

Published in final edited form as:

Trends Pharmacol Sci. 2014 November ; 35(11): 592–603. doi:10.1016/j.tips.2014.09.001.

Selective targeting of the stress chaperome as a therapeutic strategy

Tony Taldone, Stefan O. Ochiana, Pallav D. Patel, and Gabriela Chiosis

Program in Molecular Pharmacology and Chemistry and Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA

Abstract

Normal cellular function is maintained by coordinated proteome machinery that performs a vast array of activities. Helping the proteome in such roles is the chaperome, a network of molecular chaperones and folding enzymes. The stressed cell contains, at any time, a complex mixture of chaperome complexes; a majority performs “housekeeping functions” similarly to non-stressed, normal cells, but a finely-tuned fraction buffers the proteome altered by chronic stress. The stress chaperome is epigenetically distinct from its normal, housekeeping counterpart, providing a basis for its selective targeting by small molecules. Here we discuss development of chaperome inhibitors, and how agents targeting chaperome members in stressed cells are in fact being directed towards chaperome complexes and their effect is therefore determined by their ability to sample and engage such complexes. A new approach is needed to target and implement chaperome modulators in the investigation of diseases, and we propose that the classical thinking in drug discovery needs adjustment when developing chaperome-targeting drugs.

Keywords

molecular chaperone; HSP90; HSP70; HSP60; chemical tools; epigenetic regulation

The housekeeping and the stress chaperome

Normal cellular function is maintained by coordinated proteome machinery that performs a vast array of housekeeping activities. These include transmission of signaling impulses, performing metabolic activities, replicating DNA, regulating cell motility and transporting molecules from one location to another. Helping the proteome in such diversity of roles is the chaperome. The chaperome is composed of an interconnected network of molecular chaperones as well as co-chaperones and folding enzymes that assist in their function [1]. We refer to this machinery as the housekeeping chaperome. Together with the protein degradation machinery, these molecular machines maintain cellular homeostasis [2–4].

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Corresponding author Dr. Gabriela Chiosis, Program in Molecular Pharmacology and Chemistry and Department of Medicine, Memorial Sloan-Kettering Cancer Center, hiosisg@mskcc.org.

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Stresses imposed on the cell by specific imbalances and alterations in the proteome may lead to disease, which in this context, is a dysfunction of normal homeostasis. Stress on human cells, and in turn disease, is complex in nature and may result from genetic dysfunctions, invasion by a pathogen, an environmental cause or a combination of such factors. What these have in common is that they manifest by altering the proteome, which in turn perturbs the chaperome [5–10]. While often attributed to changes in molecular chaperone expression, it is now evident that expression alone is insufficient to explain the ability of the chaperome system to buffer such large variety of alterations as is characteristic of each stress [11–14]. As we are starting to appreciate, mainly from studies in cancer, chemical modifications such as post-translational modifications (PTMs) [15–18] but also biochemical modifications such as by co-chaperone and adapter protein recruitment [8, 19–24] physically alter the chaperome to modify its function. In addition to activating the chaperome, these epigenetic modifications contribute to the altered cellular location of the chaperome members, as noted in numerous diseases [21, 25–32].

We will discuss below how these epigenetic modifications provide a basis for the specific pharmacologic targeting of the chaperome in the treatment of diseases. We will also make a case that the distinct epigenetic and thermodynamic nature of the chaperome in stressed cells require a new approach into how we target and implement chaperome modulators to the investigation of diseases. We will provide lessons learned from the discovery and development of heat shock protein 90 (HSP90) inhibitors, to serve as a guideline for these efforts. Given the large array of pathologies associated with an altered function of the chaperome, we will make a case that the numerous current efforts towards the discovery of ligands, chemical tools and drugs that target chaperome members should provide both an investigational toolset for the study of the altered proteome, and in turn of diseases, and drugs to be implemented in their treatment (Figure 1).

The major chaperome members

The human chaperome is composed of a large number of proteins, encoded by as many as 169 genes [1]. While a majority of the chaperome members are referred to as heat shock protein (HSP), and categorized by their molecular size, such as HSP90, HSP70, HSP60 and small HSPs [2, 3], it is estimated that only a fifth of the human chaperome (33/169 genes) is heat inducible [1]. The most studied chaperome member to date is HSP90. It is one of the most conserved HSPs found in bacteria and all eukaryotes, and is widely expressed in all cells. In humans, there are four known HSP90 members, HSP90 α , HSP90 β , Glucose-Regulated Protein 94 (GRP94) and Tumor Necrosis Factor Receptor-Associated Protein 1 (TRAP1) [33–35]. In normal cells, HSP90 α and HSP90 β reside mainly in the cytosol with minor localization in the nucleus, whereas GRP94 is found in the endoplasmic reticulum (ER) and TRAP1 in the mitochondria. Another widely expressed HSP family is the HSP70s [36–38]. The human HSP70 family contains at least eight homologous chaperone proteins. Endoplasmic reticulum (ER) and mitochondria have specific HSP70 proteins, GRP78 (also known as BIP or HSP70-5) and HSP70-9 (mtHSP70, mortalin or GRP75), respectively. The remaining members, such as HSP70-1a, HSP70-1b, HSP70-2 and Heat Shock Cognate protein 70 (HSC70), reside mainly in the cytosol and nucleus. HSP60 (chaperonin 60 or Cpn60) typically located in the mitochondria, can also be found in the cytoplasm under

normal physiological conditions. It appears to function as an oligomer composed of monomers that form a complex arranged as heptameric rings [39]. For small HSPs, the current view is that they bind unfolding “client” proteins, and then these small HSP-client complexes interact with the large ATP-dependent HSPs [40, 41]. Other HSPs, such as the large HSP40 family, or DnaJs [42], and HSP110 [43] appear to serve a co-regulatory role, in that they participate in protein client regulation together with the major HSPs, and regulate several steps in their activity. These, however, also have independent functions [44, 45]. To perform their function, the chaperones, in turn, are aided by a large number of function- and complex-specific co-chaperones and adapter proteins [46–48].

Targeting the stress chaperome lessons from HSP90

The contribution of epigenetics to altering chaperome function is best understood for HSP90. In human cells, HSP90 exists in equilibrium between three conformational states, the apo-, the ADP and the ATP-bound states [35, 49]. Whereas nucleotide binding provides a modest stabilizing energy biasing HSP90 towards a particular conformation, it is now believed that the conformational fate of the chaperone is regulated by the presence of its co-partners and by the extent of PTMs [15, 50–52]. Together, these provide in the cell a balanced number of HSP90 species that are best primed to assist the need of a specific proteome. Indeed over 20 co-chaperones and a multitude of PTMs such as phosphorylation, sumoylation, methylation and acetylation have been identified that regulate HSP90 function in human cells [15, 52]. Furthermore, it is known that co-chaperones may undergo PTMs that alter their interaction with HSP90 [52]. The chaperoning of each client protein by HSP90 may require a distinct subset of these epigenetic modifiers, each driven by its specific thermodynamic need [53–55]. These epigenetic modifiers may guide HSP90 to sample multiple distinct conformations that ultimately determine the fate of bound substrate (i.e. proper folding and activation or degradation), and as we are now appreciating, the interaction of a small molecule with HSP90 [8, 56, 57].

While a limitation for classical genetic and biochemical approaches (Box 1), the complex presentation of the chaperome in disease represents an advantage for pharmacologic approaches (Figure 1). There are numerous ligands that over the years have been serendipitously or rationally discovered to interact with members of the chaperome (Figure 2). Perhaps a turning point in appreciating the importance of small molecule chemical tools in the investigation of the chaperome came from the serendipitous discovery that the natural product geldanamycin (GM) inhibited HSP90 [58]. Although the HSP90 protein has been known since the 1980s, initially drew little interest, especially as a target in disease. After all it is abundantly (~1–3% of total cellular protein) and ubiquitously expressed in most, if not all human cells, and is not particularly variable in expression between normal and cancer cells [14]. Knock-down of even 50% of HSP90 in cancer cells has little effect on their viability, whereas knock-out of at least the HSP90 β paralog is embryonically lethal [59]. Such findings match poorly with the belief that a good therapeutic target has to be crucial to the malignant phenotype and be of low expression in vital organs and tissues [60]. It is GM that changed our thinking. Found in a screen searching for compounds able to revert the phenotype of cells transfected with the v-src oncogene, GM was demonstrated to do so by specifically binding to the N-terminal regulatory pocket of HSP90 and by this, to inhibit its

function [58]. Low concentrations of GM were active on many cancer cells and induced differentiation, reduced cell proliferation and/or induced death, while showing no significant toxicity to normal cells [58]. Subsequent crystal structures of HSP90 in complex with GM and the regulatory nucleotides have uncovered the unique characteristics of this pocket and its high potential for druggability, further spurring the interest in HSP90 as a drug target [61]. The low toxicity seen with HSP90 inhibitors on normal cells remained unexplained for more than a decade, until Kamal *et al* proposed that, although no specific mutations differentiated HSP90 in normal and cancer cells, in cancer cells, the chaperone was found entirely in complexes of high affinity to small molecule inhibitors [62]. In normal cells, by contrast, a dynamic complex of HSP90, with low affinity for small molecule inhibitors was present. This mechanism provided a satisfactory explanation for the distinct sensitivity of normal and cancer cells to GM and other HSP90 inhibitors. It however fell short of explaining other observations, such as the little effect 50% reduction in HSP90 levels had on cancer cells. An explanation came eight years later when Moulick *et al* showed that HSP90 in cancer cells was not comprised entirely of the high affinity form, but rather it was composed of a “housekeeping HSP90” species, which had low affinity to certain small molecule inhibitors, similar to the HSP90 found in normal cells, but also of a distinct HSP90, defined as the “oncogenic HSP90” species [8]. This epigenetically distinct HSP90 comprises a functionally distinct HSP90 pool, enriched or expanded in cancer cells; cells use it to maintain the altered proteins and protein networks that are needed to drive the malignant phenotype. In this view, small molecules by their ability to interact specifically with the “oncogenic HSP90”, will primarily and selectively affect these complexes, and will act on the “housekeeping HSP90” only at higher or at saturating concentrations. By contrast, genetic targeting of HSP90 will equally reduce the expression of both “oncogenic” and “housekeeping” HSP90 pools, and thus it is conceivable that more than 50% reduction of HSP90 levels would be necessary to lower HSP90 to the threshold level required for cell survival.

Box 1

Limitations of classical approaches in the study of the chaperome in disease

The complex presentation of the chaperome species in stressed cells helps explain the limitations of classical approaches towards understanding stress, both as it relates to the chaperome and to the proteome it regulates. Most such methods, *i.e.* genetic and biochemical, treat the chaperome as a monolithic entity and thus, are unable to tackle the acknowledged contribution of epigenetics to the activity of these proteins. By not differentiating between the housekeeping and the stress chaperome species, genetic manipulations silencing the HSPs are also, often, lethal [37, 59]. Alternatively, due to feedback synthesis of one HSP family member after the knock-down of another, such studies may often lead to no observable phenotypes [32, 37, 122]. Cellular manipulations that are often conducted to investigate the function of a protein and its potential interactors, *i.e.* by transfection of mutants, tagged proteins, or overexpression systems, are also bound to lead to “false positives” for HSPs; this is of no surprise as the chaperome is the “buffer” of cellular stress, and such manipulations, which lead to

proteome stress, are likely to impose artificial interactions on HSPs with the transfected proteins. Furthermore, these chaperome complexes are likely to be cell- and type-specific, and in addition, subject to the profound implications induced by post-translational modifications. Depending on the particular cellular context, each HSP may display distinct functions such that the phenotype observed following perturbation by genetic knockdown versus small-molecule probe can be significantly different. Together, these facts help explain why information ensuing from such studies is sometimes conflicting.

The stressed cell (stress defined as a disease characterized by proteome alteration) therefore contains, at any time, a complex mixture of chaperome complexes; a majority performs “housekeeping functions” similarly to non-stressed, normal cells, but a finely-tuned fraction buffers the proteome altered in the process of chronic stress (Figure 1). These stress-induced and stress-associated species are epigenetically and thermodynamically different from the housekeeping chaperome. Such distinct feature provides the basis for their selective targeting by small molecules. Thus in a stressed cell, the functionally altered proteome becomes regulated by the stress chaperome; this in turn, can be selectively “sensed” by small molecules (Figure 1, depicted as a hand-held device). By chemically sensing the stress chaperome one may investigate its associated, disease-causing, proteome and thus investigate mechanisms associated with and causing the stress state [8, 28, 31, 32, 40]. By using small molecules selectively targeting the stress chaperome species as affinity-purification baits [8, 63, 64] or as global perturbors of the altered proteome [65], one may inquire into its nature, i.e. identify. By attacking specifically the stress chaperome, one may inactivate the altered proteome, and thus revert or slow the disease phenotype, i.e. treat [66–69]. A properly tailored pharmacologic toolset directed at the major stress-associated chaperome members, could enable such mechanistic (i.e. investigate), proteomic (i.e. identify) and therapeutic (i.e. treat) investigations in a large number of diseases such as cancer [41, 67, 69, 70], neurodegeneration [71–74], pathogen-induced diseases [75–79] but also, as it has recently been reported, in polycystic ovarian syndrome [80], alcoholism [81], obesity [82], psoriasis [83] and cystic fibrosis [84] among others.

Ligands targeting the major chaperones

HSP90 binders

To date, most known HSP inhibitors target HSP90. The most amenable domain of HSP90 to inhibitors has been the nucleotide binding pocket located in the N-terminal domain (Figure 2A). This nucleotide site is targeted by numerous inhibitors based on various chemotypes such as ansamycin (e.g. GM, 17-DMAG, 17-AAG, IPI-504), resorcinol (e.g. Radicicol, NVP-AUY922, AT13387, STA-9090/Ganetespib, KW-2478), purine (e.g. PU3, PU-H71, MPC-3100, CUDC-305/Debio 0932, BIIB021) and other interesting scaffolds (e.g. SNX-5422/SNX-2112, HSP90, XL888) [41, 66, 67] and some of these agents have reached clinical trials [61]. These inhibitors bind to the ATP binding pocket of HSP90 and prevent HSP90 from cycling between the ADP and ATP bound conformations thus impairing the chaperone activity [41]. Though N-terminal HSP90 inhibitors share a similar binding site their exact mode of binding may be different, and the result is the wide ranging effects on

biology [8, 56]. The specific binding mode as well as a discussion on the residues that many of these compounds interact with has recently been analyzed in detail [85].

While these ligands bind with comparable affinity to the four HSP90 paralogs [85], conformational differences in the HSP90s have recently enabled the discovery of ligands selective for individual paralogs (Figure 2B,C) [32, 86, 87]. Such chemical tools with a high degree of selectivity for HSP90 α/β [86, 88] and GRP94 [32] may pave the way for a better understanding of the biological role of these paralogs in specific disease contexts, as they enable parsing out their function in the context of an un-engineered human cell [32]. They may also enable the identification of disease states where inhibition of a specific HSP90 paralog may be more therapeutically advantageous than inhibition of all HSP90s.

For the less targeted C-terminal domain there are a few inhibitors reported in the literature such as celastrol and gedunin, novobiocin and derivatives such as KU174 (Figure 2A) [89]. These interact with HSP90 at non-overlapping sites as demonstrated by their ability to capture HSP90 in distinct conformational states [90].

HSP70 binders

HSP70 inhibitors reported to date target the ATP binding site, the allosteric sites in the nucleotide binding domain (NBD), the substrate binding domain (SBD) or their exact binding is still elusive (Figure 2D) [38, 41]. Adenosine-derived inhibitors were designed and synthesized using modeling and X-ray crystallographic structures leading to VER-155008 and compound 14, that, at least at biochemical level, interact selectively with HSP70 and GRP78, respectively [91, 92]. These compounds target the ATP-binding site of HSP70s, thus inhibiting their chaperone activity (blue circle, Figure 2D). Apoptozole is another compound which inhibits HSP70 activity, presumably via binding to the ATP-binding site, and this was rationalized via modeling studies to suggest that apoptozole can adopt a conformation that overlays with ATP (Figure 2D) [84]. Additionally, MKT-077 and YK5 are two compounds reported to bind to two different allosteric sites in the NBD of HSP70 [68, 93]. The allosteric site 1 (orange circle, Figure 2D) where the cationic rhodacyanine dye MKT-077 binds is negatively charged due to the presence of the side chains of Glu175, Asp199 and Asp206 [68]. On the other hand, YK5 and derivatives were rationally designed to interact with the allosteric site 2 on the NBD (green circle, Figure 2D). Additionally, myricetin [94] is also reported to bind to an allosteric site in the NBD which is adjacent to site 2. Another HSP70 domain that has been targeted is the SBD.

Phenylacetylenylsulfonamide (PES, also known as pifithrin- μ) is believed to interact with HSP70 in the SBD (red circle, Figure 2D) and inhibit chaperone activity [41, 95].

HSP60 binders

There are no crystallographic studies on human HSP60, and hence the domain identification and characterization has been thus far based on the structure of GroEL, the bacterial homolog (Figure 2E). The development of HSP60 inhibitors focused primarily on the inhibition of ATP binding or targeting of the HSP60s cysteine residues [96]. As seen for HSP90s and HSP70s, inhibition of ATP binding and hydrolysis impacts the ATP-dependent conformational changes of HSP60 that are essential for its protein folding function [96].

Compounds believed to target the ATPase activity of HSP60 are the imidazole nucleoside mizoribine and the pyrazolopyrimidine derivative EC3016 reported to block ATP binding and hydrolysis, and hence affect the folding function of HSP60 [97]. Covalent binders that target the reactive cysteines of HSP60 also inhibit the chaperone activity, and one illustrative example is epolactaene which covalently binds to Cys442 (Figure 2E, equatorial domain) [39, 98].

No two HSP inhibitors are the same

Agents that are directed towards HSPs in stressed cells are in fact being directed towards HSP complexes and their effect is therefore determined by their ability to sample and engage such complexes. As mentioned above, there are numerous variables that determine the constituency of these complexes at any time. One is conformation (Figure 3). All major HSPs are known to undergo conformational changes associated with substantial rearrangements in their structure [35, 49, 50, 99]. The pocket available for ligand binding may change during these conformational states. By interacting with the protein at different sites, inhibitors sample non-overlapping conformations of the protein or freeze the protein in distinct conformations. For example, this can manifest at the local level, such as capturing changes in the structure of the protein by rearrangements of the protein in a particular domain, or more major in nature such as reflected in the major conformations noted for the HSP90s and HSP70s. Ligand-induced or captured local changes are numerous and best understood for the HSP90s and HSP70s [32, 85, 86, 100, 101]. For example, the N-terminal domain pan-HSP90 inhibitors insert themselves into the same pocket (Figure 2A). However, they take advantage of the flexibility of the residues surrounding such pocket to result in slightly distinct interaction modes. Taldone *et al* performed a study of the major clinical inhibitors to show that three sub-pockets, presented distinctly as the amino acid residues move during lid opening and closing, are available for these agents and that these agents differ by the sub-pockets they extend into [85]. Because these sub-pockets and their presentation are distinct among the four HSP90 paralogs, this study also provided a basis to understand the paralog binding profile observed for the numerous pan-HSP90 inhibitors. Taking advantage of these distinctions, especially in the conformational flexibility of an amino acid sequence (Figure 2B, the amino acid sequence is INNLGTIA shown in red) in the N-terminal binding pocket of HSP90 α , Ernst *et al* developed a ligand selective for HSP90 α/β [86]. This sequence is identical for HSP90 α and β but differs in the first two amino acids in GRP94 (VK) and TRAP1 (VS) [88]. Testing a diverse set of HSP90 inhibitor chemotypes they noted that the compounds that use the α -helical binding conformation of HSP90 α (*i.e.* BIIB021 and SNX-2112 analogs) show the highest degree of selectivity against GRP94 and TRAP1 [88]. Using a structure-based approach they prepared novel benzolactam derivatives that were able to access this conformation (Figure 2B) of HSP90 α resulting in selective low nanomolar Hsp90 α/β inhibitors (>1000 fold selective vs. GRP94 and TRAP1) [86]. A similar approach was used by Patel *et al* to identify ligands selective for another HSP90 paralog, GRP94 [32]. To this end, Patel *et al.* used a strategy that combined library screening and structural studies to discover purine based ligands that were more than 100-fold selective for GRP94 over HSP90 α/β and TRAP1 [32]. These ligands took advantage of the conformational flexibility of GRP94 to “freeze” the protein in a state that

unveils a pocket possible uniquely in GRP94 due to a 5-amino acid (QEDGQ) insertion that induces a shift in a residue (Phe199) to provide access to this novel allosteric site (Site 1, Figure 2C). The study provided tool compounds such as PU-H54 and PU-WS13. Another reported GRP94 inhibitor is 5'-N-ethylcarboxamidoadenosine (NECA, Figure 2C), an adenosine A₂ receptor antagonist. This compound takes advantage of a distinct GRP94 conformation which opens up another cavity that is accessed by the 5'-N-ethylcarboxamido moiety of NECA (Site 2, Figure 2C) but cannot be accessed in HSP90 [100]. Other compounds reported to bind to GRP94 in a similar manner to NECA are the resorcinol based compounds Radamide [87] and the N-benzylimidazole, Compound 2 (Site 2, Figure 2C) [102] for which selectivity was proposed using docking studies.

Interestingly, these local conformational preferences of HSP90 ligands translate toward global conformational preferences, where for example some ligands were found to prefer an open conformation of the HSP90 while others a client-protein bound, closed conformation [8, 103]. As each conformation is characterized by a distinct epigenetic profile (i.e. may harbor distinct PTMs and bind different co-chaperones and adapters), it may interact with a client protein at a distinct maturation state or may carry no protein interactor at all, the inhibitor will therefore encounter a plethora of target-containing complexes [24, 104], each having a different binding affinity for the small molecule inhibitor.

Interacting with HSP90 at sites distinct from the nucleotide-binding pocket again elicits a distinct profile, as for example seen for certain C-terminal binders such as the novobiocin-derived KU174 and celastrol (Figure 2A), which elicit and/or prefer an HSP90 conformation distinct from each other and also distinct from that induced by GM [90]. A similar view is now emerging for HSP70 where, for example, certain inhibitors such as those that act through insertion into allosteric sites located in the N-terminal domain (i.e. YK5 and MKT-077, Figure 2D), appear to sample preferentially the ADP-bound conformation [93, 101, 105, 106]. These allosteric pockets become preferentially available in this conformational state, where binding of a nucleotide exchange factor such as HSP110 stabilizes a more open N-terminal domain of HSP70 [101]. Interestingly, depending on their mode of interaction with HSP70, inhibitors also preferentially act on specific complexes, as measured by their ability to inhibit the ATPase activity of HSP70 [93, 107]. Whereas, direct ATP-pocket binders such as VER-155008 inhibit the intrinsic and the HDJ1 (an HSP40) stimulated activity of HSP70 [107], others such as YK5 appear to prefer the HSP110/HSP40 stimulated ATPase activity [93], potentially because its binding pocket is only available in the HSP110-bound conformation.

Another variable that influences HSP inhibitor activity is its sampling capacity (Figure 3). For HSP90, it has been shown that chemically distinct inhibitors that target the same binding pocket are able to select for overlapping but not identical subpopulations of HSP90 [8, 56]. GM and PU-H71 are both ATP competitive inhibitors that bind to the N-terminal nucleotide binding pocket (Figure 2A), yet PU-H71 is less affected by HSP90 phosphorylation and is capable of accessing a broader range of stress HSP90 conformations, and thus complexes [56].

A yet another important variable is cellular location (Figure 3). Altered cellular location of chaperome members has been reported in numerous diseases, although it has been mainly studied in the context of cancer [21, 25–30, 32, 108]. The ability of small molecules to engage any such complexes is highly determined by their ability to permeate cellular membranes and thus access such distinct cellular locations. Unfortunately, no study was performed to address this question. On the other hand, knowledge of the altered mitochondrial location of HSP90 in certain cancers, led the Altieri group to link GM to a mitochondria-targeting sequence [109]. This compound, gamitrinib, quickly transferred into mitochondria where it engaged both HSP90 found translocated in the mitochondria in certain cancers, and its paralog TRAP1 [109, 110].

Such complexity in the cellular presentation of HSPs helps explain why, although the majority of HSP90 inhibitors interact with its nucleotide binding pocket, the phenotype that is observed in cells [56, 66] and moreover, their *in vivo* properties as measured by efficacy and therapeutic index, hardly overlap among these inhibitors [66, 111–119]. There are two factors that one may analyze to understand such paradigm [66] (Figure 3). One is how well the small molecule interacts over the time it spends in the cancer cell with the relevant active HSP90 complexes (Figure 3A). Another is how selective the small molecule is for the active HSP90 complexes over those with housekeeping functions (Figure 3B). The first concept is not obvious; after all, these inhibitors occupy the same pocket. This may imply that the sampling capacity of a ligand for the stress complexes is a direct measure of its potential biological activity, i.e. potency. Evidence for this concept comes from tumor pharmacokinetics (PK) and pharmacodynamics (PD) studies. For example, while retention in the tumor mass was observed for several HSP90 inhibitors that ultimately progressed toward clinical testing, the tumor residence time was distinct among these inhibitors [111–117]. Similarly, while an extended tumor PK was noted for most such inhibitors, this did not always correlate with the extent and duration of HSP90 client downregulation, i.e. with the PD effect. The interwoven relationship between tumor PK and PD effects for an HSP90 inhibitor has long been appreciated [120, 121]. These two factors are likely affected by the HSP90 species targeted by the inhibitor. As such, the kinetics of association (k_{on}) and more critically dissociation (k_{off}) can vary depending on the particular complexes that the inhibitor is bound to; this, in turn, has a direct influence on the resulting PD profile and ultimately, efficacy. While in studies in tissue culture one may fully saturate the HSP90 sites in the cancer cell by adding more inhibitor, this is hardly the case in mice or humans. Human patients are not test tubes where the inhibitor is added at a constant concentration over a set period of time. The residence time of a compound is influenced by its rate of biodistribution (i.e. how fast does it reach the tumor), rate of clearance (i.e. how fast is cleared from the plasma and surrounding normal tissue) and as mentioned above, its target engagement (i.e. sampling of global HSP complexes present in the cell).

Another implication of such distinct HSP species relates to the therapeutic index of HSP targeting compounds (Figure 3B). The advantage of selectively targeting the stress chaperome versus the housekeeping chaperome is two-fold in the development of drugs. First, the better the selectivity, the less the agent will be interfering with HSP functions in normal cells, and ergo it will be less toxic. Lower toxicity means more drug can be

administered to the patient, and thus, a better engagement of the target at the site of disease will be possible, ergo the better the efficacy of the agent in treating disease. Indeed, evidence from *in vivo* studies supports a distinct interaction of HSP90 ligands with the stress versus the housekeeping target. A general property of most HSP90 inhibitors that have shown promising *in vivo* activity and translated to clinic is that they are retained in tumors for prolonged periods of time while being rapidly cleared from normal tissues and plasma [111–116]. This results in long residence time of the drug selectively in the tumor and suggests that the off rate (k_{off}) of drug bound to HSP90 in tumor tissues and normal tissues is very different and that it is much lower in tumor HSP90 (tumor $k_{\text{off}} \ll$ nontumor k_{off}). The reason for the significant difference in the k_{off} rate between tumor and normal tissues is likely due to the stress HSP90 species that are found only in cancer cells (and other stressed cells).

Concluding remarks

The recent realization that the stress chaperome is epigenetically distinct from its normal, housekeeping counterpart has opened the door for its selective targeting by small molecules. The prospect of selectively targeting the ‘bad’ side of stress chaperones offers a novel and exciting therapeutic strategy for a range of diseases that are dependent upon their aberrant functions. This prospect is becoming more of a reality through our increasing understanding of the biochemical makeup of the HSPs that direct these two opposing functions and by ligands which have been shown to be able to discriminate between the two. A greater understanding of how epigenetic factors direct the function of HSPs and more pointedly, how they coerce them to function in a manner which is ultimately harmful to the organism (human) will continue to be a major avenue for research in the field.

Recently, there has been a multitude of ligands that have been reported to modulate various HSPs. These small molecule probes have played a vital role in teaching us the biology that we currently know. In fact, probes such as GM have enabled a chemical approach to our current understanding of the dual nature of HSP90 in a manner which genetic approaches are not able to. Although an HSP ligand may be defined as a chemical that interacts with an HSP and modulates its function, an HSP-directed chemical tool requires a more stringent selection, and criteria related to selectivity for the stress chaperome species are key in its use in dissecting mechanisms related to disease and in investigating the potential of the HSPs as targets in disease. The goal is now to translate these agents into clinically useful drugs. While no drugs directly targeting the HSPs are currently approved by the FDA, efforts during the last decade or so in the development of HSP90 inhibitors for cancer have taught us many valuable lessons that can serve as a platform for the development of molecules targeting other HSPs (Box 2). We now know that as a result of the heterogeneous presentation of HSPs in disease a more nuanced approach is required to drug development, one that allows for a clearer understanding of the potential of the ligand to target the aberrant species while minimally affecting HSPs engaged in normal proteostasis. Pre-clinically, this entails extensive testing in various transformed and nontransformed systems in order to gauge the potential of the molecule to discriminate between the various HSP species. Extensive pharmacokinetic evaluation of these agents is required in order to determine distribution into diseased tissues (e.g. tumor) as well as clearance from normal

tissues (e.g. noncancerous) and to get a sense of the degree of target engagement at the site of disease and to gauge the potential for toxicity at normal tissues. The translation of these agents into the clinic will require a rethinking as to how drugs are currently developed where plasma PK is often used as a surrogate for tumor drug concentrations. Therefore, alternative noninvasive methods are required in order to determine levels of drug and target engagement at the site of HSP-drug action.

Box 2

Limitations in the use of classical medicinal chemistry approaches to the development of HSP inhibitors

The normal paradigms of drug discovery and development do not necessarily apply towards the development of HSP agents. Traditionally, drug candidates have been optimized by improving affinity for the target (largely measured in a biochemical assay) and by modifying pharmaceutical properties (e.g. lipophilicity, permeability, metabolic stability, plasma protein binding, etc.) that seek to optimize *in vivo* plasma PK. Because the content-specific epigenetic modifications of HSPs cannot be recapitulated in biochemical assays, HSP drug discovery may benefit from a more elaborate approach, in which feedback between structural information, biochemical data and phenotypic assays informs on both selective and high-affinity targeting of the stress complexes. Another important factor that needs re-examining is the plasma PK. In current practice, it serves as a surrogate for tissue distribution and concentrations since it is more practical to obtain plasma samples for determination of drug concentrations. This optimization paradigm results in compounds with relatively high plasma half-life, meaning compounds remain in circulation for a long time and as such are exposed to all tissues. This is contrary to what is necessary and observed for HSP agents (HSP90 direct ATP-pocket interactors), where rapid clearance from plasma (low plasma half-life) and from normal tissues while concentrating into the tumor and engaging the tumor HSP species for prolonged period of time (high tumor half-life) are desired. Such PK profiles at the site of disease (i.e. tumors, neurodegenerative neurons) have implications for clinic, especially when it comes to the choice of dose and schedule of administration. So far, only HSP90 inhibitors that act via the ATPase pocket have moved to clinic in cancers. In preclinical tumor models, these successful candidates each show the property of area-under-the-curve ($AUC_{\text{tumor}} \gg AUC_{\text{tissue}}$), meaning the time residence of the inhibitor is much higher at the site of action than it is in the surrounding tissues. Such a property may obviate the need for continuous dosing and indicates that intermittent administration ensures that the tumor is continuously exposed to drug. Thus the therapeutic index of such agents may be improved by the choice of a schedule that prioritizes exposure of target at the site of action. How can stress HSPs be targeted selectively in practice so as to maximize the therapeutic benefit while minimizing the potential toxicity? An answer may be the residence time of a drug at the site of action, understood not as a ratio of $AUC_{\text{tumor}}/AUC_{\text{tissue}}$ but rather as the absolute value of AUC_{tumor} that is high and an AUC_{tissue} that is low. As described earlier, successful HSP90 inhibitors in general exhibit high AUC_{tumor} , however, they are likely distinguished by their relative exposures to other

tissues. In fact, this variance has been attributed as the cause for ocular toxicity in some inhibitors but not others [123].

There has never been a stronger impetus towards the identification of HSPs modulators as in the past decade. Still, despite the numerous ligands discovered we are still limited by the reduced number of chemical tools and lack of approved drugs. This landscape is poised to change in the future, should we implement our recent insights into the distinct epigenetic and thermodynamic nature of the chaperome in stressed cells. Lessons learned from the development of HSP90 inhibitors have taught us that a one size fits all approach does not apply, rather a ‘personalized’ approach is required in order to decide on the best course of action. With these lessons in mind, agents that target the stress chaperome could be poised to become the next generation of drugs used to treat various diseases.

Acknowledgments

This work was partially funded by U01AG032969, R01CA172546, R01CA155226, R01CA187490, R21AI090501 and R21CA158609.

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Highlights

- The stress and the housekeeping chaperome are epigenetically distinct
- Small molecules may “sense” these epigenetic and thermodynamic differences
- Epigenetic modifications of the stress chaperome provide the basis for selective targeting
- Development of stress chaperome inhibitors require a new, stress-specific, paradigm

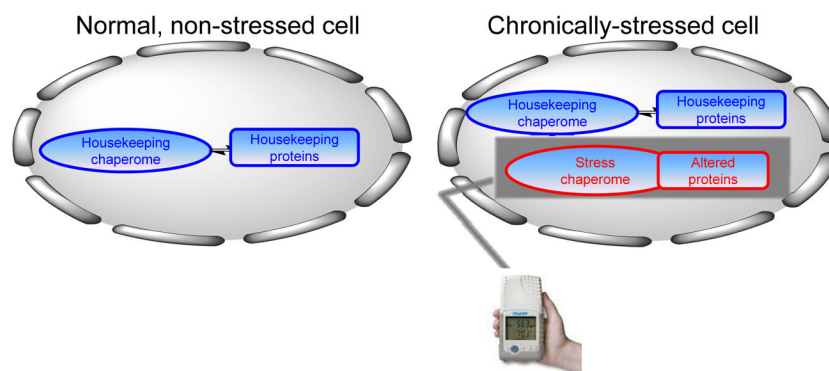
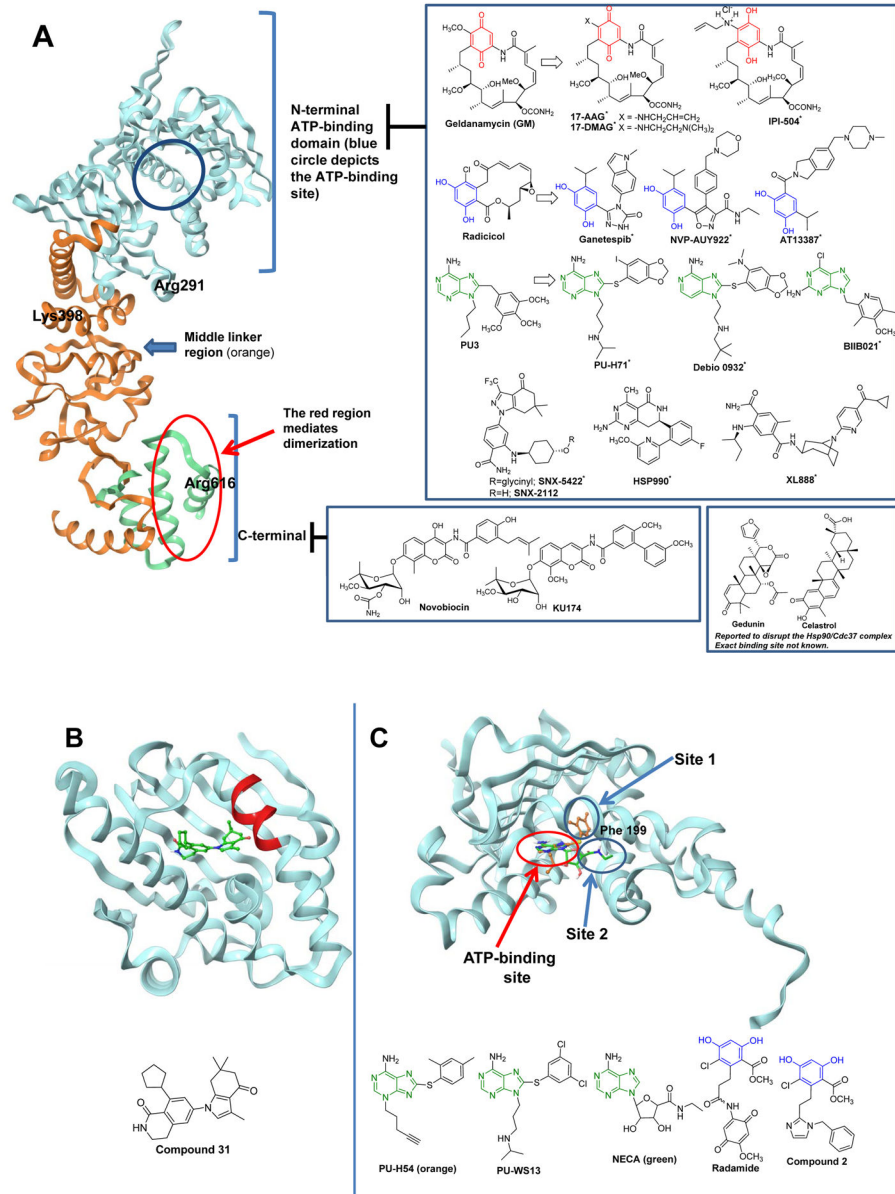


Figure 1.

The stress-associated cell contains at any time a mixture of chaperome complexes. Some retain the nature of the “housekeeping” complexes of non-stressed, normal cells, in that they are characterized by a dynamic association between the chaperome and the proteome it regulates. Others, epigenetically and thermodynamically distinct from the “housekeeping” species, are characterized by an increased association between the chaperome and the altered proteins. Such distinct features of the stress chaperome provide the basis for their selective targeting by small molecules. Pharmacologic (chemical) tools may therefore be designed to specifically target the stress chaperome. These stress chaperome-specific chemical tools act as “sensors” of stress (depicted by the hand-held sensor device) and therefore enable to investigate, identify and treat stress-associated cellular states.



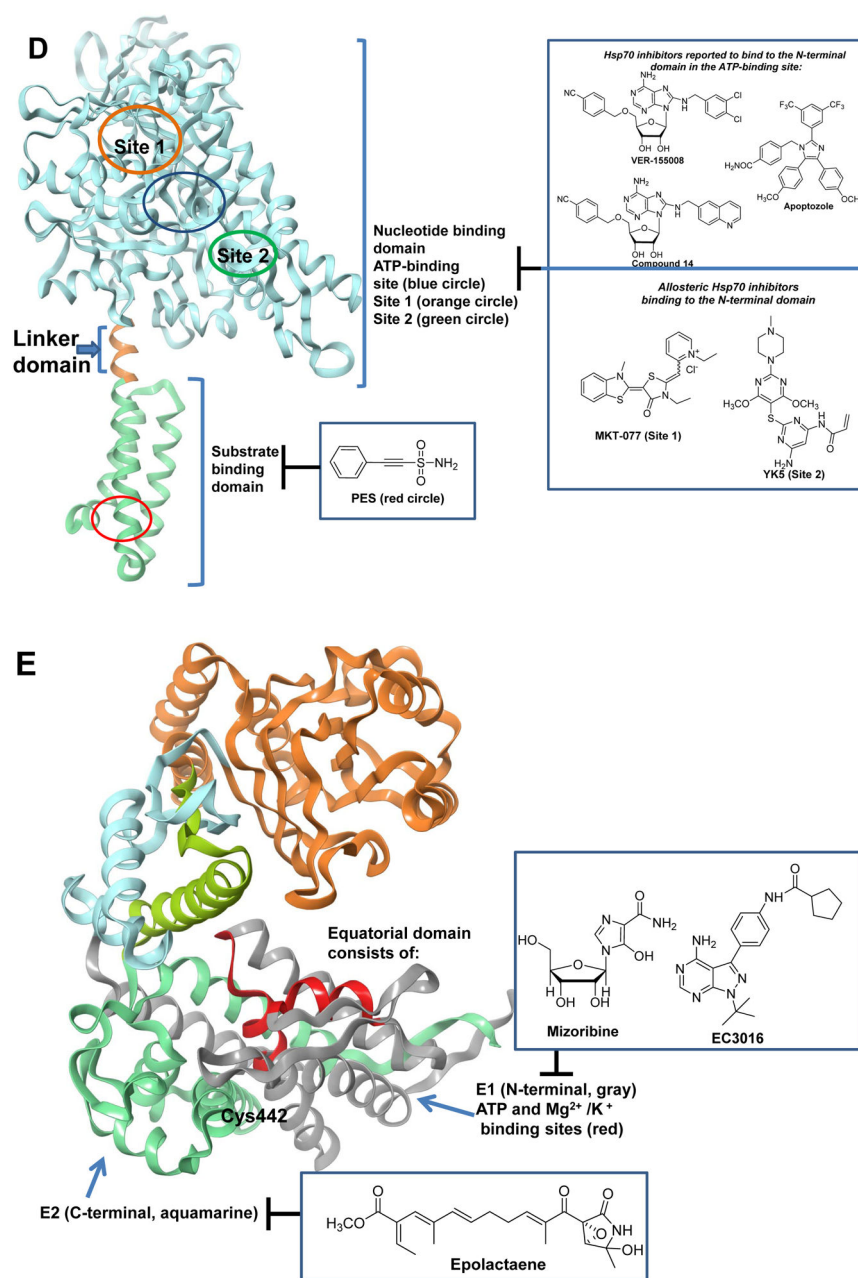


Figure 2.

Representative HSP binders and their demonstrated or proposed mode of interaction with the protein. **A.** Modulators of HSP90. The ribbon representation of the full-length monomer HtpG (*E. coli* Hsp90, PDB: 2IQO) is shown. Chemical structures: red, the benzoquinone/hydroquinone core; blue, the resorcinol core; green, the purine and purine-like core. *These compounds have advanced to clinical studies. **B.** Ribbon view of the N-terminal domain of human Hsp90 α in complex with the selective modulator compound 31 (PDB: 4O0B). **C.** Ribbon view of the N-terminal domain of GRP94 (*Canis lupus familiaris*, PDB: 3O2F) in complex with the selective binder PU-H54 (Site 1). The binding mode of NECA (Site 2) and the structure of other proposed GRP94 ligands are also shown. For NECA, its complex with

GRP94 (PDB: 1U2O) was overlaid with that of PU-H54 to indicate the distinction between the two binding sites. **D.** Various HSP70 modulators and their proposed sites of interaction with HSP70. The ribbon view of the HSP70 homolog DnaK (*E. coli*, PDB: 4B9Q) is shown. **E.** Ribbon view of GroEL (*E. coli*, PDB: 1XCK) and the proposed binding domains for reported HSP60 modulators. Cysteine residues (e.g. Cys442) are present only in human HSP60; Cys442 was added to indicate the potential interaction site of the ligand with the human protein.

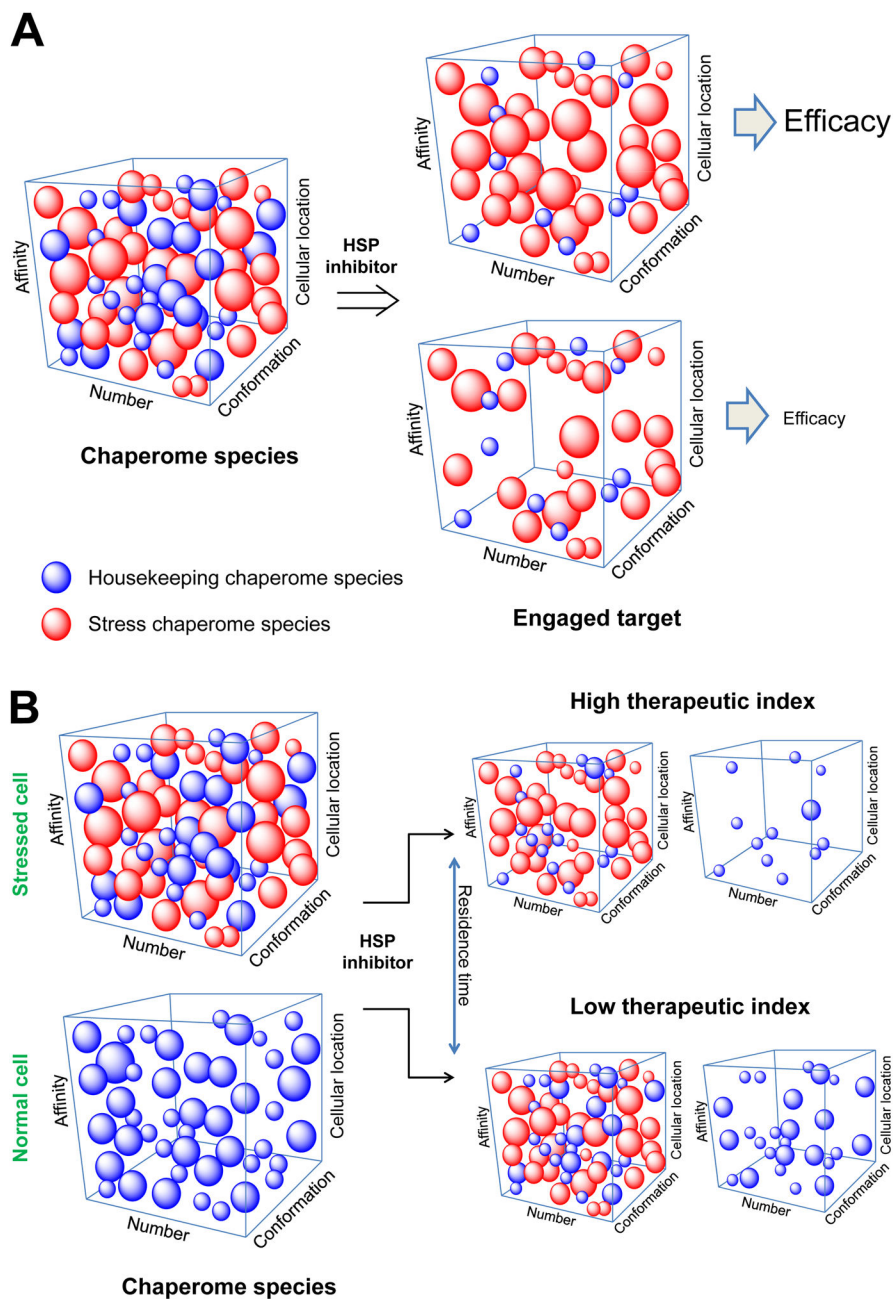


Figure 3. Features that influence the efficacy (**A**) and the therapeutic index (**B**) of chaperome inhibitors. Each cell is characterized by a complex mixture of chaperome complexes; normal cells maintain the “housekeeping” chaperome complexes, whereas stressed cells are enriched in “stress” chaperome species. The constituency of these complexes is driven by the proteome, resulting in a plethora of chaperome species differentiated by affinity, conformation, number and cellular location. The conformation the HSPs adapt and the epigenetics that they display (defined as both chemical modifications by PTMs and biochemical modifications by co-chaperones and adapter proteins) results into chaperome

complexes of distinct thermodynamic nature, i.e. affinity for the small molecule ligand. The proteome also dictates the number of chaperome complexes present in a cells, as well as their cellular location; these change as needed, in order to appease (or buffer) alterations as they occur in the proteome. Small molecules sample and engage these complexes over the time they spend in the cell. These characteristics help explain the two major factors that may determine the efficacy (**A**) and the therapeutic index (**B**) of chaperome inhibitors. Efficacy is regulated by how well the small molecule interacts with the relevant active stress chaperome species over the time it spends in the stressed cell (**A**). Therapeutic index is determined by how selective the small molecule is for the stress over the housekeeping chaperome species (**B**).