provide little rationale for these interactions. For example, Hsp90 does not recognize an amino acid sequence that is common among the vast array of putative client proteins, nor do proteins within the same family that are structurally similar interact with Hsp90 in a comparable manner, such as is the case with epidermal growth factor receptor and Her2. Due to the many criticisms that have been offered for the various approaches of identifying the Hsp90–client interaction, whether by immunoprecipitation, yeast two-hybrid assays or mass spectrometry analysis, a recent study attempted to circumvent previous obstacles by expressing tagged potential client proteins (i.e. kinases, ligases and transcription factors) with essential co-chaperones in order to study the interactions in a quantifiable manner. While no specific recognition sequence or structure was determined, the researchers concluded that a co-chaperone, Cdc37 in this case, provided a recognition of an as yet undefined fold and the thermal and conformational stability determined the extent of the interaction of Hsp90 with many of its kinase clients [25]. Cynically, one could also conclude from this study that any denatured protein is more likely to interact with Hsp90 than properly folded ones. Certainly the observation that inclusion of protein kinase inhibitors generally reduced binding to Hsp90 supports such a notion. Expressed protein kinases are generally stabilized in the presence of ATP competitive inhibitors as reflected by an increase in thermal stability. In our experience, studies that use assays relying on affinity pull-downs to study protein–protein interactions are fraught with artifacts and designing the appropriate controls to prove that interactions are real is not easy. Our own experience in the development of affinity resins targeting Hsp90 revealed the importance of carrying out appropriate controls in discriminating proteins that bind directly to Hsp90 versus those that are non-specific (Fig. 3). Figure 3 shows that changing the linker that is used to immobilize an Hsp90 inhibitor can dramatically affect the patterns of proteins recovered from a cell extract. One could conclude for example from the resin shown in the decane linker lane that this resin recovers a large amount of Hsp90 clients in addition to Hsp90. However, by blocking binding of Hsp90 itself to the affinity medium by including a free Hsp90 inhibitor in the cell extract prior to mixing with the affinity resin shows that, although recovery of Hsp90 is blocked, none of the other proteins are affected, showing that these are artifacts and have nothing to do with Hsp90 [47]. Ultimately this has led us to develop a medium that clearly recovers only Hsp90 and very little else including co-chaperones. We are somewhat perplexed by the absence of the Hsp90 interactome with this medium, despite the observation that it clearly recovers Hsp90 in a competitive manner. Our own conclusions from these studies is that the Hsp90 interactome should be revisited using more rigorous controls such as including Hsp90 inhibitors in control extracts to eliminate non-specific binding to the medium surface. We feel that such studies are likely to shorten the stable interactome considerably.

Having suggested that affinity based approaches might lead to misidentification of clients, other approaches examining client fate in the presence of an Hsp90 inhibitor may be more informative. Many studies that try to establish Hsp90 client associations evaluate the fate of

the putative client after Hsp90 inhibition. Most often cited is the degradation of a given client, while those proteins that persist are thought not to be chaperoned by Hsp90. The best example of this phenomenon is the degradation of Her2 in breast cancer cell lines treated with Hsp90 inhibitors. By western blot we can confirm recovery of Her2 with Hsp90 on our most selective Hsp90 resin [47]. Importantly, this recovery is blocked by the inclusion of an Hsp90 inhibitor in the cell extracts prior to mixing with the affinity medium. We note, however, that the ratio of active drug bound Hsp90 recovered relative to Her2 is at least two orders of magnitude higher (Hsp90 : Her2). As suggested in studies by Lindquist and colleagues, Hsp90 interacts with its clients transiently or with low affinity, at least in the drug bound state [25]. Client degradation approaches also have their caveats; for example it is common knowledge that the fate of unfolded proteins varies—some are degraded with differing kinetics and others form more stable aggregates when improperly folded. Thus, the lack of sensitivity to Hsp90 inhibition cannot fully explain the interactions or discredit client interactions. Additional caveats in establishing clients is that only a fraction of Hsp90 is considered active due to its ability to bind inhibitors in its ATP-binding pocket leaving the remaining Hsp90 pool to continue its chaperoning functions.

With many uncertainties in the reported clients and no clear way to establish a transient interaction for a protein that is otherwise stable without the chaperoning functions of Hsp90, the nature of defining a general activated state of Hsp90 in cancer will continue to be difficult to attain. However, much progress is being made in the differences in cancer Hsp90 compared with normal tissue Hsp90 with regard to its post-translational modifications. These modifications are now demonstrating their effects in ATPase activity, as well as localization, which in turn are affecting the association Hsp90 has with other proteins. For a comprehensive list of reported modifications see Table 1. Hsp90 modifications that influence localization are highlighting some of the stronger differences that are being observed in cancer cells over normal tissue.

Localization

The emphasis of distinguishing tumorigenic Hsp90 from normal Hsp90 is being placed in the field of client association. Post-translational modifications are thought to influence the association of Hsp90 with its clients, and in the past decade the community of Hsp90 has begun to reveal how post-translational modifications affect the localization of Hsp90. More importantly, they have shown that ectopic localization can lead to the progression of a more malignant phenotype of most cancers.

No longer is Hsp90 being considered to reside exclusively in the intracellular milieu, but it can be found on the surface membrane of a variety of cancer cells, as well as being secreted into the extracellular space [60,69]. The fact that cell surface Hsp90 is higher in some cancer cells than normal cells makes it an even more attractive target to destabilize metastatic pathways that are dependent on surface Hsp90 for invasion and migration. Since the initial screen in 2004 that implicated Hsp90 in cell invasion and migration, many researchers have shown that blocking or neutralizing secreted Hsp90 has an inhibitory effect on these metastatic behaviors [70,71]. While we still do not understand the mechanisms responsible for Hsp90 extracellular expression, certain environmental stresses and growth factors have been shown to stimulate its secretory pathways [72,73]. The secretion of Hsp90 also appears to be influenced by post-translational modifications, such as acetylation and phosphorylation [74,75].

The presence of Hsp90 outside the cell was first implicated in 1986 when a mouse tumor-specific antigen was found to be a heat shock protein, now recognized as Hsp90 [76]. In 2004, it was published that Hsp90 was discovered in a functional screen looking at cell

surface proteins that are necessary for cell invasion, but due to the cellular abundance of Hsp90 it was first viewed as an artifact. However, the screen was validated and Hsp90 was determined to be biologically important in the mechanisms of cell invasion as it was established that it interacts with and activates matrix metalloproteinase-2 (MMP2) [70]. Also in 2004, it was observed that in flow analysis of malignant melanoma isolated tumor cells exhibited surface expressed Hsp90 [60]. In the same year it was discovered that surface Hsp90 plays a role in the migration of developmental neurons. Both Hsp90 and are expressed on the surface of rat primary neural cells and antibodies against Hsp90 inhibited cell motility and lamellapodia formation. In this study crude measurements were made to determine the relative expression of surface Hsp90. Ratios of surface to total levels of Hsp90 were obtained and surface Hsp90 represented < 10% of total cellular Hsp90 [77]. Others have shown that tumor cell lines excrete Hsp90 into the conditioned medium and the lack of other classical intracellular proteins such as actin and tubulin argue that the Hsp90 does not come from lysed cells. In one study of dying cells, acrylamide-induced necrosis led to extracellular release of Hsp90 and increased HSF1 activity. Extracellular Hsp90 could be considered a danger signal to the immune system that something is detrimental to the cells [78]. However, it is unknown what external cues trigger the excretion of Hsp90 in necrotic cells.

Because intracellular Hsp90 plays a key role in proteomic homeostasis, it begs the question, are its chaperoning functions required outside the cell or does it play a different role altogether? It is known that intracellular misfolded proteins have three possible fates: chaperoning, proteolysis or aggregation. It appears that extracellular proteins require chaperoning as well. Compared with intracellular fluid, extracellular fluids have a lower protein concentration, 6% in plasma and 2% in interstitial fluid, as opposed to 30% in cytosol [79]. It is thought that extracellular Hsp90 functions with the co-chaperones Hsp70, Hsp40, Hip, Hop and p23 to assist in the cleavable activation of MMP-2 and can do it independently of ATP, an important feature for performing its function in an environment where ATP is drastically reduced [80].

Normal cells secrete Hsp90 but only when they are subjected to a compromised environment, such as heat, reactive oxygen species, -irradiation and injury released growth factors, whereas tumor cells constitutively secrete Hsp90 [72]. Early researchers proposed that Hsp90 is secreted by a non-canonical secretory pathway because Hsp90 lacks the conventional signal peptides in secretory proteins as well as post-translational modifications such as N-glycosylation. Also Hsp90 is not localized to the endoplasmic reticulum and Golgi apparatus and Hsp90 secretion is resistant to Brefeldin A treatment, a classical inhibitor of endoplasmic reticulum/Golgi dependent secretion [81]. Indeed it has been demonstrated that Hsp90 is excreted through the exosome pathway [72,82]. McCready *et al.* showed that when exosomes harvested from MDA-MB-231 cells were applied to other invasive cell lines in a wound healing assay both exogenous exosomes and recombinant Hsp90 stimulated faster migration into the scratched area. Conditioned medium was immunoprecipitated for Hsp90 and 10 client proteins were identified by mass spectrometry. The complex of interest was Hsp90 : AnnexinII : tPA : plasminogen, which results in the activation of plasmin, a key protein in tumor invasion and cancer metastasis [82]. Why, however, is some Hsp90 targeted for exosomal secretion while the majority remains in the cell? Additionally, secretion of Hsp90 does not appear to be isoform specific as first speculated, with the recent finding of Stellas *et al.* who showed that breast cancer cells also secrete the isoform [70,72,73,83].

Some speculated whether or not the numerous possibilities for protein modification distinguish Hsp90 for secretion. Knockdown of histone deacetylase 6 (HDAC6) results in increased acetylation which in turn decreases ATP binding and chaperone activity in Hsp90.

Seven lysine residues on Hsp90 revealed hyperacetylation by mass spectrometry (Table 1), and acetylation increased 17-AAG binding to Hsp90, whereas K294 acetylation in the middle domain affects co-chaperone binding. Hyperacetylated Hsp90 was found extracellularly and promoted *in vitro* breast cancer cell invasion. Additionally, an antiacetyl Lys69 Hsp90 antibody markedly inhibited the cell's invasiveness. P300 is the putative histone acetyl transferase responsible for acetylating Hsp90. The Hsp90 K292Q mutant showed increased binding to ATP whereas the other K to Q mutations at the other sites showed decreased binding. However, all the acetylated mimetics demonstrated increased binding to biotinylated geldanamycin [74]. While acetylation appears to play a role, others suggest that it is the phosphorylation of T5 and T7 of Hsp90 and others suggest that it is a C-terminal truncation [75,84]. It was shown that the secreted Hsp90 is a C-terminal truncated form and its secretion was regulated by the C-terminal EEVD motif via interacting with proteins containing TPR domains. It was also demonstrated that the secretion of Hsp90 was determined by the phosphorylation status at residue Thr90, regulated by protein kinase A and PP5. It was further demonstrated that the secretion of Hsp90 is a prerequisite for its pro-invasive function and blocking the secreted Hsp90 resulted in significant inhibition of tumor metastasis. Meanwhile, the level of plasma Hsp90 was positively correlated with tumor malignancy in clinical cancer patients [84].

While the precise mechanisms by which Hsp90 is targeted for exosomal secretion remain unclear, it is well established that extracellular Hsp90 plays pivotal roles in driving a non-motile tumor cell to become motile and invasive. Hsp90 facilitates each step in the three-step model for tumor invasion: degradation of the extracellular matrix, adhesion and migration of the cancer cells [85]. These events most probably are coordinated with intracellular chaperoning functions that drive the cell to become more malignant. These observations were made in the context of Hsp90 inhibition and its effects on cytoskeletal architecture and remodeling during cell motility and cell invasion. Hsp90 inhibition led to decreased cell motility associated with a decrease in filopodia, lamellipodia and tough cortical actin bundles and resulted in less ruffling. The Hsp90 inhibition was also associated with a loss of Diaphanous-related formin-2 and a decrease in Ras homolog gene family member A, both necessary for the formation of lamellipodia and generation of contractile force. Hsp90 inhibition stimulated an increase in the pull-down of actin in the soluble fraction with Hsp90. These observations suggested an increased interaction of Hsp90 with the soluble form of actin (G-actin) and β -crystallin upon Hsp90 inhibition, which might be responsible for the decreased actin treadmill at the cell periphery. Actin-enhanced cyan fluorescent protein and actin-enhanced yellow fluorescent protein showed a 50% reduction in fluorescence resonance energy transfer when inhibited with 17-AAG, and 17-AAG treatment resulted in an 80% decrease in inverse cell invasion for MDA-MB-231-GFP cells. Inhibition of Hsp90 also leads to a decrease in surface Hsp90 expression [86]. In light of the many advances in studying extracellular Hsp90, several questions remain. How do the extracellular cues communicate with the exosomal trafficking machinery to send Hsp90 out of the cell? Do the extracellular cues selectively cause Hsp90 secretion and leave other exosome-housed proteins behind or do they simply trigger secretion of the exosome vesicles as a whole? What percentage of total intracellular Hsp90 gets secreted?

One of the most important questions that we can ask pharmacologically is whether the surface expression of Hsp90 can be exploited to increase the safety threshold of future Hsp90 inhibitors. Our laboratory is currently exploring the hypothesis that expression of surface Hsp90 may contribute to the tumor selectivity of many Hsp90 inhibitors. This hypothesis is driven by recent data using cell impermeable Hsp90 inhibitors tethered to fluorophores showing that a portion of ectopically expressed Hsp90 is re-internalized causing drug accumulation (Barrott *et al.*, unpublished results). By extension of this observation, it is conceivable that many leading inhibitors of Hsp90 preferentially enter

tumor cells through a similar mechanism and may have been fortuitously optimized to do so. Such a statement is not without possibility. From our own experience in the discovery of Hsp90 inhibitors, although our initial high throughput leads were identified by elution of native Hsp90 bound to an ATP affinity column, all molecules were subsequently evaluated for biological activity in cell-based assays [41]. These assays comprised a combination of growth inhibition and evidence of the mechanism of action including inhibition of Her2 expression, induction of Hsp70 and S6 phosphorylation. Generally at this stage compounds that caused overt cell toxicity would have been considered second tier and not pursued further. Such a path would most likely have been pursued by other groups developing Hsp90 inhibitors. It will therefore be interesting to test whether Hsp90 inhibitors that have higher *c* Log *P* values, and are thus more freely diffusible, are more toxic to normal cells compared with our current armamentarium of clinically active Hsp90 inhibitors. This finding would have great significance because the development of Hsp90 inhibitors that only penetrate cells through interactions with surface Hsp90 could have vastly improved safety profiles enabling the therapeutic window of this class of drugs to be extended.

Combination therapy

Typically when Hsp90 inhibitors are successively dosed to animals bearing human tumors, the tumors stop growing. However, when the inhibitor is withdrawn the tumors tend to start growing again (Fig. 4). Generally the same phenomenon occurs in patients with solid tumors being treated with a variety of structurally unrelated Hsp90 inhibitors. These observations are consistent with the role of the protein in maintaining tumor growth and suppression of these pathways by Hsp90 inhibitors. For these reasons Hsp90 inhibitors may have limited use as a mono-therapy, although more recent observations suggest that certain tumor types may be more responsive to Hsp90 inhibition than others and mono-therapy may be sufficient to promote tumor reduction and even cellular death. In recent phase II studies with ganetespib alone, an encouraging response rate of 50% in non-small-cell lung carcinoma patients whose tumors contained ALK translocations was observed [87]. Instead, however, there is great excitement in the field for using Hsp90 inhibitors in combination with either existing chemotherapeutics or more cutting-edge drugs like Herceptin, lapatinib or Gleevec. Figure 4 shows that a combination of the drug SNX5422 and Herceptin not only shrinks the tumors dramatically and persistently (even after withdrawal), but it is also synergistic. The argument for combination therapy is in the evidence that genome sequencing of human cancers reveals numerous driver mutations within any one individual cancer [88,89]. Intratumor heterogeneity may foster tumor evolution and adaptation and hinder personalized-medicine strategies that depend on results from single tumor-biopsy samples [90]. Hsp90 inhibitors can play a unique role in preventing drug resistance in tumors because oncogenes rely heavily on Hsp90 to chaperone their otherwise unstable conformation due to their mutations. This dependence has been termed oncogene addiction. Because numerous mutant oncoproteins are 'addicted' to Hsp90 activity, an inhibitor to Hsp90 has the ability to affect multiple targets and pathways, which can prevent oncogene switching, a major mechanism for developing resistance [91,92].

Hsp90 inhibitors have the potential to circumvent or diminish the drug resistance seen in the use of target-based therapies, but because cancers evolve, resistance to Hsp90 inhibition is also a real possibility. However, the silencing of co-chaperones such as p23, Aha1 or Cdc37 has been shown to cause dramatic sensitization to Hsp90 inhibition [93–95]. Because Hsp90 inhibition allows the trimerization and activation of HSF1, and because elevated HSF1 is linked to stages of oncogenesis, there are efforts to find inhibitors to HSF1 that can be used in conjunction with Hsp90 inhibitors to minimize the negative effects of Hsp90 inhibition [96,97]. As a result of HSF1 activation, Hsp70 transcription is dramatically upregulated, which is often used as readout in cell-based assays for efficacy of an Hsp90 inhibitor.

However, Hsp70 induction is another chaperone that can drive oncogenesis in many tumors. Researchers have shown a therapeutic value for targeting Hsp70 because tumors overexpress the inducible form over the constitutive form [98]. Therefore, combination therapy that inhibits both Hsp90 and the inducible Hsp70 could be a formidable method of treating cancer by eliminating the unwanted effect of Hsp70 induction.

Conclusion

While some are still not cognizant to the advances of Hsp90 inhibitors in the clinic, others, including members of the Hsp90 community, still question whether or not any of the now 17 Hsp90 inhibitors will successfully exit clinical trials and enter the market. We think the day will come when Hsp90 inhibitors will be commonplace in the clinic and that in the oncological application of Hsp90 inhibitors these drugs will be used as first line chemotherapeutics in combination with other potent anti-cancer therapies to prevent or prolong the development of drug resistance. In the immediate term combination therapy involving an Hsp90 inhibitor and cutting-edge targeted therapies clearly holds the greatest promise. If the consistent synergisms in efficacy and tumor elimination observed in animal combination studies with drugs such as Herceptin hold true in human trials, perhaps we are well on the way to seeing actual ‘cures’ rather thinking about merely halting disease progression over the shorter term. Beyond combination therapy, as we have discussed, other aspects of Hsp90 biology may render it even more druggable. Exploring the differences in Hsp90 between cancer cells and normal tissues may reveal additional strategies on how to target Hsp90 and how to improve its therapeutic window. One of these in our sights is the role of ectopically expressed Hsp90 in metastatic progression. Although yet to be demonstrated in human biopsy samples, clearly, if there is a correlation between expression of ectopic Hsp90 and metastatic progression, this is likely to be of both diagnostic and therapeutic importance. If expression of ectopic Hsp90 correlates with poor outcomes it could be used as an early biomarker to discriminate indolent forms from aggressive disease. We are particularly excited by the prospect of developing Hsp90 inhibitors that only target ectopically expressed Hsp90 as a means to improve the selectivity of Hsp90 inhibitors and deliver tumor-killing payloads to tumor cells. On top of all these tangible possibilities for extending the therapeutic potential of Hsp90 is the co-chaperone/ chaperone machinery of associated proteins regulating and regulated by Hsp90. Once *bona fide* clients are established and validated, one could envision strategies that could selectively inhibit these protein–protein interactions, thus potentially making Hsp90 therapy highly targeted and disease specific.

Finally, from a pharmacologist’s perspective, there are lessons to be learned from the development of Hsp90 as a cancer target that are pertinent to 21st century drug discovery. Clearly, new therapies to treat cancer are not always going to be obvious as has been the case in the past, i.e. one must not assume that a ‘hallmark of cancer’ involves a point mutation and that alone signifies a usable target. Not all targets are going to be ‘Gleevec type stories’. If we make the assumption that this is the case we are likely to miss the boat on some great opportunities. A second lesson is that although an Hsp90 drug has yet to be brought to market, there is clearly a ground swell of independent data that strongly indicate that this will happen soon, despite some side effect concerns such as retinal toxicities. This ground swell is self-evident in the form of the numbers of independently derived inhibitors that are currently being advanced clinically. Within these independent paths we count an extraordinary six distinct structurally diverse scaffolds upon which the current clinical armamentarium of Hsp90 inhibitors is currently derived. In future drug discovery efforts involving unconventional targets where we do not have obvious genetic data to validate a target we should look for similar trends to assure pharmaceutical companies that such targets should not be ignored.

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Abbreviations

Aha1	activator of heat shock 90 kDa protein ATPase homolog 1
Cdc37	cell division cycle 37
ER±	estrogen receptor positive or negative
Grp94	glucose regulated protein 94
HDAC6	histone deacetylase 6
Her2	human epidermal growth factor receptor 2
Hip	Hsp70-interacting protein
Hop	Hsp70–Hsp90 organizing protein
HSF	heat shock factor
Hsp90	heat shock protein 90
MMP2	matrix metalloproteinase-2
PP5	protein phosphatase 5
tPa	tissue plasminogen activator
TPR	tetratricopeptide repeat
Trap1	tumor necrosis factor receptor associated protein 1

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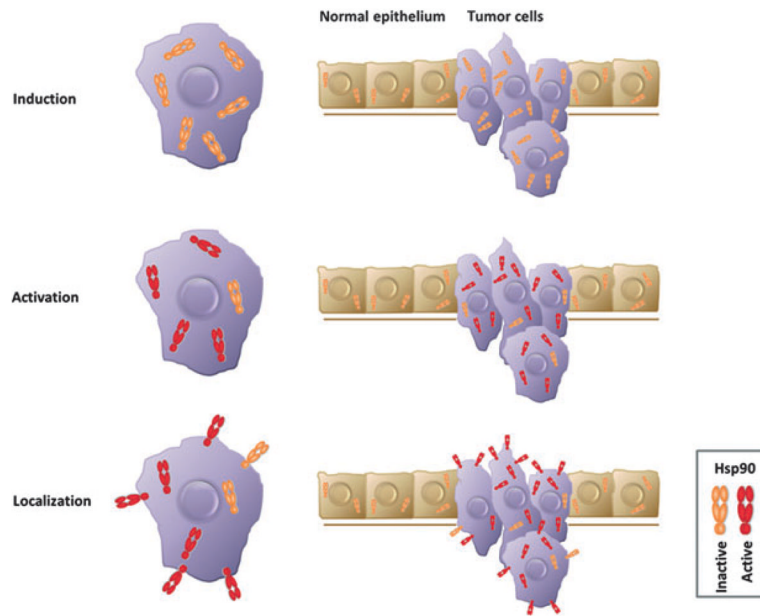


Fig. 1. Hsp90 differences between tumor cells and adjacent normal cells. Hsp90 is elevated in tumor cells, induction. Hsp90 is activated (red) in tumor cells and localizes to the cell surface, whereas in normal cells Hsp90 resides exclusively in the cytosol.

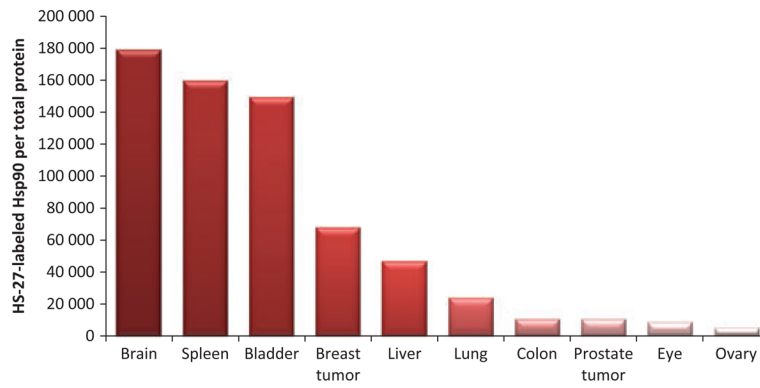


Fig. 2. Labeled Hsp90 in various lysed mouse tissues normalized to total protein. The fluorescently labeled small molecule inhibitor HS-27 detects active Hsp90, and surprisingly two xenograft tumor samples did not exhibit the highest active Hsp90 to total protein ratio.

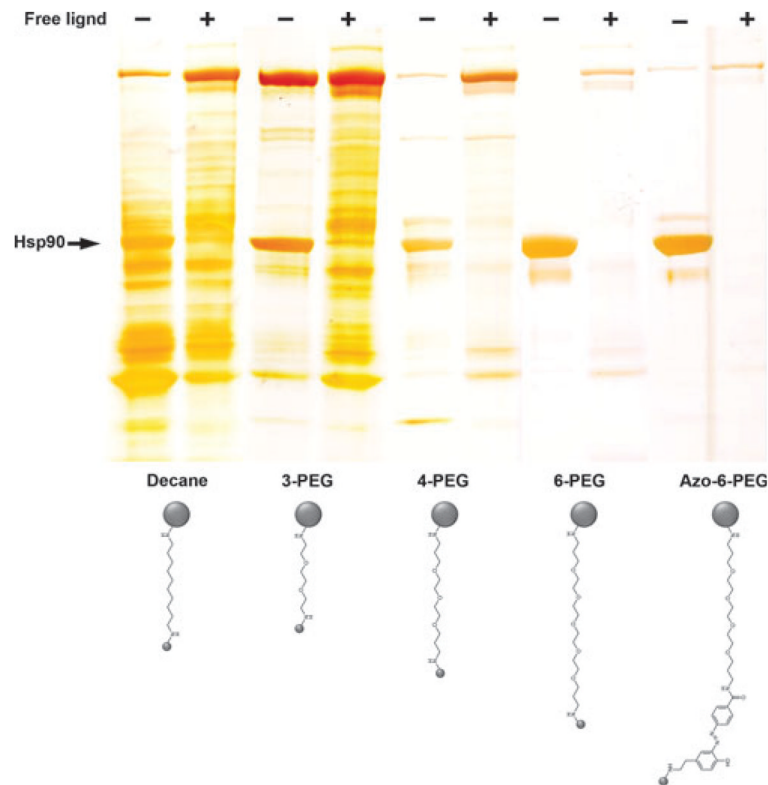


Fig. 3. Directed chemical evolution of a selective affinity medium for Hsp90. SDS/PAGE silver stain showing the effects of different side chain modifications on Hsp90 recovery and recovery of nonspecifically bound proteins. Selectivity towards Hsp90 was demonstrated by inclusion of 1 mM of the free ligand (+) in the tissue extract prior to mixing with affinity resin. Mass spectrometry was used to define all of the bound protein. In the azo-6-PEG resin bound proteins were eluted with 25 mM sodium dithionite in NaCl/P_i. PEG, polyethylene glycol. Figure reproduced from [47].

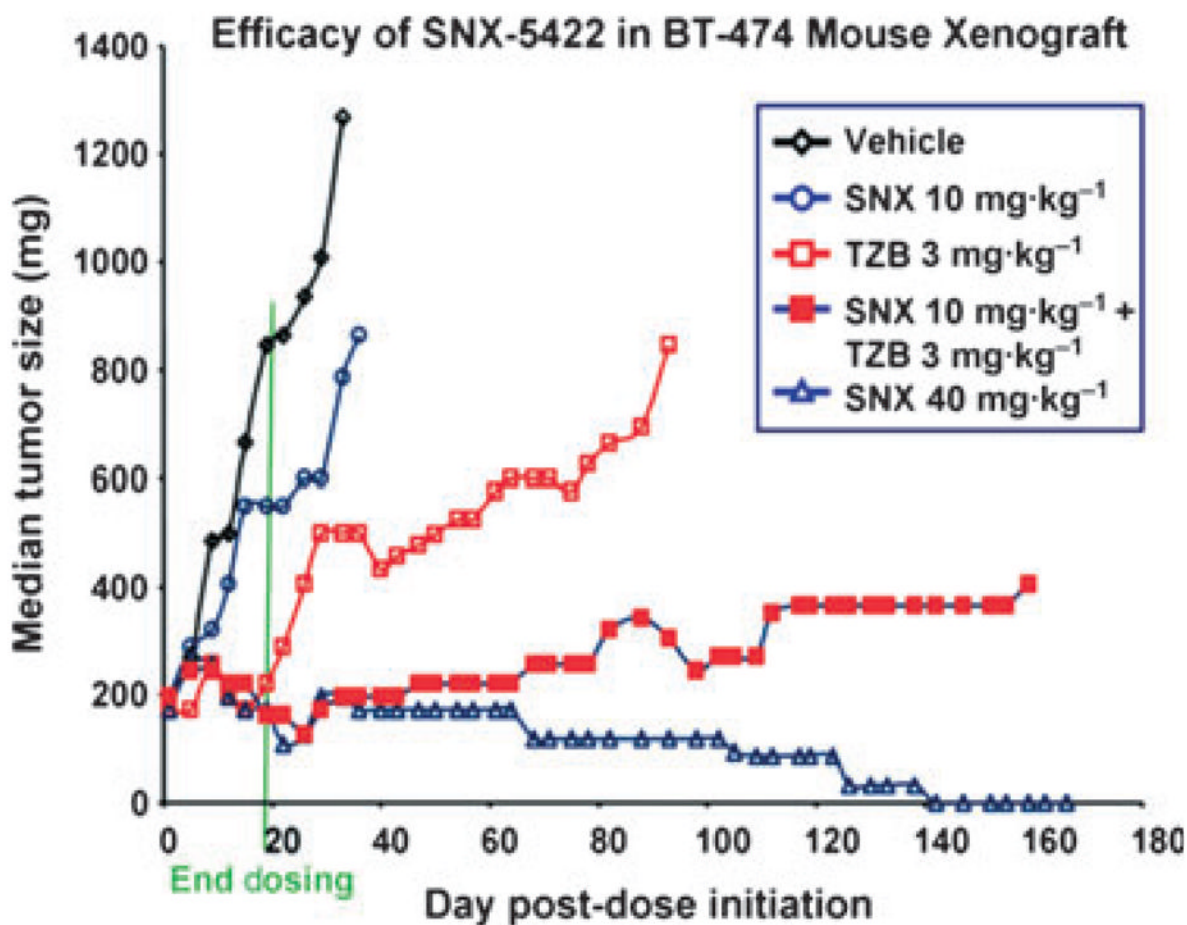


Fig. 4. *In vivo* pharmacology of SNX-5422. Effects on BT-474 tumor-bearing nude mice ($n = 10$) dosed with vehicle or (1) 10 mg·kg⁻¹ SNX-5422 (SNX) by mouth three times per week, (2) trastuzumab (TZB) 3 mg·kg⁻¹ intravenously twice per week, (3) 10 mg·kg⁻¹ SNX by mouth three times per week and TZB 3 mg·kg⁻¹ intravenously twice per week or (4) 40 mg·kg⁻¹ SNX three times per week. Dosing in all drug-treated groups was for only the first 3 weeks. All groups were followed for 6 months or until sacrifice due to tumor burden. Figure reproduced from [41].

Table 1

Post-translational modifications of Hsp90. p300, histone acetyl transferase; CK2, casein kinase II; PKA, protein kinase A; DNA-PK, DNA-dependent protein kinase; eNOS, endothelial nitric oxide synthase.

Modification	Hsp90 residue ()	Enzyme	Result	Reference
4-HNE, 4-ONE	C572		Decreased client association	99
Acetylation	K294	HDAC6	Decreased client association	100
Acetylation	K69, K100, K292, K327, K546, K558	p300, HDAC6	Increased inhibitor binding, increased extracellular expression	74,101–104
Dephosphorylation		Ppt1/PP5	Decreased chaperone function	105
Phosphorylation	Y309	SRC	VEGFR2-induced angiogenesis	106
Phosphorylation	S231, S263	CK2	Increased apoptosome formation, decreased client association	107,108
Phosphorylation	Y38	Swe1/Wee1	Increased client association, decreased inhibitor binding	109,110
Phosphorylation	T36	CK2	Decreased ATPase activity, increased inhibitor binding	111,112
Phosphorylation	Y197	Yes	Decreased co-chaperone association	113
Phosphorylation	Y309	Yes	Increased co-chaperone association	113
Phosphorylation	T5, T7	DNA-PK	Increased extracellular expression	75,114
Phosphorylation	T		P2X ₇ receptor repressor	115
Phosphorylation	S263	B-Raf	Unknown	116
Phosphorylation	S460	PKA	Unknown	117
Phosphorylation	T90	PKA	Increased extracellular expression, decreased client association	84,118
S-nitrosylation	C597	eNOS	Decreased chaperone function, decreased ATPase activity	119,120
Ubiquitination			Decreased client association	121