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Alternative approaches to Hsp90 modulation for the treatment of cancer

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Abstract

Hsp90 is responsible for the conformational maturation of newly synthesized polypeptides (client proteins) and the re-maturation of denatured proteins via the Hsp90 chaperone cycle. Inhibition of the Hsp90 N-terminus has emerged as a clinically relevant strategy for anticancer chemotherapeutics due to the involvement of clients in a variety of oncogenic pathways. Several immunophilins, co-chaperones and partner proteins are also necessary for Hsp90 chaperoning activity. Alternative strategies to inhibit Hsp90 function include disruption of the C-terminal dimerization domain and the Hsp90 heteroprotein complex. C-terminal inhibitors and Hsp90 co-chaperone disruptors prevent cancer cell proliferation similar to N-terminal inhibitors and destabilize client proteins without induction of heat shock proteins. Herein, current Hsp90 inhibitors, the chaperone cycle, and regulation of this cycle will be discussed.

Background

Molecular chaperones are an evolutionarily conserved class of proteins that prevent aggregation and assist in the conformational maturation of other cellular proteins (referred to as client proteins). Heat shock proteins (Hsps) are a group of molecular chaperones that are ubiquitously expressed under non-stressed conditions and upregulated upon exposure to cellular stress, including elevated temperature. Hsp90 is the most abundant heat shock protein and represents 1–2% of cellular proteins in unstressed cells [1–3]. There are four human isoforms of Hsp90; the cytosolic isoforms Hsp90 α and β , Grp94 (localized to the endoplasmic reticulum) and TRAP1 (localized to the mitochondria). Hsp90 facilitates the conformational maturation of Hsp90-dependent proteins via the Hsp90 chaperone cycle, in which the Hsp90 homodimer forms a large, multiprotein complex that relies upon co-chaperones, immunophilins, and partner proteins to fold nascent polypeptides, as well as the rematuration of denatured proteins [4–6]. The Hsp90 heteroprotein complex folds these

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substrates through a series of conformational transitions at the middle and N-terminal domain of Hsp90 that facilitate ATP hydrolysis at the N-terminus [7,8]. Inhibition of the Hsp90 protein folding machinery results in client protein ubiquitinylation and subsequent degradation by the proteasome, which can ultimately result in cell death [9–12]. Many Hsp90-dependent client proteins (e.g., ErbB2, B-Raf, Akt, steroid hormone receptors, mutant p53, HIF-1, survivin and telomerase, among others) are associated with the six hallmarks of cancer. Therefore, oncogenic client protein degradation via Hsp90 inhibition represents a promising approach toward anticancer drug development [13–15].

Originally, small molecule inhibitors of Hsp90 were designed to perturb the ATPase activity located at the N-terminus and include derivatives of geldanamycin, radicicol and purine [16–18]. N-terminal Hsp90 inhibitors are effective at inhibiting Hsp90 function and lead to antiproliferative activity through client protein degradation; however, Hsp90 N-terminal inhibition also leads to induction of the heat shock response (HSR) [19–21]. N-terminal inhibitors displace the Hsp90-bound transcription factor, HSF-1 [22,23]. Upon displacement, HSF-1 trimerizes, translocates to the nucleus and binds the heat shock element, which leads to increased levels of the Hsps, including Hsp90. The HSR is a prosurvival response to conditions that cause the denaturation of proteins. Hsp27, Hsp40, Hsp70, and Hsp90, among other Hsps, are overexpressed to refold denatured proteins. The consequence of inducing a prosurvival response concomitantly with inducing client protein degradation is typically, cytostatic activity. However, this induction also leads to dosing and scheduling problems in the clinic, as N-terminal inhibitors induce expression of the target they inhibit. Therefore, Hsp90 inhibitors with novel mechanisms of action are sought to take advantage of the dependence that client protein-driven cancers have upon Hsp90, without concomitant induction of the prosurvival, HSR.

Two alternative strategies for inhibiting the function of Hsp90 include disruption of the Hsp90 heteroprotein complex and disruption of the Hsp90 C-terminal dimerization domain. Disruption of the Hsp90 heteroprotein complex has emerged as an effective strategy to prevent client protein maturation without induction of the HSR [24]. More specifically, disruption of interactions between Hsp90 and co-chaperones, such as Cdc37, or direct inhibition of co-chaperones and immunophilins, such as p23, F₁F₀ ATP synthase and FKBP52, prevent the maturation of Hsp90 clients at concentrations that do not induce the HSR [25–28].

By contrast, novobiocin was the first Hsp90 C-terminal inhibitor identified, and was found to weakly inhibit Hsp90 in SKBr3 cells at approximately 700 μ M concentration [29]. As a result, derivatives of the coumarin-containing natural product have been intensely sought and compounds manifesting improved activity identified. These C-terminal inhibitors prevent cancer cell proliferation at concentrations similar to N-terminal inhibitors and induce degradation of Hsp90-dependent client proteins without induction of the HSR [30,31]. In addition to novobiocin and novobiocin analogs, epigallocatechi-3-gallate (EGCG), silybin and cisplatin have been reported to bind the Hsp90 C-terminus [32].

Herein, recent progress in Hsp90 structure elucidation and dynamic aspects of the Hsp90 chaperone cycle will be discussed. Alternative strategies for Hsp90 inhibition that include

direct and indirect targets of Hsp90, as well as the current state of traditional small molecule Hsp90 inhibitors will be summarized. Lastly, post-translational modifications (PTMs) to Hsp90 and partner proteins will be described, as well as the effect of these modifications on client protein maturation, stability and activity in normal and transformed cells.

Hsp90 chaperone cycle

Hsp90 exists primarily as a homodimer. Each homodimer is composed of monomers that contain three domains; an N-terminal domain with an ATP-binding pocket, a C-terminal dimerization domain, and a middle domain that connects the N- and C-termini through a highly-charged, flexible linker. The Hsp90 protein folding cycle is complex and is illustrated in Figure 1. Many conformational switches and changes are needed to obtain the catalytically active state of Hsp90, and various co-chaperones are needed for regulation of the protein folding cycle [33]. Most of the co-chaperones that interact with Hsp90 at the beginning of the chaperone cycle bind the EEVD motif located at the C-terminus. Co-chaperone Hsp90-Hsp70 organizing protein (HOP) is responsible for delivering substrates from Hsp70 to Hsp90, and binds Hsp90 to stabilize the open conformation (a brief overview of co-chaperone binding to Hsp90, and its effects are highlighted in Table 1) [34]. HOP interacts directly with the EEVD motif through its tetratricopeptide repeat (TPR) domain.

After HOP binding, Hsp70 and the bound client protein stabilize the open conformation of Hsp90, upon which, various immunophilins (specifically, those with prolyl isomerase activity), co-chaperones, and other partner proteins bind to form a heteroprotein complex. When immunophilin co-chaperones, such as peptidyl-prolyl isomerase (e.g., FKBP51, FKBP52, and so on), bind Hsp90, an asymmetric complex is formed [34]. This asymmetric structure is often referred to as the ‘late complex’ and promotes the chaperone cycle through stabilization of the open conformation [35]. After formation of the late complex, activator of Hsp90 ATPase, Aha1, assembles with the heteroprotein complex, and dissociates co-chaperones, such as HOP and Hsp70 [35]. Buchner and coworkers demonstrated that Aha1, the most effective activator of ATPase activity, is needed for HOP release, and not p23, which had been previously reported [35]. They found that Aha1 and co-chaperone Cpr6 bind Hsp90 in a synergistic manner to stabilize the Hsp90 closed conformation.

Hsp90-catalyzed ATP hydrolysis is not only required for folding the bound substrate, but also release of the properly folded client. In order to achieve ATP hydrolysis, Hsp90 must adopt a closed conformation [33]. To form the closed state, Aha1 binds in a sequential manner, followed by ATP. Structural reorganization ensues to facilitate closure of the N-terminal ATP lid. Lid closure stabilizes the catalytic loop, which is required for ATP hydrolysis. In addition, the ATP lid establishes contacts between specific residues of the catalytic loop and ATP, in addition to the adjacent Hsp90 monomer, resulting in further stabilization of the Hsp90 complex. This conformational change represents the rate-limiting step in the protein folding cycle, not ATP hydrolysis [33].

In addition to establishing that Aha1 and Cpr6 bind Hsp90 in a synergistic manner, it was demonstrated that Aha1 association with Hsp90 accelerates the conformational change required for attainment of the closed state. In fact, the conformation formed upon Aha1 binding is different from the state wherein ATP hydrolysis occurs, as illustrated in Figure 1

[35]. In the closed state with Aha1, ATP can exchange readily. However, upon p23 displacement of Aha1, a second closed conformation is formed, which is followed by ATP hydrolysis [35]. The resulting complex disassembles and the folded substrate is released to promote regeneration of the catalytic cycle.

p23 is a well-established regulator of Hsp90 ATPase activity and has been shown to inhibit ATP hydrolysis and client release [36,37]. McLaughlin *et al.* demonstrated that p23 interacts with Hsp90 in the presence and absence of ATP; however, affinity for the p23–Hsp90 complex formation increased when ATP was present. Mass spectral analysis determined that one molecule of p23 bound to each Hsp90 monomer and p23 interacted with truncated fragments that lack the C-terminus. These data indicate that Hsp90 dimerization is not required for p23–Hsp90 association. Collectively, these data suggest that p23 locks Hsp90 subunits during the ATP-bound state, which exhibits a high affinity for protein substrates.

Another co-chaperone, carboxyl terminus of Hsp70-interacting protein (CHIP), regulates the release of properly folded client proteins, or targets them for degradation [38,39]. CHIP contains a ubiquitin ligase domain and thus, provides a direct link to protein degradation via the proteasome [40]. CHIP interacts with Hsp90 or Hsp70 through its TPR domain, and when bound to Hsp90, CHIP can displace HOP and p23. In the presence of Hsp90 inhibitors, CHIP mediates ubiquitinylation of substrates and ultimately directs them for degradation via the proteasome [41].

Other co-chaperones have less defined roles in the inhibition or promotion of Hsp90 ATPase activity; however, proper function of these co-chaperones and direct interaction with Hsp90 appear important for client protein maturation. Small glutamine-rich tetratricopeptide repeat-containing protein- α (SGTA) is linked to several cellular processes including cell division, mitosis, cell cycle checkpoint activation, and viral infection. SGTA interacts directly with Hsp70 and may interact with Hsp90 via its TPR domain to facilitate maturation of the growth hormone and androgen receptors [42]. It has also been proposed that SGTA participates in the sequestration of client proteins in an inactive state.

F₁F₀ ATP synthase was proposed to possess co-chaperone function by interacting with Hsp90 in several different cancer cell lines [28,43]. Furthermore, inhibition of F₁F₀ ATP synthase directly affected client protein maturation. F₁F₀ ATP synthase is a macromolecular machine that produces the majority of cellular ATP and is localized to the mitochondria and the cell surface in certain cancers [44–47]. F₁F₀ ATP synthase was shown to directly interact with Hsp90, specifically the Hsp90 α isoform [48]. A selective inhibitor of F₁F₀ ATP synthase, as well as general inhibitors of ATP synthases, have been shown to disrupt interactions between F₁F₀ ATP synthase and Hsp90 α , and ultimately result in client protein degradation [48,49].

Client-specific Hsp90 co-chaperones

While Hsp90-co-chaperone interactions continually change throughout the chaperone cycle to either inhibit or promote ATP hydrolysis, co-chaperones also exhibit client specificity during the chaperone cycle and promote the maturation of client protein classes [50]. The mechanism by which co-chaperones assist in the delivery of clients to Hsp90 may occur via

interactions with specific clients or by interactions with Hsp90 that prime the chaperone machinery for selective loading of protein substrates.

The co-chaperone activity manifested by cell division cycle 37 homolog, or Cdc37, is required for the maturation of all Hsp90-dependent kinases [25,51]. This co-chaperone inhibits ATPase activity, but does not affect accessibility to the ATP binding site [52–54]. Sgt1 (suppressor of G2 allele of *skp1*) is required for activation of nucleotide-binding leucine-rich repeat receptors [55]. The co-chaperones Tah1 and Pih1 are associated with chromatin remodeling and temporarily halt the Hsp90 ATPase cycle to prepare Hsp90 for the maturation of small nuclear ribonucleoproteins [56].

Other co-chaperones that exhibit client protein specificity are the peptidyl-prolyl isomerases (PPIases), and include FK506 binding protein (FKBP) 51, FKBP52, and cyclophilin (Cyp)40 [57,58]. These remodeling PPIases are commonly utilized in Hsp90-steroid hormone receptor complexes. Interestingly, these PPIases are selective for specific hormone receptor clients. FKBP51 in concert with protein phosphatase 5 (PP5) is present during the maturation of glucocorticoid receptor complexes; however, isolated FKBP51 is found associated with progesterone receptor, whereas Cyp40 associates with the estrogen receptor.

While it remains unknown as to how co-chaperones selectively interact with clients, co-chaperones involved in the maturation of certain clients, or substrate classes, provide an opportunity to selectively prevent the maturation of these client(s) by targeting interactions between Hsp90 and the co-chaperone.

Disrupting Hsp90 co-chaperone interactions

Disruption of interactions between Hsp90 and co-chaperones has been a successful strategy for the degradation of client protein substrates. Small molecules can disrupt these interactions and manifest properties unlike N-terminal Hsp90 inhibitors. Celestrol and gedunin (Figure 2) were originally suspected to disrupt interactions between Hsp90 and Cdc37 and induce degradation of kinase client proteins [59–61]. The mechanism by which these compounds affect the Hsp90 chaperone cycle has since been explained. Celestrol is a natural product that exerts potent anti-cancer activity via multiple mechanisms of action. One of these mechanisms is disruption of Hsp90–Cdc37 interactions. The natural product gedunin was shown to disrupt Hsp90-co-chaperone interactions through binding p23.

NMR studies by Sreeramulu, *et al.* demonstrated that celestrol binds and covalently modifies cysteine residues located at the N-terminus of Cdc37 via Michael adduct formation and therefore, does not directly bind Hsp90 [62]. In addition, Chadli, *et al.* reported that celestrol inhibits the Hsp90 chaperone cycle by destabilizing p23, resulting in the degradation of steroid hormone receptors [63]. Therefore, celestrol appears to exhibit a multifaceted mechanism for inhibition of the Hsp90 protein folding cycle. Gedunin was also reported to bind the co-chaperone, p23, and inhibit p23 chaperone activity while blocking interactions between p23 and Hsp90 [27]. Gedunin also inactivated p23 via caspase 7 activation, which led to cleavage of the p23 C-terminus, resulting in apoptosis. Interestingly, treatment with gedunin did not increase Hsp27 levels and only slightly elevated Hsp70 levels were observed, suggesting this approach may not induce the HSR.

Hsp90 requires ATP for chaperone activity and inhibition of ATP synthase with pan ATP synthase inhibitors such as oligomycin A, 2-deoxy-D-glucose, antimycin A and efrapeptins prevent Hsp90-dependent client maturation, which destabilizes the client–Hsp90 complex, leading to client degradation via the proteasome (Figure 3) [43,48,49]. ATP synthase inhibitors do not induce the HSR, as oligomycin A and efrapeptins manifested little to no increase in Hsp90, Hsp70 and Hsp27 levels. Furthermore, inhibition of the singular ATP synthase, F₁F₀ ATP synthase, with the selective inhibitor, cruentaren A, retained this activity and resulted in client protein degradation through destabilization of F₁F₀ ATP synthase–Hsp90 α interactions (Figure 3) [48,64,65]. Hsp90 and p23 in complex with hTERT, the catalytic subunit of telomerase, is required for the nuclear localization of telomerase, a protein whose unregulated function contributes to unlimited replicative potential. The promiscuous natural product, curcumin, induced cytoplasmic accumulation and degradation of hTERT through disruption of p23–hTERT interactions as one of its many mechanisms of action (Figure 3) [66]. Curcumin decreased association between p23 and hTERT, but did not prevent hTERT from interacting with Hsp90. By contrast, the N-terminal Hsp90 inhibitor, geldanamycin, disrupted both the Hsp90–hTERT and p23–hTERT complexes.

San A-amide, a derivative of the natural product sansalvamide A, induces apoptosis in various cancer cell lines, including pancreatic, colon, breast and prostate (Figure 3) [67,68]. Vasko *et al.* reported that San A-amide-mediated apoptosis in the HCT-116 colon cancer cell line occurred through displacement of inositol hexakisphosphate kinase-2 (IP6K2) and FKBP52 from the Hsp90 C-terminus [69]. San A-amide binds the N-middle domain of Hsp90 and appears to disrupt the structural equilibrium of this region, which appears to alter the substrate binding site. By contrast, San A-amide has no effect on the N-terminal client, Her2. This mechanism of action is unique to San A-amide, as the Hsp90 N-terminal inhibitor 17-allyl-aminogeldanamycin (17-AAG) does not affect IP6K2 and FKBP52 binding, but does affect Her2 maturation. Furthermore, San A-amide exhibits no effect on Hsp90 ATPase activity, which supports its role as an allosteric modulator and its potential to selectively disrupt Hsp90 C-terminal binding interactions.

N-terminal Hsp90 inhibitors

Hsp90 inhibitors that bind the N-terminus compete with ATP and perturb ATPase activity, thereby disrupting client protein maturation. ATP adopts a unique conformation when bound to Hsp90, due to the presence of a Bergerat fold in the protein structure, which is characteristic of the GHKL (Gyrase, Hsp90, Histidine, Kinase, MutL) subgroup within the ATPase/kinase superfamily [70,71]. Compounds that bind the N-terminal ATP-binding pocket adopt a bent conformation and afford selectivity for Hsp90 over other ATP-binding proteins. All Hsp90 inhibitors under clinical investigation are N-terminal inhibitors and include derivatives of geldanamycin (GDA), radicicol (RDC), and purine (Figure 4). N-terminal inhibitors have been studied in great detail and comprehensive reviews are present throughout the Hsp90 literature [72–76].

GDA belongs to the benzoquinone class of ansamycins and was originally pursued as an antibiotic until it was shown to reverse v-src oncogenic transformation by destabilization of

the Hsp90–src complex [77]. Although GDA is a potent anticancer agent, it did not undergo clinical evaluation due to its poor drug-like properties. However, several derivatives of GDA have entered clinical investigation and include 17-allyl-17-demethoxygeldanamycin (17-AAG), 17-desmethoxy-17-N,N-dimethylaminoethylaminogeldanamycin (17-DMAG), 17-allylamino-17-demethoxygeldanamycin hydroquinone hydrochloride (IPI-504), and 17-desmethoxy-17-aminogeldanamycin (IPI-493) (Figure 4A). RDC is a macrocyclic lactone that contains a resorcinol moiety as well as an allylic epoxide and an $\alpha,\beta,\gamma,\delta$ -unsaturated ketone. Although RDC is unstable in serum, the resorcinol moiety of RDC has been utilized to develop several compounds that are under clinical investigation, such as STA-9090, NVP-AU922/VER-52269, KW-2478, and AT-13387 (Figure 4B). The crystal structure of ADP bound to Hsp90 provided an opportunity to design inhibitors that take advantage of the unique Bergerat fold. Purine-containing compounds, such as PU-3, CNF 2024/BIIB021, MPC-3100, Debio 0932 (CUDC-305), and PU-H71 are also under going clinical evaluation (Figure 4C).

Despite the effectiveness of N-terminal inhibitors as anticancer agents, several concerns have emerged as a consequence of N-terminal inhibition. Induction of the HSR has resulted in dosing and scheduling problems during clinical trials and spurred the development of Hsp90 inhibitors that are devoid of this response, such as C-terminal inhibitors and allosteric modulators of the chaperone cycle. In addition, gastrointestinal distress, hepato- and ocular toxicities have been observed during preclinical and clinical trials [78]. This may be the result of off-target effects, metabolic instability and/or pan inhibition of all four Hsp90 isoforms. Recently, it was shown that the hERG channel depends upon the Hsp90 α isoform for its maturation [79]. Hsp90 inhibitors have been shown to disrupt proper trafficking and function of hERG [80]. Therefore, cardiotoxicity has also been a concern during the development of Hsp90 inhibitors. All Hsp90 N-terminal inhibitors in the clinic exhibit pan Hsp90 inhibitory activity and target all four isoforms. It appears that other clients (oncogenic or not) may also exhibit a preference for individual Hsp90 isoforms [79,81,82]. Therefore, deciphering the role played by each isoform will be important for the development of future Hsp90 inhibitors.

Despite the high sequence similarity and identity possessed among the N-termini of each Hsp90 isoform, inhibitors have recently been developed that manifest selective inhibition. An inhibitor derived from the ansamycin scaffold has been developed that targets Hsp90 α and β , but not Grp94 (Figure 5A) [83]. Likewise, resorcinol- and purine-containing inhibitors have also been reported to selectively inhibit Grp94 based on subtle differences within the N-terminus of Grp94 (Figure 5B & C) [81,82].

C-terminal binding pocket

The C-terminus of Hsp90 contains a second nucleotide-binding site. Matts and coworkers, were the first to note that molybdate inhibited geldanamycin binding to the N-terminus and that molybdate protected the C-terminus from proteolysis [84]. Additional evidence for a second binding site was presented by Neckers and coworkers, when they determined that novobiocin, a coumarin antibiotic, bound the C-terminus of Hsp90 [29]. The researchers found that novobiocin bound a carboxy-terminal Hsp90 fragment [29]. Later, they were able

to demonstrate that the same C-terminal binding site bound ATP and novobiocin, both within amino acids 542–732. More specifically, they found amino acids 657–677 to be essential, as removal of these residues resulted in diminished affinity for novobiocin [85]. In addition, these amino acids revealed that novobiocin bound to a site that is important for Hsp90 dimerization and co-chaperone binding [85]. The ability to interrupt dimerization was therefore, proposed to represent a useful strategy to prevent client protein maturation.

Matts and coworkers demonstrated that novobiocin binding altered the EEVD motif and consequently impacted Hsp90 interactions with various co-chaperones [86]. Upon further exploration, they showed that novobiocin binding resulted in a conformational change that ultimately led to dissociation of bound kinases and co-chaperones [86]. Csermely and coworkers found the anticancer drug, cisplatin, also bound the C-terminus and strengthened interactions between Hsp90 and Hsp70 [87]. Their studies suggested that the C-terminal ATP binding site only opens after occupancy of the N-terminal binding site [87]. Peyrot and coworkers provided additional evidence for a C-terminal nucleotide-binding site using isothermal calorimetry, scanning differential calorimetry, and fluorescence spectroscopy [88].

It was proposed that the C-terminus contains a Rossmann fold between amino acids 490 and 630, which could also exhibit NADH reductase activity [88]. However, Csermely, and coworkers demonstrated that nicotinamide adenine dinucleotides only interacted with the N-terminus and therefore, no such NAD-binding site existed [89]. It was found that both purine and pyrimidine nucleotides bound the C-terminus, which is less specific than the N-terminal binding site that only binds nicotinamide adenine dinucleotides and diadenosine polyphosphate. In fact, GTP and UTP also bind to the C-terminal binding site [89]. In addition to exploring the differences in nucleotide specificity of the C-terminal binding site, they determined that a ligand must contain both a charged group as well as a large hydrophobic moiety to effectively bind this region [89].

Agard and coworkers further examined the intra and intermonomer interactions of Hsp90, and demonstrated that dimerization is required for ATP hydrolysis [90]. Removal of the C-terminal dimerization domain resulted in reduced ATPase activity (~6–10-fold). However, it was shown that dimerization could be replaced via a disulfide bond between two monomers, which restored ATPase activity [90]. Additional work by Buchner and coworkers identified a switch point at the C-terminus, in which mutants of alanine 577 caused both higher ATPase activity and increased activation of client proteins [91]. Interestingly, these results are consistent with amino acids that are known to stabilize β -sheets. In support of this observation, they showed that mutation of asparagine, which is known to destabilize β -sheets, produced lower affinity for N-terminal nucleotides [91].

Most recently, Matts and Blagg provided additional information about the location of the C-terminal binding site by the use of proteolytic fingerprinting and photo affinity labeling [92]. They showed that Hsp90 C-terminal inhibitors stall the complex into an open conformation, which results in protection from proteolytic cleavage (Arg620 and Lys615), and prevention of N-terminal occupation. Importantly, these results were in agreement with the work of Hugel and coworkers as discussed below [92,93]. These findings were further supported by

Retzlaff and coworkers, who demonstrated that C-terminal inhibitors interact with and stabilize a region about the C-terminal pivot point to prohibit the conformational changes required for chaperone activity [91,92]. This work demonstrated that binding to the C-terminus prevents formation of the closed conformation [92].

Recent investigation of the Hsp90 dynamic cycle by Hugel and coworkers highlighted conformational activity within the C-terminus that may explain the different biological outcomes observed with C-terminal inhibitors [93]. They observed that the Hsp90 C-terminus opens and closes, which is in contrast to prior models. They noted an anticorelation between the N- and C-terminal domains, in which the Hsp90 dimer was found to open and close several times before dimer dissociation. Interestingly, their data demonstrated that the C-terminus opens and closes regardless of the N-terminal state, which was confirmed by the use of AMP-PNP, which causes Hsp90 to remain in the closed state. Their results, as illustrated in Figure 6, showed that when no nucleotide is bound to the N-terminus, the C-terminus exists in an open conformation 43% of the time, and in a closed conformation 57% of the time. However, when ATP is bound to the N-terminus, the C-terminal states reversed. Reversal of the C-terminus state upon ATP binding is caused by destabilization of the C-terminus. Since the N-terminal ATP-binding pocket is far from the C-terminal domain, the protein must communicate through its structure, which is important when considering the implication of C-terminal inhibition [93]. With Hugel's data and model, one could propose that binding to the C-terminus can occur when the domain is open, which would prevent dimerization, or after dimerization, which would prevent occupation of the N-terminal binding pocket.

Hsp90 C-terminal inhibitors: novobiocin & novobiocin analogs

Since both Hsp90 and DNA gyrase bind ADP and geldanamycin in a bent conformation, it was proposed that a DNA gyrase inhibitor could also inhibit Hsp90 [29]. Originally it was proposed that the coumermycin antibiotics – specifically novobiocin (structure illustrated in Figure 7), chlorobiocin, and coumermycin A1 – could bind the N-terminal nucleotide-binding pocket due to structural similarities within the binding site. Indeed, novobiocin was able to compete with radicicol and geldanamycin for Hsp90 binding, but neither geldanamycin nor radicicol could compete with novobiocin [29]. Amino-terminal and carboxy-terminal fragments of Hsp90 were prepared for further investigation. As expected, geldanamycin bound the N-terminal fragment, however, novobiocin was found to bind the C-terminal fragment [29].

Although novobiocin was found to bind the C-terminus, it manifested low efficacy in cellular models (~700 μ M in SkBr₃). To improve upon this low activity, structure–activity relationship studies were pursued. The first novobiocin analog reported was A4 (illustrated in Figure 7), in which the benzamide side chain was replaced with an acetamide and lacked the 4-hydroxyl and the 3'-carbamate [94]. A4 induced client protein degradation at approximately 10 μ M in the LNCaP prostate cancer cell line (a mutated androgen receptor-dependent prostate cancer cell line) [94]. However, A4 induced a robust HSR at concentrations approximately 1000-fold lower than that needed for client protein degradation [94]. Segregation of the HSR and client protein degradation had not been

previously observed. Recall that, N-terminal inhibitors induce client protein degradation at the same concentration they induce the HSR. Since A4 exhibited heat-shock induction without the degradation of client proteins, it has been evaluated as a neuroprotective agent [94].

4-deshydroxynovobiocin (DHN1) and 3'-descar-bamoyl-4-deshydroxynovobiocin (DHN2), illustrated in Figure 7, were synthesized to examine whether the 4-hydroxy was detrimental to Hsp90 inhibitory activity [95]. These studies confirmed that removal of the 4-hydroxy resulted in decreased DNA gyrase activity, but improved Hsp90 inhibitory activity [95]. Importantly, these compounds induced client protein degradation at concentrations in which no HSR was observed. Further modifications to the coumarin core were designed to mimic the guanosine nucleus via the addition of hydrogen bond acceptors placed at the 5-, 6-, and 8-positions, since GTP had been shown to bind selectively to the C-terminus [96]. The most potent compound, KU-174 (illustrated in Figure 7), was subjected to a NCI60 screen and showed activity against multiple cancer cell lines [97]. Other investigational studies on the coumarin core (compound 2) are illustrative of work in this area [30,96,98–100]. Most recently, it was demonstrated that the coumarin core could be replaced with a biphenyl moiety (compound 3 represents such an example). Analogs containing the biphenyl moiety manifest good activity against the SkBr3 and MCF-7 cell lines, and represents a new scaffold for further exploration of the Hsp90 C-terminal binding site [101].

In addition to the coumarin core, novobiocin contains a noviose sugar and a benzamide side chain. A biaryl ring system that contains a *meta*-methoxy was found to exhibit comparable activity to the benzamide side chain present in KU-174 [102]. Additional studies on replacements of the benzamide side chain have also been reported [102,31,103,104]. Extensive SAR studies have been carried out on noviose [105–108]. It was determined that the 2'- and 3'-hydroxy moieties are essential for activity, whereas, the 6'-gem-dimethyl group and the 5'-methoxy are not [105]. Additional SAR demonstrated that N-methyl piperidine represents a suitable replacement for the noviose sugar (compound 1 in Figure 7).

EGCG & silybin

There are two other natural products that have been shown to bind the Hsp90 C-terminus, (–)-EGCG and silybin (Figure 7). EGCG is the major component of green tea and silybin is the major component of silymarin, which is extracted from the seed of milk thistle [109,110]. Through the use of proteolytic footprinting, immunoprecipitation, and an ATP-argarose pull-down assay, Gasiewicz and coworkers demonstrated that EGCG binds the same amino acids (538–728) as novobiocin [109]. Inhibition of Hsp90 via EGCG binding causes client protein degradation (telomerase, multiple kinases, and the aryl hydrocarbon receptor), and therefore anticancer activity [111,112].

Historically, silybin has been used for the treatment of liver and gallbladder disorders [113]. However, silybin was also shown to manifest cytotoxic activity against various cancer cell lines in addition to enhancing the efficacy of other chemotherapeutic agents. Silybin was identified as an Hsp90 C-terminal inhibitor and Hsp90-dependent client proteins were degraded in a concentration-dependent manner, while Hsp90 levels remained constant [114].

Overlay of silybin and novobiocin suggest the coumarin and flavonone ring could be interchanged, which led to compounds that exhibited improved Hsp90 inhibitory activity [115].

PTMs of the Hsp90 chaperone cycle

The Hsp90 chaperone cycle can be fine-tuned to quickly adapt to changes in the intracellular environment through PTMs. Several comprehensive reviews describe these covalent modifications, which include phosphorylation, acetylation, S-nitrosylation, oxidation, ubiquitinylation and SUMOylation. These PTMs can affect ATPase activity, stabilization of Hsp90–client interactions, co-chaperone interactions, and in some cases, target client proteins for maturation or degradation [116–118]. A summary of some Hsp90 PTMs and original references are provided in Table 2. PTMs of the Hsp90 chaperone cycle can enhance or diminish the effects of small molecule Hsp90 inhibitors, such as GDA. Targeting these PTMs can provide an opportunity to enhance the efficacy of Hsp90 inhibitors and gain further insight into the mechanism by which PTMs regulate the Hsp90 protein folding machinery.

Co-chaperones Cdc37, Sgt1, p23, and FKBP52 are susceptible to phosphorylation by casein kinase 2 (CK2) [119–124]. CK2 phosphorylates Ser13 of Cdc37. This modification is required for Cdc37's chaperoning of numerous kinase clients as well as for the binding of Cdc37 to Hsp90. De-phosphorylation of Cdc37 at Ser13 by the phosphatase, PP5/Ppt1, negatively affects the chaperone cycle and prevents maturation of these clients. Phosphorylation of Sgt-1 at Ser361 negatively impacts Sgt-1 dimerization and prevents kinetochore assembly, a process required for chromosome segregation during eukaryotic cell division. CK2-mediated phosphorylation of p23 at Ser113 and Ser118 was shown to be important for prostaglandin synthase activity and required for formation of the Hsp90-p23-CK2 complex. CK2 also phosphorylated Thr143 of FKBP52. While this modification does not affect FKBP52 from binding FK506, phosphorylated FKBP52 does not bind Hsp90.

Several kinases are capable of phosphorylating Hsp90 and have varying effects on the chaperone cycle. Double-stranded DNA protein kinase (DNA-PK) phosphorylates Thr5 and Thr7 of the Hsp90 α N-terminal domain and may play a role in DNA damage. Client kinases also phosphorylate Hsp90 and may regulate their own chaperoning via a feedback mechanism. B-Raf was shown to phosphorylate Ser263 of Hsp90 in melanoma and Akt to phosphorylate Hsp90 α and Hsp90 β , Grp78 and Grp94, Hsp70, and protein disulfide isomerase (PDI). c-Src phosphorylates Hsp90 β at Tyr301 in response to VEGF receptor-2 (VEGFR-2) activation and increases association between Hsp90 β and eNOS. Protein kinase A (PKA) phosphorylates Thr90 of Hsp90 α and reduces association with eNOS, which results in both diminished eNOS activity and nitric oxide production. Phosphorylation of Thr90 of Hsp90 α via PKA was also shown to mediate cellular secretion of Hsp90 α and may play a role in wound healing and/or metastasis. Swe1^{Wee1} kinase phosphorylates Tyr38 of the N-terminus of Hsp90 α when Hsp90 is in the 'open conformation' and positively affects the ability of Hsp90 to chaperone a select group of client kinases (ErbB2, Raf-1, and Cdk4). However, phosphorylation of this residue also negatively affects GDA binding to the Hsp90 N-terminus.

CK2 phosphorylation of Ser231 and Ser263 in the charged linker of Hsp90 α , as well as the equivalent residues in Hsp90 β (Ser226 and Ser255) occurs in normal cells, but not leukemic cells. Several leukemogenic kinases, such as Bcr-abl, FLT3/D835Y, and Tel-PDGFR β , suppressed phosphorylation of Hsp90 β at these residues, which resulted in stable interactions between Hsp90 β and apoptotic peptidase activating factor-1 (Apaf-1). Ultimately, this activity led to inhibition of apoptosome function and may contribute to resistance in leukemia. CK2 phosphorylation at these sites also disrupts the Hsp90-aryl hydrocarbon complex and destabilizes the aryl hydrocarbon receptor protein. CK2 was also shown to phosphorylate Thr36 of Hsp90 α . Mutation of this residue to a phosphomimetic, aspartate, resulted in decreased chaperoning of several clients and reduced interactions between Hsp90 α with Aha1.

Acetylation/deacetylation of Hsp90 has been investigated using HDAC inhibitors [125–127]. Acetylation of Hsp90 by p300 resulted in diminished interactions between Hsp90 and clients and ultimately, resulted in client instability and degradation. Mutation of lysine to acetylated lysine was shown to decrease interactions between Hsp90 and several clients/co-chaperones as well as to reduce ATP-binding. De-acetylation of Hsp90 has been shown to occur via several HDACs including HDAC6, HDAC1 and HDAC10, which correlate with the stabilization of Hsp90-client interactions and increased chaperone activity [128–131].

Other PTMs include S-nitrosylation of Hsp90 α at Cys597 via nitric oxide, oxidation of Cys572 by the oxidized lipid, 4-hydroxy-2-nonenal (4-HNE; is formed during cellular oxidative stress), ubiquitinylation, and more recently, SUMOylation. S-nitrosylation, oxidation, and ubiquitinylation decrease chaperone function by perturbing ATPase activity or destabilizing Hsp90-client interactions, which results in client degradation. Alternatively, SUMOylation of the conserved lysine residue, Lys191, initiated recruitment of the ATPase activating co-chaperone, Aha1. In addition, SUMOylation facilitated binding of several Hsp90 N-terminal inhibitors and sensitized yeast and mammalian Hsp90 to inhibition.

Future perspective

Tumor dependency on pathways regulated by Hsp90 provides clear rationale for the development of Hsp90 inhibitors. Hsp90 inhibition offers a multifaceted treatment strategy, which is in contrast to current cancer therapies that target a single signaling pathway. Despite the potential of Hsp90 as a drug target, no Hsp90 inhibitor has been US FDA approved. Alternative methods to target Hsp90 include inhibition of the C-terminus or allosteric modulation of the chaperone machinery.

Recent advances toward elucidation of the Hsp90 structure and intricacies of the chaperone cycle have provided details on the conformational changes required for heteroprotein complex formation during various stages of the chaperone cycle. PTMs of proteins involved in the chaperone cycle have shed light on how quickly Hsp90 adapts to changes in the cellular environment. Alternative methods to modulate Hsp90 may provide an opportunity to select for oncogenic client degradation, while avoiding some of the negative consequences of N-terminal inhibition. In conclusion, the Hsp90 chaperone cycle and inhibitor development have become a diverse and dynamic field of study. As details of the

chaperone cycle emerge, more information regarding the regulation of chaperone machinery in normal versus transformed cells will be discovered. Such studies will lead to the development of tailored strategies for modulation of the Hsp90 chaperone machinery for the treatment of cancer as well as other diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Key terms

Hsp90 heteroprotein complex	An asymmetric protein complex that enhances or perturbs Hsp90 ATPase activity
Heat shock response	A prosurvival upregulation of heat shock proteins in response to cellular stress
C-terminal dimerization domain	The location within the C-terminus responsible for Hsp90 dimerization, certain co-chaperone interaction and small molecule binding
Post-translational modification	Covalent modifications of Hsp90 and co-chaperones post-translation that effect maturation, stability and activity of client proteins
Hsp90 closed conformation	A sequential, two-step dimerization of the Hsp90 N-terminus required for ATP hydrolysis and client protein folding
Allosteric modulators	Small molecules that affect client protein maturation via interactions with different components of the heteroprotein complex versus the N-terminal ATP binding site
NCI60	A 60 cancer cell panel screen for small molecules offered by the NIH-NCI

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Executive summary

Background

- Inhibition of Hsp90 is a promising strategy for the development of anticancer chemotherapeutics due to the dependence of cancers on the Hsp90 protein folding machinery. Hsp90 inhibitors that have undergone clinical evaluation target the Hsp90 N-terminus; these include derivatives of geldanamycin, radicicol and purine. Although N-terminal inhibitors induce tumor digression, N-terminal inhibition results in an increase in heat shock proteins via induction of the prosurvival heat shock response.

The Hsp90 chaperone cycle

- The chaperone cycle of Hsp90 is facilitated by the association of many different co-chaperones. Association of Aha1 stabilizes the first closed conformation in the cycle. This is followed by p23 displacement of Aha1 to form the second closed conformation which leads to ATP hydrolysis.

Alternative modulation of Hsp90 activity

- Disruption of the Hsp90 C-terminus and interactions between Hsp90 and proteins of the chaperone cycle (co-chaperones) have emerged as alternative strategies to disrupt oncogenic client protein maturation.

Disrupting Hsp90 co-chaperone interactions

- Disruption of interactions between Hsp90 and co-chaperones, such as Cdc37, FKBP52 and p23, via small molecules has resulted in client protein destabilization and degradation without induction of the heat shock response. Disruption of select Hsp90 co-chaperone interactions results in the degradation of client protein classes, as some rely upon selective co-chaperones for maturation.

C-terminal binding pocket

- The C-terminal binding site overlaps with the Hsp90 dimerization domain and the EEVD motif, which is important in co-chaperone association. Recently, the C-terminus has been shown to open and close, and exhibits an anticorelation to the N-terminus. Therefore, an inhibitor binding the open C-terminus would prevent dimerization, and an inhibitor binding the closed C-terminus would prevent occupation of the N-terminal binding pocket.

Hsp90 C-terminal inhibitors

- Novobiocin, the first C-terminal inhibitor, provided a starting point for extensive structure-activity relationship studies leading to new analogs which have improved anti-cancer activity. Importantly, these C-terminal inhibitors induce client protein degradation at concentrations that do not induce the heat shock response.

Post-translational modifications of the Hsp90 chaperone cycle

- Post-translational modifications of co-chaperones and Hsp90 allow the chaperone cycle to quickly adapt to changes in the cellular environment. These post-translational modifications can vary between cancerous and normal tissues and may provide an opportunity to select for cancer cells, thereby avoiding disruption of post-translational modifications required for normal cell function.

Figure 1. The Hsp90 chaperone cycle

The two closed conformations of Hsp90 are highlighted in the figure. The first closed conformation is stabilized by Aha1. p23 then displaces Aha1, to form the second closed conformation.

Figure 2.
Celastrol and gedunin.

Figure 3.
Allosteric modulators of the Hsp90 chaperone cycle.

Figure 4. N-terminal inhibitors of Hsp90

(A) Geldanamycin and derivatives, **(B)** radicicol and derivatives, as well as **(C)** Pu-3 and purine-containing analogs.

Figure 5. Isoform selective inhibitors of Hsp90

Structures of (A) Hsp90 α/β inhibitor, (B) resorcinol-containing Grp94 inhibitor and (C) purine-containing Grp94 inhibitor.

Figure 6. Effect of nucleotide binding to N-terminus on C-terminal dimerization

The percentages under each Hsp90 dimer reflect the likelihood of the C-terminus being either opened or closed.

Figure 7.
C-terminal inhibitors of Hsp90.

Table 1

Hsp90 co-chaperones and effects on Hsp90 conformation and ATPase activity.

Co-chaperone	Interaction site in co-chaperone	Interaction site in Hsp90	Effect on Hsp90 conformation	Effect on Hsp90 ATPase activity
Hop/Sti1	TPR2A, TPR2B	N, M, C	Stabilizes open state	Inhibition
Cdc37	M, C	N	Stabilizes open state	Inhibition
Sgt1	CS domain	N	Not determined	No effect
Tah1	TPR	C	Not determined	Weak inhibition by Tah1–Pih1 complex
Aha1	N, C	N, M	Stabilizes closed state	Acceleration
Ppt/PP5	TPR1, TPR2, TPR3	C	Not determined	No effect
Cpr6, Cpr7/Cyp40	TPR	C	Not determined	Weak acceleration
FKBP51/FKBP52	TPR	C	Not determined	No effect
p23/Sba1	N	N	Stabilizes closed state	Inhibition

C: C-terminal domain; CS: CHORD (cysteine and histidine-rich domain)-containing protein and Sgt1 domain; TPR: Tetratricopeptide repeat; M: Middle domain; N: N-terminal domain.

Table 2

Post-translational modifications of human Hsp90.

Modification	Catalytic protein or moiety	Amino acid residue	Effect on Hsp90 chaperone activity	Ref.
Phosphorylation/de-phosphorylation	DNA-PK	Thr5 and Thr7 phosphorylation	Unknown	[132]
	B-Raf	Ser263 phosphorylation of Hsp90 α	Unknown	[133]
	Akt	Thr and Ser phosphorylation of Hsp90 α , Hsp90 β , and Grp94	Unknown	[134]
	c-Src	Try301 phosphorylation of Hsp90 β	Increased eNOS activity and NO production	[135]
	PKA	Phosphorylation of Ser452 of Hsp90 β and Thr90 of Hsp90 α	Phosphorylation of Hsp90 α decreased eNOS activity and NO production. Also stimulates cellular secretion of Hsp90 α	[136,137]
	CK2	Phosphorylation of Ser231 and Ser263 of Hsp90 α and Ser226 and Ser255 of Hsp90 β in untransformed cells but not leukemic cells	Phosphorylation of Hsp90 α caused dissociation of Hsp90 and the aryl hydrocarbon receptor	[138,139]
	Swe1 ^{Wee1} Kinase	Tyr38 phosphorylation of Hsp90 α	Increased chaperoning activity of select clients. Decreased GDA binding to Hsp90	[140]
S-nitrosylation	NO	Cys597 nitrosylation	Inhibited ATPase activity	[141,142]
Oxidation	4-HNE	Cys572 oxidation	Decreased chaperone activity	[143]
Ubiquitinylation	Ubiquitin pathway	Lys ubiquitinylation	Destabilized Hsp90 client interactions and resulted in client degradation	[144,145]
SUMOylation	SUMOylation pathway	Lys191 SUMOylation	Initiated recruitment of Aha1 and facilitated binding of N-terminal inhibitors	[116]