



Published in final edited form as:

J Mol Biol. 2015 September 11; 427(18): 2931–2947. doi:10.1016/j.jmb.2015.05.010.

The chemical biology of molecular chaperones - Implications for modulation of proteostasis

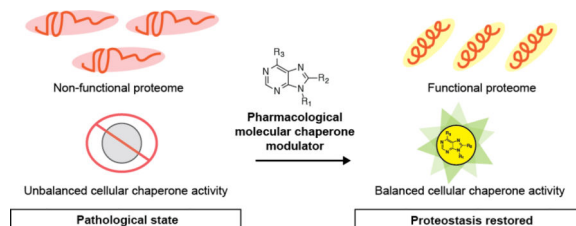
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Abstract

Protein homeostasis (proteostasis) is inextricably tied to cellular health and organismal lifespan. Aging, exposure to physiological and environmental stress, and expression of mutant and metastable proteins can cause an imbalance in the protein folding landscape, resulting in the formation of non-native protein aggregates that challenge the capacity of the proteostasis network (PN), increasing the risk for diseases associated with misfolding, aggregation and aberrant regulation of cell stress responses. Molecular chaperones have central roles in each of the arms of the PN (protein synthesis, folding, disaggregation, and degradation), leading to the proposal that modulation of chaperone function could have therapeutic benefits for the large and growing family of diseases of protein conformation including neurodegeneration, metabolic diseases, and cancer. In this review, we will discuss the current strategies used to tune the PN through targeting molecular chaperones and assess the potential of the chemical biology of proteostasis.

Graphical abstract



Keywords

Protein folding; aggregation; heat shock protein; small-molecule modulators; pharmacology

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Introduction

All free-living prokaryotes and eukaryotes rely on the proteostasis network (PN), a conserved cellular machinery to support all aspects of protein biogenesis, to adapt to a complex and changing environment, and to determine organismal lifespan. Quality control processes are central for biological fidelity, and together with cell stress responses that detect and react to acute and chronic imbalances are essential for evolutionary robustness. For proteins, this is exemplified by proteostasis, the process that integrates signals and regulates flux from synthesis through folding, transport, and clearance, which altogether ensures cellular, tissue, and organismal health [1].

At the core of the PN are the molecular chaperones that detect fluctuations in protein conformation and can determine the functional properties of client substrates. Many molecular chaperones were discovered as heat shock proteins (HSPs) whose expression is induced by transient exposure to acute elevated temperatures and other forms of environmental and physiological stress [2]. The HSPs were initially subdivided into six groups based upon their molecular weight: sHSP, HSP40, HSP60 (chaperonins), HSP70, HSP90 and HSP100, although an updated classification based upon function has been recently proposed [3]. In addition to the role of molecular chaperones in macromolecular assembly and disassembly of proteins, interactions of chaperones with clients can confer alternative conformational intermediates that influence function.

An intrinsic feature of protein folding is metastability and the sampling of alternative conformational states. This freedom to sample structural conformations, therefore, allows for the probability that non-functional and potentially toxic folds are accessed during aging and disease. Adjusting the cellular balance of chaperones to drive folding equilibrium towards functional conformers or to promote degradation of non-functional states compensates for perturbations in the protein folding landscape. As modern medicine identifies an increasing number of diseases that result from the aberrant expression of metastable proteins, strategies that modulate the PN could have broad beneficial consequences. Furthermore, the composition of, and need for, PN components varies among tissues and changes over time, which infers opportunities to tune and re-tune the PN to achieve tissue-specific effects.

The concept that molecular chaperones could be targeted for therapeutic benefit was initially met with concern because of their evolutionary conservation, ubiquitous expression, high cellular abundance, and central roles in all aspects of protein biogenesis. However, the therapeutic value of shifting the protein folding landscape through pharmacological effects on molecular chaperones is highlighted by the recent development of small-molecule proteostasis regulators for neurodegeneration diseases and cancer. Much emphasis has been placed upon finding small-molecules that regulate the expression of multiple molecular chaperones simultaneously or by directly influencing the activity of isolated chaperone proteins.

From a rational ligand-design perspective, (Fig. 1) current approaches to directly target molecular chaperones with small-molecules largely fall into three classes: 1) ATP-

competitive inhibition, 2) modulation of substrate binding and 3) modulation of co-chaperone interactions. Small-molecule binding of the ATP pocket disrupts the hydrolysis event that allosterically regulates the conformation of the substrate-binding domain in chaperones such as HSP70, HSP60, and HSP90 (Fig. 2). Conversely, substrate binding and co-chaperone interactions must be modulated through small-molecule targeting of protein-protein interactions. Fewer small-molecule modulators have been identified from these latter approaches due to inherent difficulties of targeting sites that are typically flat and solvent-exposed, but targeting proteins via such sites is becoming increasingly feasible [4, 5]. Although modulating chaperone activity through targeting co-chaperone binding offers the opportunity to selectively enhance or inhibit chaperone function, an important consideration is that this may have unintended consequences on the folding landscape of other chaperone clients.

Finally, the development of novel chemical tools to modulate the protein-folding landscape will help unravel the cellular roles of various PN components. This represents an essential step toward efficacious pharmacological modulation of the PN. In this review, we draw from the recent literature to highlight small-molecules with “drug-like” properties that are reported to affect specific chaperone targets in human and other relevant model systems, with a focus on the HSP90, HSP70 and HSP60 machines. We present examples of available chemical strategies, followed by discussion of how these probes shape the current outlook on the pharmacology of molecular chaperones, and highlight, where appropriate, underdeveloped areas. Other strategies for pharmacological modulation of proteostasis, such as targeting stress-inducible transcription factors (i.e. HSF-1) [6], will not be discussed and are beyond the scope of this review.

HSP90

Function

HSP90 molecular chaperones are amongst the most abundant cellular proteins and correspond to approximately two percent of the cellular proteome under normal conditions of cell growth. The human HSP90 family is comprised of four members that exhibit distinct subcellular localization, with two isoforms HSP90AA1 (inducible) and HSP90AB1 (constitutive) in the cytosol and nucleus, GRP94 (94-kDa glucose-regulated protein) localized to the endoplasmic reticulum, and TRAP1 (tumor necrosis factor receptor-associated protein 1) in mitochondria. HSP90 interacts with a wide spectrum of protein clients that includes many protein kinases [7–9], nuclear receptors [10–12], and transcription factors [13–16] and thus has a pivotal role in cell signaling. For example, HSP90 regulates transcriptional responses through the chaperone-dependent assembly of steroid aporeceptors such as the glucocorticoid receptor [17] and chaperone-regulated control of HSF-1 and the heat shock response [16].

Structure

HSP90 is comprised of a C-terminal dimerization domain that is coupled to an ATPase cycle directed by the N-terminal domain [18, 19], and a middle regulatory domain that links the termini [19–21] (Fig. 3). The capacity for substrate binding is dependent upon HSP90

cycling between “open” or “closed” conformations, which requires a large conformational transition that is ATP-dependent [22–24]. ATPase cycling is regulated by co-chaperones that influence the hydrolysis reaction rate [25–27] and facilitate substrate association as observed with the HSP90-progesterone receptor interaction that requires HOP [28], and additionally in the case of protein kinases, which are delivered to HSP90 via the co-chaperone CDC37 [29].

Co-chaperones

The largest family of HSP90 co-chaperones are tetratricopeptide repeat (TPR)-containing proteins [26], although not all of these proteins have been analyzed for co-chaperone activity. TPR co-chaperones do not directly bind non-native proteins, but have an integral role in directing the activity of HSP90, for example HOP serves as an adaptor and facilitates the shuttling of non-native substrates from HSP70 to HSP90 [30]. The N-terminal TPR domain (TPR1) of HOP is responsible for interaction with the C-terminus of HSP70, whereas the C-terminal TPR domain (TPR2) facilitates interaction with HSP90 [31]. An EEVD motif in the C-termini of HSP90 and HSP70 anchors the TPR-chaperone interaction in the case of both TPR1 and TPR2, with specificity conferred by residues on the N-terminal side of the EEVD sequence [32]. TPR1 and TPR2 form helical bundles that cradle the EEVD-containing peptide substrates in defined grooves [32].

Roles in pathology and disease

Given its central role, it is unsurprising that HSP90 is implicated in many human pathologies. Deregulation of HSP90 supports oncogenic transformation [33], which is largely attributed to its role in the stabilization of signaling proteins involved in cell growth and proliferation [34], evasion of apoptosis [35] and metastasis [36]. HSP90 expression is increased in tumor cells, and high expression is correlated with poor prognosis [37]. The degradation of the mutant cystic fibrosis transmembrane conductance regulator (CFTR) protein is also dependent upon HSP90, as demonstrated in a yeast-based model for cystic fibrosis [38, 39]. In neurodegenerative diseases, such as Alzheimer’s disease, Parkinson’s disease and the polyglutamine diseases (ie. Huntington disease, spinobulbar muscular atrophy, dentatorubral-pallidoluysian atrophy and spinocerebellar ataxias), HSP90 has been observed to stabilize the associated toxic oligomers associated with these conditions [40], presumably through inhibition of HSP70-promoted ubiquitination [41].

Pharmacological targeting of HSP90

The small-molecule natural product geldanamycin, which was initially identified for antimicrobial activity, was shown to have potent cytotoxic activity against two cancer cell lines [42] and later shown to be broadly active in a screen against a panel of 60 cancer cell lines (NCI-60) [43]. Although early investigations suggested that geldanamycin was a tyrosine kinase inhibitor, this turned out to be an indirect consequence of protein kinases being an HSP90 client [44]. Subsequent biochemical [45] and crystallographic [46] studies established that geldanamycin binds the ATP-pocket in the N-terminal domain of HSP90. These findings stimulated the development of many synthetic analogs based upon geldanamycin’s ansamycin pharmacophore [47–49] (Fig. 3) as well as the search for other

scaffolds that inhibit HSP90, some of which are in clinical trials for the treatment of cancer [50, 51].

As there is a substantial literature on small-molecules that target HSP90 [52–54], we will comment on only a few examples. Of the numerous small-molecules that have been found to bind HSP90, many of these prevent folding activity through the interactions in ATP pocket. In addition to the natural product radicicol [55] (and its derivatives [56–58]), other classes of small-molecules that target the HSP90 N-terminal ATP-binding site include the synthetic heterocycles including purine ATP-mimetics [59–63], pyrazoles [64–67], and isoxazoles [68, 69], among other scaffolds [51, 70–74]. The HSP90 ATP pocket has proven to be quite amenable to small-molecule binding, and many inhibitors bind this region with high affinity (comparable to geldanamycin's nanomolar binding constant).

Although the HSP90 ATP pocket has received the most attention, a complimentary approach to targeting this chaperone is via the C-terminus. The coumarin antibiotic, novobiocin [75], was the first small-molecule observed to bind the HSP90 C-terminus, as assessed by the ability of an immobilized derivative to selectively interact with this domain [76–79]. These observations elicited efforts to identify other C-terminal HSP90 interactors, many of which are based upon the novobiocin scaffold (Fig. 3). In contrast to inhibitors that bind the N-terminus, novobiocin derivatives that target the C-terminal domain slow the growth of cancer cells and promote degradation of HSP90 clients without inducing the heat shock response, which is an important and somewhat unexpected distinction [80]. Other non-ATP competitive HSP90 inhibitors are known, but most are non-selective, which limits their *in vivo* use; examples include epigallocatechin gallate [81] (inhibits several non-chaperone targets [82–86]), cisplatin [87] (damages DNA [88]), and silybin [89] (inhibits P-glycoprotein [90] and cytochrome P450 [91]).

A particularly interesting class of small-molecules is capable of modulating co-chaperone access to the EEVD-motif in the HSP90 C-terminus. The macrocyclic peptide “compound 2” [92] (Fig. 3) was discovered in a structure-activity-relationship study on the Sansalvamide A pharmacophore, and was shown to allosterically prevent binding of HSP90 co-chaperones IP6K2, FKBP38, FKBP52, and HOP in a biochemical assay using purified proteins [93]. In a separate study, a high-throughput screen that used an *in vitro* assay to monitor the HSP90-TPR2A protein-protein interaction, revealed a small-molecule, C9, which has a 7-azapteridine core, that directly binds several TPR-containing co-chaperones [94, 95]. C9 directly binds the TPR2A domain of HOP, as assessed by fluorescence-polarization and isothermal titration calorimetry, presumably via the peptide groove where the co-chaperone-chaperone protein-protein interaction occurs. The feasibility of targeting HSP90 co-chaperones is further supported by the discovery that the tetranortriterpenoid natural product, gedunin [96], mediates apoptotic cancer cell death through binding p23 [97].

Application of small-molecule HSP90 modulators

Since the early observation that geldanamycin has cytotoxic activity in cancer cell lines, significant effort has been expended to develop HSP90 inhibitors for the treatment of cancer [51], but these efforts have yielded limited success. Despite many clinical trials there is still

not a single FDA-approved HSP90 inhibitor, which is largely due to the toxicity of candidate compounds. Although the early clinical trials may sustain the initial concerns raised with respect to pharmacological modulation of chaperones, many of the clinical candidates exhibit only modest selectivity among HSP90 isoforms [98], and perhaps modulators with greater isoform fidelity may prove less toxic. Additionally, because HSP90 family members are expressed in each subcellular compartment, it could be argued that increased regulatory control may come from the development of isoform-selective inhibitors.

The development of isoform-selective HSP90 inhibitors is challenging because of the high degree of structural similarity among the four human paralogues, however recent reports suggest progress on paralogue-specific inhibitors [99, 100]. Some of these paralogue-specific inhibitors exhibit different phenotypic outcomes relative to their non-selective counterparts, for example that GRP94 is disproportionately involved in the chaperoning of the HER2 protein in SKBr3 breast cancer cell lines relative to the other HSP90 isoforms [101]. Likewise, inhibitors optimized for HSP90 α/β specificity displayed lower toxicity than pan-inhibitors when characterized in a cell-based model that monitored mutant Huntingtin clearance [102].

While conceptually promising, the treatment of neurodegenerative diseases using HSP90 inhibitors, has been complicated by activation of the heat shock response, and the fact that inhibition of HSP90 disfavors association and stabilization of the oligomerization-prone clients, which in-turn promotes degradation [103]. This highlights an important consideration of HSP90 inhibitors, that general inhibition of HSP90 client interaction will have both positive and negative effects on cell protective mechanisms because of its central role in many cellular processes. Development of HSP90 inhibitors with differential effects on clients, however, could have selective effects on diseased tissues.

Altogether, the application of HSP90 inhibitors toward modification of the PN in cancer has hinted that a highly conserved chaperone system may be targeted in living cells, but this must be carefully controlled. Preliminary indications suggest that small-molecule HSP90 modulators could have broad benefits in disease. Targeting the ATP-binding site has successfully afforded many potent HSP90 inhibitors, but selectivity is usually only modest, in large part due to high structural similarity in this region. The targeting of areas outside the ATP pocket is promising for increasing the selectivity of HSP90 modulators, and in some cases, provides divergent phenotypic profiles relative to their ATP-competitive counterparts [104], therefore even though these sites are more difficult to target, further development is warranted. Additionally, the targeting of co-chaperone interactions remains a largely undeveloped approach for reasons similar to the C-terminal binders, but this may provide a way to finely adjust HSP90 function.

HSP70

Function

HSP70 corresponds to another highly conserved and abundant chaperone that functions in the context of co-chaperones (i.e. J-domain, Bag, Hsp110) to regulate multiple diverse

folding processes, including disaggregation, transport, and clearance of proteins. The human HSP70 family includes at least 17 members, represented by HSC70 [105, 106] that is expressed constitutively and HSP70s [107, 108] that are inducibly regulated by stress conditions. HSP70s are localized to all major subcellular compartments including HSC70 and HSP70 in the cytosol and nucleus, BiP (GRP78) in the endoplasmic reticulum, [109, 110], and GRP75 in the mitochondria [111].

HSP70 employs cycles of holding and folding with a diverse array of clients through transient interactions with hydrophobic motifs that are typically confined to the core of a properly folded protein. Consequently, a prominent class of HSP70 substrates are nascent polypeptides [112, 113] whose folding is promoted via the ribosome-associated complex [114]. Non-native and misfolded mature clients rely on HSP70 to guide refolding or redirect to the degradative machineries (through both lysosomal [115] and proteasomal [116] pathways), moreover the appearance of aggregates by metastable and aggregation-prone species is suppressed through promoting solubility through chaperone interactions [117]. In addition to interactions with a multitude of clients throughout the process of protein biogenesis, the HSC70 isoform also acts to disassemble clathrin triskelia in clathrin-mediated endocytosis and affords a key regulatory step in receptor recycling and endocytic trafficking [118].

Structure

HSP70-substrate interactions occur in the C-terminal domain and are dependent upon ATP hydrolysis, which occurs in the N-terminal domain (Fig. 4). The ATP-bound form has low (micromolar) affinity for its substrates, which allows for rapid exchange, whereas the ADP-bound form has higher affinity for its substrates and exchange rates are retarded [119]. Several hydrolysis cycles can be necessary for complete folding to a native state. *In vitro*, HSP70 has very low ATPase activity [120], which disfavors the binding and dissociation events that are necessary for the folding cycle. The ATPase activity of HSP70 is amplified through association with HSP40/DnaJ proteins, and through nucleotide exchange factors, such as BAG domain-containing proteins, which complete the cycle through reloading the chaperone with a new ATP molecule.

Co-chaperones

Functions of HSP40 include directing non-native clients to the HSP70 machine, and acting as an HSP70 co-chaperone to promote hydrolysis of ATP to ADP. HSP40 proteins are J-domain proteins, which are generally divided into three classes. The type I and type II classes are defined by a multidomain structure comprised of an N-terminal J-domain, a Gly/Phe rich region, and a C-terminal domain; the type I class additionally contain a cysteine-rich region (zinc-finger domain) insert in the C-terminal domain. The type III classes include proteins that have a J-domain but do not fall into the type I or type II categories. The J-domain is responsible for interactions with HSP70 [121, 122], whereas the C-terminus, which varies widely among members of the J-domain family, has chaperone activity and can act as a dimerization interface [123, 124].

Roles in pathology and disease

Given the fundamental role of HSP70 in protein biogenesis and quality control, the aberrant regulation and function of HSP70 has been implicated in many diseases. For example, HSP70 expression is highly elevated in essentially all tumor types and is proposed to be strongly predictive of patient prognosis [125]. HSP70 can contribute to tumorigenesis by inhibition of apoptosis via both caspase-dependent and independent pathways [126, 127], and escape of senescence [128]. Additionally, HSP70 has also been proposed to contribute to the development of drug resistance in cancer; for example, chronic myeloid leukemia patients with resistance to the protein-kinase-inhibitor imatinib consistently display higher HSP70 expression relative to imatinib responsive patients [129].

Likewise, the HSP70 machine is additionally entwined in the etiology of many neurodegenerative protein-misfolding diseases including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington disease. The cytotoxicity of both A β and tau are influenced by HSP70 because it facilitates disaggregation [130], degradation and elimination of A β [131, 132], inhibits hyperphosphorylation of tau [133], and prevents aggregation and promotes degradation of abnormal tau [133, 134]. Elevated expression of Hsp70 has also been shown to alleviate polyglutamine-related toxicity in *Drosophila* models [135, 136]. Furthermore, dopaminergic neuron loss associated with alpha-synuclein toxicity is also mitigated in a *Drosophila* model for Parkinson's disease [137].

Pharmacological targeting of HSP70

Relative to HSP90, efforts to pharmacologically modulate HSP70 are less well developed. There are crucial biochemical and structural differences between these chaperones that could impact their pharmacology. First, HSP70 binds ATP up to 300-times more tightly than HSP90 [138]. Second, there is a major divergence in the shape of the ATP-binding sites of HSP90 and HSP70, with the HSP70's site being more solvent exposed, which naturally disfavors small-molecule binding [138]. However, the success of targeting the N-terminal ATP-ase site of HSP90 has nonetheless fueled the exploration of an analogous approach to HSP70 modulation.

Among the early examples of ATP-competitive HSP70 inhibitors is apoptozole, which was discovered as an inducer of apoptosis in a cell-based phenotypic screen, that monitored both the presence of phosphatidylserine on the cell membrane and membrane permeability [139]. Through the use of affinity chromatography and liquid chromatography-mass spectrometry, the action of apoptozole was attributed to binding the ATP-pocket of HSC70, and surface plasmon resonance analysis indicated a nanomolar dissociation constant. Using a cell-based model, apoptozole was subsequently shown to influence the membrane presence of mutant CFTR, through a decrease in HSP70-dependent degradation of mutant CFTR by the ubiquitin-proteasome system [140].

Another achievement in ATP-competitive HSP70 inhibition was the development of VER-155008 (Fig. 4), an ATP-mimic that binds HSP70 with a mid-nanomolar *in vitro* dissociation constant, as assessed by a fluorescence polarization assay [141]. Although VER-155008 is poorly selective among the HSP70 isoforms, a promising subsequent

structure-activity-relationship study identified one derivative of the parent scaffold that displayed some selectivity for HSP70 over Grp78, an isoform with its own respective role in cancer [142], through exploiting a single amino acid difference in the ATP-binding site (Thr vs Ile) [143]. Given that the HSP70 isoform distribution is not uniform, both in terms of cellular compartmentalization and tissue distribution, it would be advantageous to have selective inhibitors for the individual isoforms, and this is an encouraging step toward achieving this in the highly conserved ATP pocket.

An orthogonal route to HSP70 modulation, which may yield small-molecules with improved selectivity, is through targeting areas outside of the ATP-pocket because these regions have lower sequence conservation [144]. A recent example of this approach is an allosteric inhibitor (HS-72, Fig. 4) of ATP binding for the inducible HSP70 isoform, which was identified using a fluorescence-linked enzyme chemoproteomic approach [145]. HS-72 was shown to have high selectivity for inducible HSP70 over other HSP70 isoforms including Hsc70, Grp78, and Grp75. Another report unveiled a class of irreversible inhibitors that bind an allosteric pocket in the N-terminal domain and show higher selectivity for Hsp70 over other proteins based upon the ability of an immobilized analogue to specifically pull down Hsp70 from a SKBr3 cell extract [146].

The HSP40-HSP70 interface represents another site outside of the ATP-pocket that has been successfully targeted, particularly in the analogous prokaryotic complex. A set of small-molecules with a core dihydropyrimidinone scaffold were identified using an *in vitro* assay, and were found to perturb DnaJ binding to DnaK (the bacterial homologs of HSP40 and HSP70) and influence ATPase activity through distributed allosteric effects [147–151]. Small changes in the dihydropyrimidinone scaffold appear to toggle inhibition to activation, which bolsters the hypothesis that targeting such sites may be used to fine-tune chaperone function [151, 152]. These dihydropyrimidinones are improved structural analogs of the immunosuppressant HSP70 modulator 15-deoxyspergualin [153] (which has limitations as a HSP70-selective probe due to interactions with HSP90 [154] and possibly other non-chaperone targets [155, 156]). A related high-throughput screen additionally revealed small-molecules that bind DnaK and allosterically modulate its interaction with the GrpE nucleotide exchange factor [157].

Direct targeting of the HSP40 co-chaperones provides yet another approach to pharmacologically modulate HSP70 activity. However, there is only one example of a small-molecule that directly binds Hsp40 (Fig. 4). The inhibitor, “compound 6”, was found as part of a small-molecule screen using an assay that monitors HSP70 luciferase refolding [158]. Subsequent characterization of “compound 6” using a thermal denaturation assay revealed that the observed effect was through direct binding of the HSP40 co-chaperone. Further development of this class of inhibitors could be another step forward in small-molecule modulation of chaperone function, however, this may be challenging given the numerous potential combinations of HSP70-HSP40 interactions.

Application of HSP70 modulators

Altogether, the relatively small collection of potent and selective HSP70 modulators limits our knowledge with respect to the scope and utility of this strategy. Some HSP70 inhibitors

have displayed promising preliminary results in the treatment of cancer (ie VER-155008 [159], MKT-077 [160] and PES [161]), but toxicity and off-target effects remain an issue. An improved analog of the rhodacyanine HSP70 inhibitor MKT-077 (YM-1) has a promising toxicity profile in cell-based experiments [162], and aided in overcoming resistance in treating tamoxifen resistant MCF7 cells, but the clinical relevance of this compound will require further investigation.

Although pursued to a lesser degree, application of HSP70 modulators are producing exciting preliminary results in models for neurodegenerative diseases [163]. There are two general ways to think about pharmacological modulation within the context of these diseases. First, increasing activity through induction of HSP70 expression appears beneficial for mitigating aggregation in both cell-based and metazoan models [6]. A second approach argues that, in the context of directly targeting chaperones (the focus of this review), specific inhibition of certain aspects of Hsp70 activity may be beneficial, for example small-molecule inhibitors that effectively lock Hsp70 in the ADP-bound conformation stimulate substrate binding and increase CHIP-dependent ubiquitination and promote polyglutamine-expanded androgen receptor clearance in a cell-based model for spinobulbar muscular atrophy [136]. Clearly, the understanding of how HSP70 contributes to these diseases is not yet complete, and its roles in both substrate stabilization and triaging require further elucidation.

Although an impressive starting toolbox of chemical probes has been developed for HSP70, the chemical biology of this chaperone remains less refined than HSP90. Most of the available HSP70 modulators are inhibitors of only moderate potency, and mainly target the ATP pocket. The specificity of current modulators is generally modest or unknown, although promising preliminary studies indicate that isoform selectivity is achievable with further medicinal chemistry optimization. Very few small-molecules that directly activate HSP70 are known, but promisingly, selective activation seems feasible based on early experiments with 15-deoxyspergualin, which increases Hsc70 activity but has no effect on BiP [164]. Targeting areas outside of the HSP70 ATP-pocket has been met with some success, but there is a paucity of inhibitors that bind the C-terminus. Examples of small-molecules that bind the C-terminus have been reported, such as PES [165], but they appear to be reactive and may have off-pathway targets in *in vivo* settings.

HSP60/chaperonins

Function and structure

The chaperonins represent a third major class of chaperones that engage protein clients by encapsulation within a self-contained folding chamber, to fold post-translational intermediates to native states [166], assist in oligomeric protein assembly [167], and import protein substrates into mitochondria [168]. Much has been learned of the structure of chaperonins from the study of the bacterial homolog, GroEL [169], which exists as an ~800 kDA complex that has a cylindrical shape composed of two rings that respectively contain seven subunits [170, 171] (Fig. 5). Non-native substrates are accommodated in the central cavity of the chaperonin, which has dimensions of 6 nm in diameter and 7 nm in height [172]. Upon entry of a non-native substrate into the chamber, the subsequent folding process

is associated with major conformational changes in the central cavity driven by ATP-hydrolysis [173]. Chaperonins correspond to two major classes based upon folding mechanism, the type I chaperonins (GroEL, mitochondrial HSP60, and the Rubisco subunit binding protein) require a separate co-chaperonin (ie GroES or HSP10) to seal the folding chamber. GroES is expressed in the cytosol of bacteria and in organelles of mitochondria [174] and chloroplasts [167]. In contrast, the type II chaperonins have a built-in lid and are expressed in archaeobacteria [175] and the eukaryotic cytosol [176].

Roles in pathology and disease

Human chaperonins have been implicated in motor neuron diseases, including hereditary spastic paraplegia, in which a mutant form of mitochondrial HSP60 with reduced ATPase activity is incapable of forming a productive complex with HSP10, as assessed in an *E. coli* model [177, 178]. McKusick-Kaufman syndrome is associated with mutations within a putative subunit of the eukaryotic cytosolic chaperonin CCT [179], and the related Bardet-Biedl syndrome is also associated with deregulation of the same putative chaperonin protein [180]. Mutations in CCT are also correlated with hereditary sensory neuropathy in rat models [181]. Cancer cell survival additionally relies upon HSP60 activity [182], and overexpression is associated with evasion of apoptosis through down-regulation of a p53-promoted pathway [183], loss of senescence that is potentially due to a role in cell cycle regulation [184], and uncontrolled proliferation [185].

Pharmacological targeting of chaperonins

Small-molecule modulators of chaperonins, as a whole, have been elusive. Early efforts directed at targeting the chaperonin ATPase site have been unproductive, and only a few inhibitors, including mizoribine [186] and pyrazolopyrimidines [187] (Fig 5) were reported to bind this region with modest affinity. However, a recent high-throughput screen identified several promising new ATP-competitive leads for GroEL, including “compound 1” [188], which potently inhibits GroEL/GroES-mediated refolding in a biochemical assay. In a separate high-throughput screen that was designed to identify pharmacological activators of HSF1, a small-molecule hit, HSF1A, was found to bind CCT through pull-down experiments [189, 190], and further investigation using this small-molecule demonstrated that CCT directly binds HSF-1 to inhibit transcription [190].

Examples of small-molecules that bind outside of the chaperonin ATP-binding site are sparse, but exist. Epolactaene [191] inhibits GroEL through electrophilic reaction with Cys442, β -lactams are proposed to interact with a loop in the N-terminal region based upon docking simulations [192], and the sesquiterpene natural product suvanine [193] acts as a non-ATP-competitive chaperonin inhibitor, but its mechanism of action has not been fully characterized. The low availability of small-molecules that target areas outside of the chaperonin ATPase site, such as the protein-folding chamber, makes pursuit of such pharmacological modulators attractive because of the conceptually unique mechanism of action.

Application of chaperonin modulators

Altogether, the chaperonins have been less tractable to small-molecule modulators for reasons that are not entirely clear, a likely therapeutic application will be as anti-bacterials, which may help address the growing concern of resistance. Chaperonin inhibition may also be used for cancer treatment [194] as efforts develop for inhibitors of the human homologues.

Tabular summary

As a resource, we have included a table with a representative sample of the pharmacological chaperone modulators that have been discussed within this review (Table 1). An emphasis has been placed on biochemical affinity because of the inherent variability of potency assessment in cell-based experiments. Chaperone modulators without adequate biochemical affinity data were excluded.

Prospectives

The integral role of molecular chaperones in the PN makes them an intriguing pharmacological target, and since the serendipitous discovery of geldanamycin, there has been a growing interest in the potential therapeutic value of small-molecule chaperone modulators. Currently, there are 22 open clinical studies related to HSP90 inhibitors in the United States [195], and there is much optimism regarding this approach based upon ongoing clinical studies [196]. However, despite this optimism, toxicity remains a significant issue, and concerns regarding the highly conserved and ubiquitous nature of these proteostasis constituents still loom.

Perhaps proceeding with a renewed focus on selectivity, a feature that has largely been ignored (or underreported) in the development of chaperone modulators may circumvent these toxicity issues. Development of probes with high fidelity for a given chaperone will allow for fine adjustment the PN and the protein-folding landscape, and will help elucidate the individual functions of the chaperone machines. The field may possibly benefit from the development of standardized chaperone selectivity panels, as found in the case of validated pharmaceutical targets such as protein kinases (i.e. KINOMEscan [197], KiNativ [198], KinaseSeeker [199]), which may allow for a more focused pursuit of the most clinically relevant chaperones.

We postulate that selectivity may be difficult to achieve using the prevailing approach of targeting the highly conserved ATPase site of the chaperone machines. Non-ATP competitive chaperone modulators offer higher selectivity, but additionally display phenotypic activity that diverge from their ATP-competitive counterparts, such as the C-terminal binding HSP90 inhibitors and allosteric modulators of HSP70. While we appreciate the difficulty of pharmacologically targeting these unconventional sites, we expect to see further elaboration of these approaches based upon these preliminary observations.

Looking forward, in addition to streamlining how chaperones are targeted, it will also be important to continue to broaden how they are used. We would like to point out that all of the clinical trials based upon chaperone modulation are exclusively for the treatment of

cancer, and with increasing evidence that chaperones are valuable targets for several neurodegenerative diseases, hopefully clinical evaluation of this approach will begin in the near future. Furthermore, most chemical probes targeting molecular chaperones are inhibitors, which limits the capacity to pharmacologically modulate the PN via a targeted approach. This disparity also has clinical relevance given that small-molecules that directly bind molecular chaperones and increase their associated activities may have great utility in the treatment of disease.

Acknowledgements

This work was supported by the NIH (NIA, NIGMS, NIMH), the Chicago Biomedical Consortium, the Ellison Medical Foundation, and the Daniel F. and Ada L. Rice Foundation. We would like to thank Dr. Johnathan Labbadia for thoughtful comments on the manuscript.

Abbreviations

PN	proteostasis network
HSP	heat shock protein
TPR	tetratricopeptide repeat

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Highlights

The proteostasis network (PN) has many pharmacological targets that are relevant to many protein-misfolding diseases.

Molecular chaperones are a major pharmacological target within the PN.

HSP90 has received the most attention as a pharmacological target, but HSP70, the chaperonins and the various co-chaperones of the PN are increasingly becoming viable targets through the application of a variety of strategies.

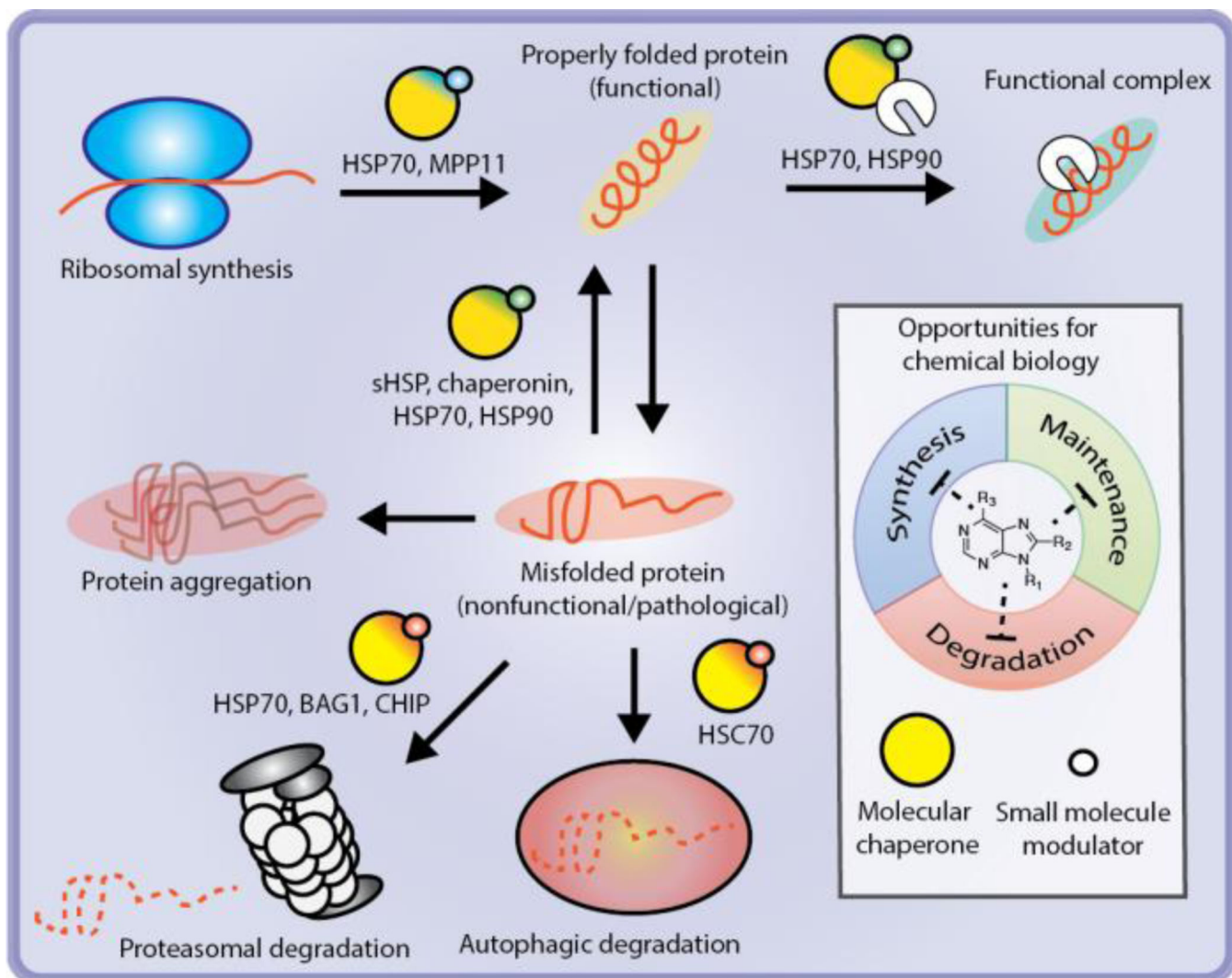


Figure 1. Molecular chaperones offer a multi-dimensional approach to pharmacological modulation of the proteostasis network (PN)

Representative molecular chaperones that respectively support each of these components are illustrated. Molecular chaperones are generically represented as yellow circles. The smaller blue, green or red circles represent a pharmacological reagent that impacts synthesis, maintenance, or degradation respectively.

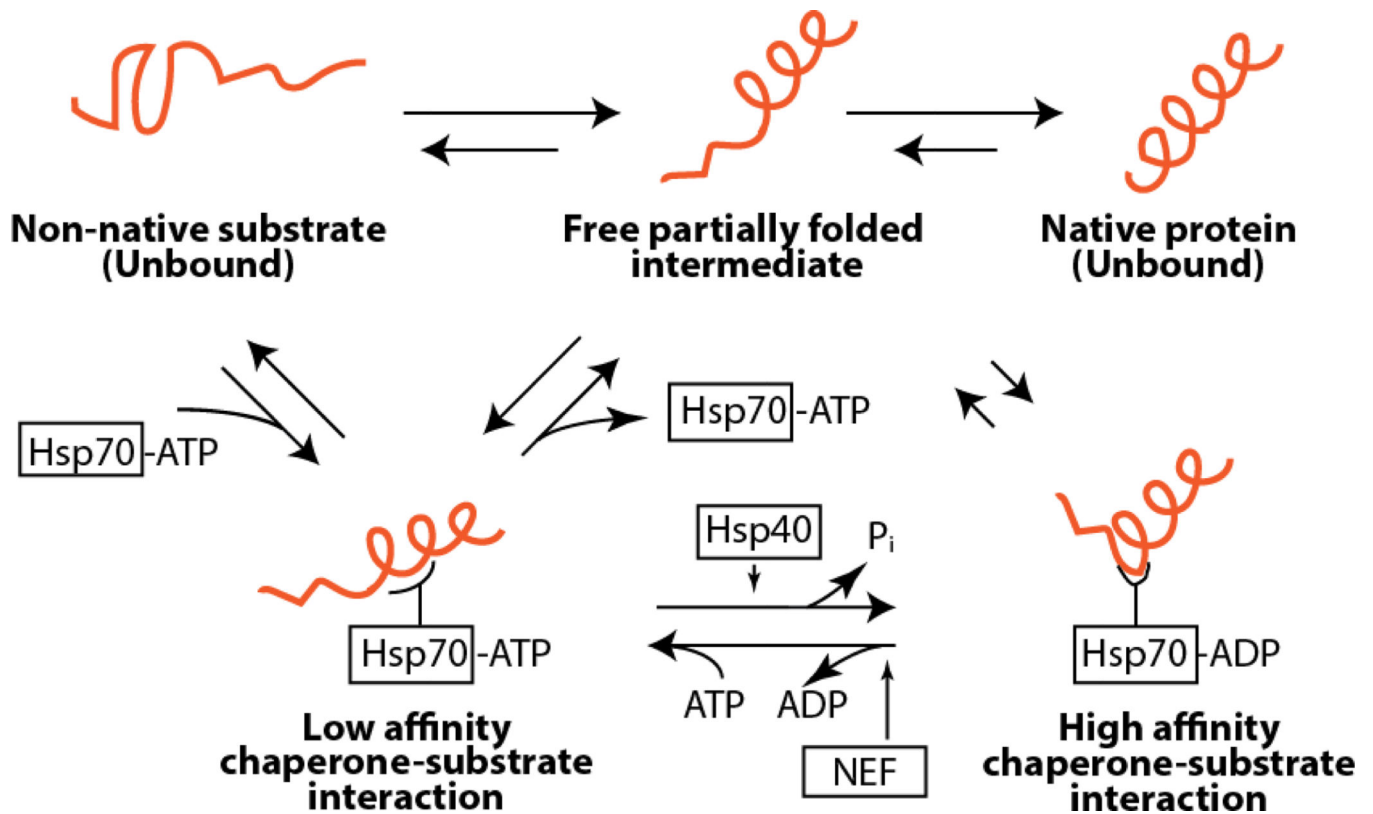


Figure 2. The Hsp70 folding cycle

Hsp70 relies on a complex collection of co-chaperones and nucleotide exchange factors (NEFs) to determine both activity level and substrate specificity. The relative ratio of co-chaperones and NEFs serves as a means to fine-tune the PN, and therefore targeting these protein-protein interactions remains an attractive approach to pharmacological modulation.

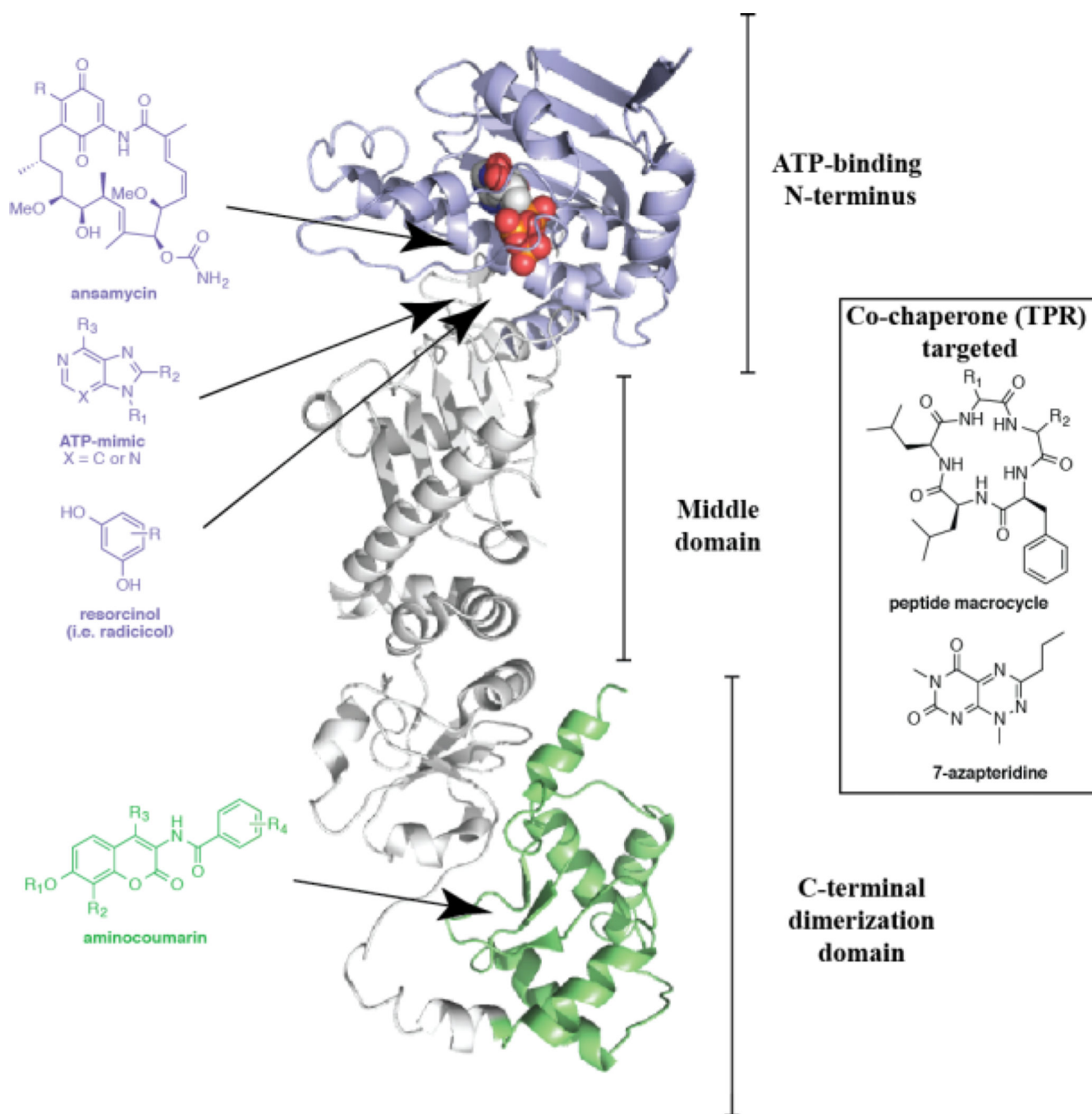


Figure 3. Hsp90 and its pharmacological modulators

The structure of the yeast Hsp90 homolog in ribbon presentation. The image represents half of the Hsp90 dimer, which is shown in complex with an ATP molecule. The three respective domains are labeled and color-coded. The ATP molecule is represented in sphere form. The image was generated with Pymol using structure PDB 2CG9. Representative chemical scaffolds for Hsp90 modulators are included, and arrows indicate binding sites.

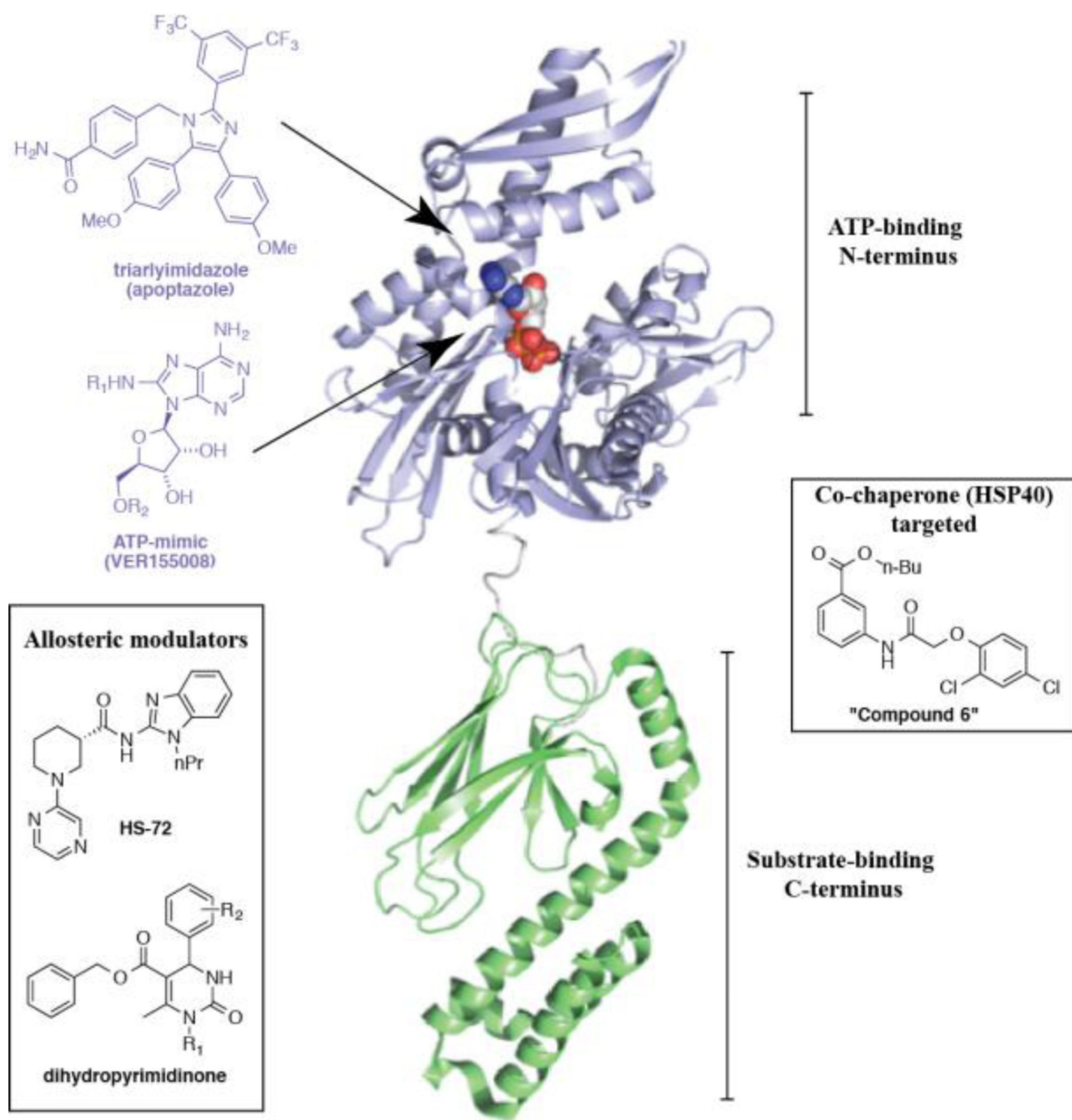


Figure 4. Hsp70 and its pharmacological modulators

The structure of DnaK, the bacterial homolog of Hsp70 in ribbon presentation, bound to ADP. The ATP- and substrate-binding domains are labeled and color-coded. ADP is represented in sphere form. The image was generated with Pymol using structure PDB 2KHO. Representative chemical scaffolds for Hsp70 modulators are included, and arrows indicate binding sites.

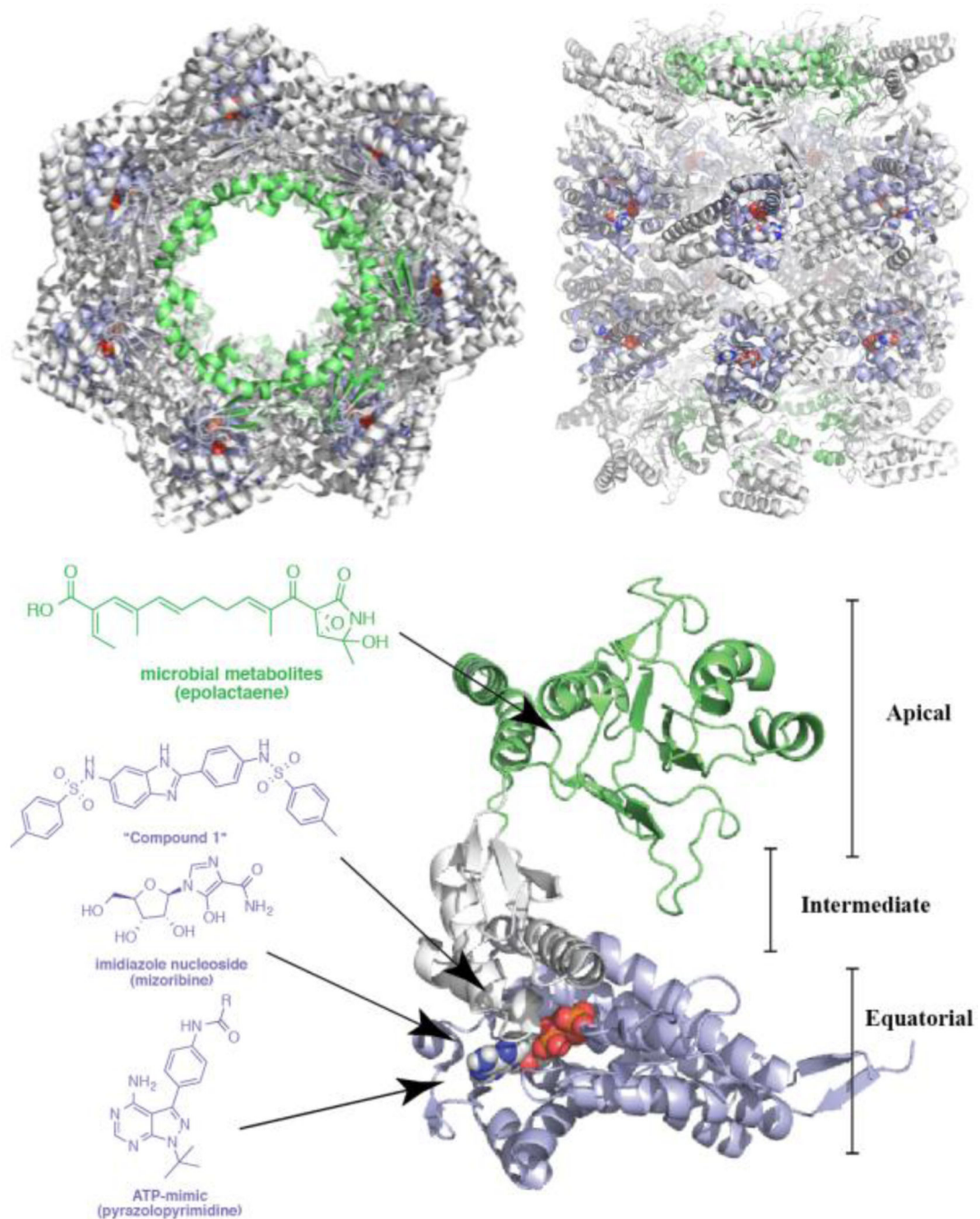


Figure 5. The chaperonin family and its pharmacological modulators

The structure of GroEL, the bacterial homolog of Hsp60 in ribbon presentation. ATP is represented in sphere form. The image was generated with Pymol using structure PDB 4AB2. The top-left portion of the panel displays an overhead view that highlights the substrate-binding cavity. The top-right portion of the panel displays a side view, which more clearly shows the ATP-binding site. The structure of an individual subunit of GroEL is

shown in the bottom right. Representative chemical scaffolds for chaperonin modulators are included, and arrows indicate binding sites.

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Table 1

Summary of selected pharmacological chaperone modulators discussed within this review.

Target class	Name	Pharmacophore	Binding site	<i>in vitro</i> potency (nM)
Hsp90	geldanamycin	benzoquinone ansamycin	ATP	$K_D = 780^a$
Hsp90	17-AAG	benzoquinone ansamycin	ATP	$IC_{50} = 6^b$
Hsp90	radicicol	resorcinol	ATP	$K_D = 3^a$
HSP90	PU3	ATP-mimic	ATP	$EC_{50} = 13,000^c$
HSP90	BIIB021	ATP-mimic	ATP	$IC_{50} = 1.7^d$
HSP90	HSP990	other	ATP	$IC_{50} = 0.6^e$
TPR	C9	other	Protein-protein interaction	$K_D = 16,000^f$
TPR	“compound 2”	Sansalvamide A derivative	Protein-protein interaction	$K_D = 3,600^g$
HSP70	VER155008	ATP-mimic	ATP	$K_D = 300^h$
HSP70	apoptozole	triarylimidazole	ATP	$K_D = 210^i$
HSP70	116-4G	dihydropyrimidinone	Allosteric	$IC_{50} = 120,000^j$
HSP40	“compound 6”	other	Protein-protein interaction	$IC_{50} = 130^k$
chaperonin	mizoribine	other	ATP	$K_D = 600^l$
chaperonin	“compound 1”	other	ATP	$IC_{50} = 110^m$

^a binding affinity for isolated N-domain [55]^b inhibition of Hsp90 from BT474 breast carcinoma cell lysate [200]^c competition for HSP90 against immobilized geldanamycin [201]^d competition for HSP90 against FITC-labeled geldanamycin [202]^e AlphaScreen binding assay [203]^f isothermal titration calorimetry [95]^g competitive binding assay [92]^h competition assay for fluorescein-labeled ATP [141]ⁱ SPR binding assay [139]^j ATPase assay (malachite green) [149]^k luciferase refolding assay [158]^l SPR binding assay [186]^m dMDH refolding assay [188].