Hsp90: the vulnerable chaperone

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The molecular chaperone Hsp90 has emerged as an important target in cancer treatment because of its roles in maintaining transformation and regulating the function of proteins involved in apoptotic, survival and growth pathways. Many Hsp90 inhibitors function by binding to the N-terminal ATP pocket, but the chaperone has many other vulnerable points. Agents that interact with its C-terminus or modify its post-translational status represent additional ways of interfering with chaperone activity. This review will discuss several emerging classes of Hsp90 inhibitors and their modes of action.

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▼ Molecular chaperones are proteins that are responsible for maintaining the correct folding, function and stability of client proteins. Of these, heat shock protein 90 (Hsp90) has recently emerged as a focus of interest because of its role in regulating proteins that are responsible for malignant transformation. Several natural products that inactivate Hsp90 function have anti-tumor effects in *in vitro* and *in vivo* models of cancer. However, due to the role of Hsp90 in normal cellular homeostasis, it remains unclear whether Hsp90 inhibitors will be sufficiently specific for use as anticancer agents. Early clinical results with 17AAG, the first Hsp90 inhibitor to enter clinical trials, suggest that these fears could be unfounded. These studies confirm that Hsp90 is a promising target for novel cancer therapeutics and pave the road for the introduction of Hsp90 inhibitors in the treatment of cancers.

Regulation of Hsp90 function

Functional analysis has revealed that the amino and carboxyl termini of Hsp90 are separated by a charged region. The dynamic interrelationship of these domains, coordinated by nucleotide and co-chaperone binding, determines the conformation of the chaperone and, thus, its activation state. Association of Hsp90 with client proteins is regulated by the activity of the N-terminal ATPase domain, which binds and hydrolyses ATP to mediate a series of association–dissociation cycles between Hsp90 and client substrates. The activity of Hsp90 is further regulated by binding of co-chaperones, which promote the interconversion of the ATP- and ADP-bound states and modulate the formation of client-specific complexes [1–3]. Recent evidence suggests that in several tumor cell lines, Hsp90 might be exclusively complexed with co-chaperones in a state of high affinity for ATP/ADP or ligands of this regulatory pocket (i.e. ATPase inhibitor drugs), whereas in normal tissues, Hsp90 might exist primarily in a latent, uncomplexed, low affinity state [4,5]. Although no direct experimental evidence has yet been presented, post-translational modifications of Hsp90 might also regulate the 'activation' state of Hsp90 complexes. Overall, these observations suggest that Hsp90 is present in cells in equilibrium between a low chaperoning activity 'latent state' and an 'activated state', with increased chaperoning efficiency (see Figure 1). The shift in equilibrium might be dictated by the amount of 'stress' on the system, such as mutated and dysregulated proteins, hypoxia or a low nutrient concentration environment. Thus, the effects of inhibiting Hsp90 function could depend more on the 'activity' and degree of involvement of the co-chaperone–protein–Hsp90 complexes and less on its cellular levels. Collectively, the above data suggest that Hsp90 inhibitor concentrations can be identified that will disrupt crucial chaperone functions in a transformed cell but that might not be toxic to normal cells.

Hsp90 super-chaperone involvement in maintaining and driving transformation

Anti-apoptotic/survival and growth promoter

Hsp90 is known to be required for the activity of several key regulators of apoptosis, and through these associations the chaperone might confer survival advantage to tumor cells (Figure 2). The function of survivin, a

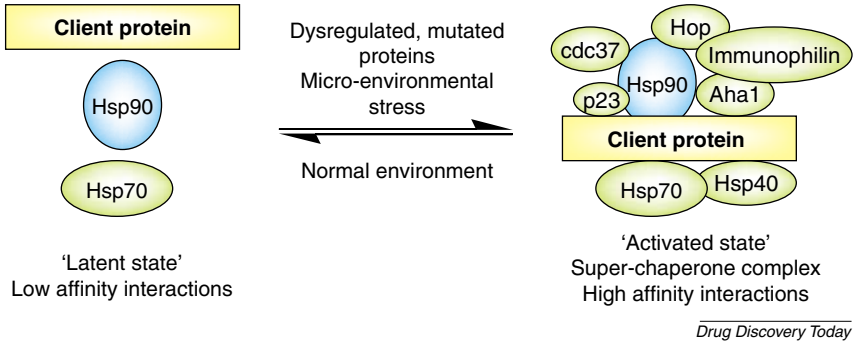

Figure 1. Hsp90 exists in an equilibrium between an 'activated state' prevalent in transformed cells and a 'latent state' predominant in normal cells. The activation state of the chaperone is regulated by co-chaperones and, possibly, post-translational modifications. These co-chaperones include heat-shock protein 70 (Hsp70), Hsp70-organizing protein (Hop), p23, cdc37, immunophilins and Aha1. The composition of the complexes is client-protein-specific and dependent on its maturation state. The figure schematically depicts Hsp90 and its possible interaction partners and does not represent timely complexes.

dual regulator of cell proliferation and cell death, which is overexpressed in virtually all human tumor types, is chaperoned by Hsp90 and it is believed that this association helps tumor cells elevate their anti-apoptotic threshold and promote proliferation [6]. The Akt-mediated survival-signaling [7] and the Raf-MAPK growth regulatory pathway are also regulated by Hsp90 [8]. In addition, Hsp90 plays important antiapoptotic roles through binding to RIP and the kinase domain of IKKα/β [9]. A recent study demonstrated that Hsp90 can suppress tumor necrosis factor α (TNFα)-induced apoptosis in Hsp90-overexpressing NIH3T3 cells by preventing the cleavage of Bid [10]. Furthermore, Hsp90 also interferes with the intrinsic caspase apoptotic pathway by forming a cytosolic complex with Apaf-1 and thereby inhibiting the formation of the active apoptosome [11].

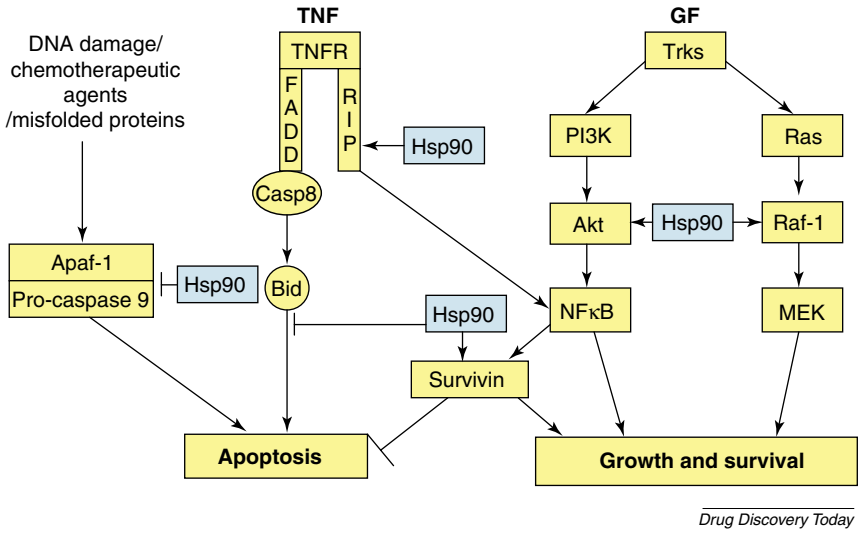

Figure 2. Hsp90 acts in regulating proteins involved in both the intrinsic and extrinsic apoptotic pathways. Abbreviations: FADD, Fas-associated death domain; GF, growth factor; Hsp70, heat-shock protein 70; Hop, Hsp70-organizing protein; MEK, mitogen-activated protein kinase kinase; PI3K, phosphatidylinositol 3-kinase; RIP, receptor-interacting protein; TNF, tumor necrosis factor.

Involvement in buffering mutation (see Box 1) and in transformation

In addition to its mutation buffering and survival promoting roles, Hsp90 helps to maintain the transformed state in several cell types. Almost 50 proteins have been identified as clients of Hsp90 (see reviews, [12–14]). Most of these proteins play important roles in the control of cell cycle, growth and apoptosis and their dysregulated function might lead to transformation. Examples include Her2, Raf-1, Akt, Cdk4, cMet, mutant p53, the estrogen and androgen receptors, mutant B-Raf, Bcr-Abl, Flt-3, Polo-1 kinase, HIF-1 α and hTERT. Inhibition of Hsp90 results in degradation of these client proteins via the ubiquitin proteasome pathway. This, in turn, leads to growth arrest and apoptosis in cancer cells *in vitro*, and to inhibition or regression of tumor growth in animals. It is appealing to hypothesize that the clientele regulated by Hsp90 includes, in each cell, the subset of proteins involved in driving transformation. A ramification of this observation is that by inhibiting one target, Hsp90, it might be possible to shut down multiple transforming signaling pathways in cancer cells, potentially resulting in wide-range anti-cancer effects [5,13]. Studies have shown that such effects can be obtained in *in vitro* and *in vivo* models of cancer.

The optimal characteristics of a successful Hsp90 inhibitor for use in patients remains to be determined. Increased interest in Hsp90 as a target has led to the identification of several chemical classes of Hsp90 inactivators. It will be important to

study the pharmacokinetic and pharmacodynamic properties of these agents in detail to understand the extent to which they differ and also to develop them optimally in their own right. It is our assignment in this review to present these agents. Only further study will determine the utility of each of these agents as cancer treatments.

Inactivators of Hsp90 function

Binders to the N-terminal ATP/ADP pocket of Hsp90

The N-terminal region of the chaperone holds a regulatory pocket that binds and hydrolyzes ATP [15]. While bound to Hsp90, the nucleotide adopts a bent shape found only in ATPases belonging to the GHKL family (G = DNA gyrase subunit B, H = Hsp90, K = histidine kinases and L = MutL). These enzymes share the same left-handed β–α–β fold, called the Bergerat fold. This fold is neither observed in the high affinity binding sites of kinases (which adopt a P-loop motif) nor in other chaperones such as Hsp70 [16]. This suggests that Hsp90 inhibitors are likely to adopt a bent conformation when inside the pocket to achieve high affinity binding. It also suggests that it is possible to discover compounds with a high degree of selectivity by identifying those that specifically bind to Hsp90 via the N-terminal ATPase pocket.

Ansamycins – GM, 17AAG, 17DMAG and GM dimmers: The ansamycin antibiotics geldanamycin (GM) and herbimycin (HA) (Figure 3a) were originally isolated based on their ability to revert the phenotype of v-src oncogene transformed cells. Further studies have demonstrated that these agents exert their activity by binding to the regulatory pocket in the N-terminal domain of Hsp90 [17,18]. By occupying this site, the ansamycins alter chaperone function by preventing the dissociation of Hsp90 client proteins from the chaperone complex. As a consequence, the trapped proteins do not achieve their mature functional conformation and are degraded by the proteasome [19]. Growth arrest by GM is followed by differentiation and/or apoptosis in a cell-line-dependent manner [20,21]. Despite their cellular potency, the use of these natural products as clinical agents has been limited by their associated liver toxicity and/or cellular instability. Hepatotoxicity in the ansamycin class is believed to be caused by the benzoquinone functionality because radicicol (RD), a structurally different natural product that has biological activity similar to that of ansamycins, is not hepatotoxic. A derivative of GM, 17AAG (Figure 3a), has similar cellular effects [22] but lower hepatotoxicity than the parent compound. 17AAG is highly potent in cell killing assays *in vitro* but commonly fails to affect the same cells xenografted *in vivo* [23]. Nevertheless, the drug has activity at non-toxic doses in a subset of breast, prostate, colon and non-small cell lung cancer (NSCLC) animal models [24,25].

Based on its biological activity, 17AAG has entered clinical trials in cancer patients in the USA and UK. Toxicity of 17AAG is schedule dependent. With daily 5× or daily 3× dosing schedules, hepatic toxicity is limiting. This

Box 1. Role of Hsp90 in buffering mutations

When Hsp90 was mutated or pharmacologically impaired in *Drosophila*, a widespread phenotypic variation was observed leading to the conclusion that Hsp90 might have an important role in buffering mutations [59]. These findings suggest intriguing possibilities for the role of Hsp90 and its inhibitors in cancer. First, it is possible that Hsp90 is the primary chaperone responsible for stabilizing the mutations that lead to cellular transformation, suggesting that inhibition of Hsp90 might be an effective approach for targeting such mutations. Furthermore, the role of Hsp90 as a capacitor of otherwise potentially lethal mutations suggests that Hsp90 inhibitors might be toxic to cells containing a burden of mutated proteins.

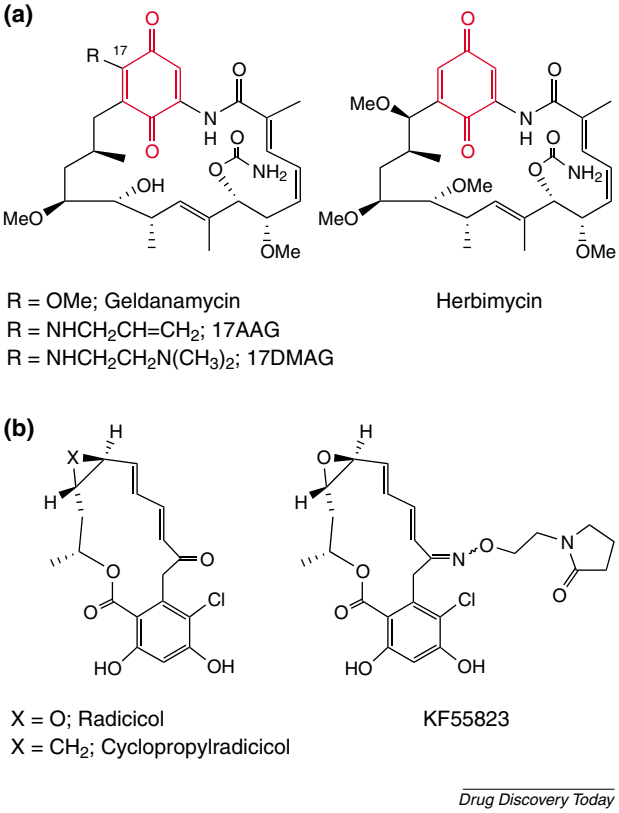

Figure 3. Natural product inhibitors of Hsp90 ATPase activity. Ansamycins' benzoquinone moiety is depicted in red.

side-effect is less prominent with intermittent dosing schedules. Odor and nausea, primarily attributed to DMSO, which is used as a solvent, remain a major issue with the current formulation. Based on these observations, weekly and twice weekly schedules have been recommended for further Phase II studies that are now ongoing in melanoma. Downregulation of client proteins including Raf-1, cdk4 and Akt in lymphocytes has been reported at well-tolerated doses in patients treated in the Phase I clinical trials [26,27]. Furthermore, Workman and colleagues have reported early evidence of therapeutic activity in melanoma patients where stable disease has been seen for over two years [26]. Investigators at Memorial Sloan-Kettering Cancer Center (http://www.mskcc.org) have also observed prolonged stable disease in patients with renal cancer and tumor regressions with the combination of 17AAG and docetaxel [28].

Despite these early promising results and its key role as a proof-of-principle Hsp90 inhibitor, 17AAG has several potential limitations. Most prominent are its limited solubility and cumbersome formulation [27]. Moreover, it appears to undergo extensive metabolism, which could result in the generation of toxic species with little antitumor activity [29]. For unknown reasons, 17AAG is also relatively inactive in tumor cell lines lacking functional Rb expression, such as small cell lung cancer cells (SCLC) (Solit, D., pers. commun.). Efforts to improve the solubility and bioavailability of 17AAG have led the National Cancer Institute (http://www.nci.nih.gov) and Kosan Biosciences (http://www.kosan.com) to develop 17DMAG as a second-generation alternative. 17DMAG has similar *in vivo* and *in vitro* activity to 17AAG but is water soluble and potentially orally bioavailable [30–32]. It also does not appear to undergo extensive metabolism in pre-clinical models [29]. The agent has now entered Phase I clinical trials in patients with advanced cancers.

Recently, Conforma Therapeutics (http://www.conformacorp.com) has reported the identification of a GM dimer (EC4) with an extended duration of action [23]. Although the nature and length of the linker has not been disclosed, it is likely that in concordance with permissible chemical modifications in the ansamycin class, joining of the two GMs occurs at C17. When compared to 17AAG, this agent was more potent in killing cells with defects in apoptosis pathways and exhibited increased *in vivo* activity [33,34].

Despite the potential advantages of these novel ansamycins over 17AAG, it is not possible to eliminate the benzoquinone feature of these compounds without affecting activity. Thus, hepatic toxicity is likely to remain a limiting factor in the clinical use of these agents. For this reason, the identification of synthetic small-molecule inhibitors of Hsp90 with unique structural characteristics is a major focus of interest in the field.

Radicicol and derivatives: Radicicol (RD), a natural product isolated from the fungus *Monosporium bonorden* is structurally unrelated to GM. The agent also potently inhibits Hsp90 function by binding to its N-terminal ATP pocket [35]. The crystal structure of RD bound to Hsp90 shows that RD interacts differently with Hsp90 when compared to GM, but it too adopts the bent, C-shaped conformation [36]. Addition of RD to tumor cells results in cellular effects that resemble those of ansamycins. Despite its promising *in vitro* activity, RD is inactive *in vivo* due to its instability in serum. Efforts directed at modifying its structure have led to several oxime-derivatives (see KF55823, Figure 3b), which have been extensively studied by Kyowa Hakko Kogyo Co. (http://www.kyowa.co.jp). These derivatives have potent activity *in vitro* and *in vivo*. Unlike ansamycins, the radicicol oximes do not cause serious liver toxicity indicating that the liver toxicity observed with the ansamycins is not caused by Hsp90 inhibition *per se* [37]. Despite their promise, the clinical development of these agents was not pursued. Danishefsky and colleagues have synthesized a RD derivative that has the epoxide exchanged for a cyclopropyl ring (Figure 3b). Although cyclopropyl-RD is almost as potent as RD in tissue culture [38], it remains to be determined if it lacks the drawbacks (toxicity profile and poor solubility) of the existent RD-derivatives.

PU-class (purine-scaffold derivatives): To address the limitations of natural product Hsp90 inhibitors, Chiosis and colleagues have explored novel pharmacological scaffolds [5,39–41]. Making use of the peculiar shape of Hsp90 inhibitors and of existent Hsp90 crystal data, they were able to design and synthesize small molecules that bind to Hsp90. These molecules (the PU-class, illustrated in Figure 4a), use purine as a scaffold. The first described member of the PU-class, PU3 (Figure 4a), inhibits the binding of Hsp90 to immobilized GM and mimics the cellular effects of ansamycins, although with modest potency [39]. Changes in the variables X_1, X_2 and X_3 resulted in a first generation small library of 70 PU-compounds and the identification of PU24FCl, a compound with an affinity for Hsp90 nearing GM (Figure 4a) [40,41]. PU24FCl has 10–50-times higher affinity for Hsp90 from transformed cells compared to normal tissue [5]. Hsp90 from all tested tumor cells shows similar affinity for PU24FCl. MutL ATPase activity is insensitive to inhibition by PU24FCl at concentrations as high as 5 mM. ADE2 SAICAR purine synthetase, an enzyme involved in purine metabolic processing and previously shown to be inhibited by 17AAG with nanomolar potency, is also inert to PU24FCl up to 2 mM [5]. In

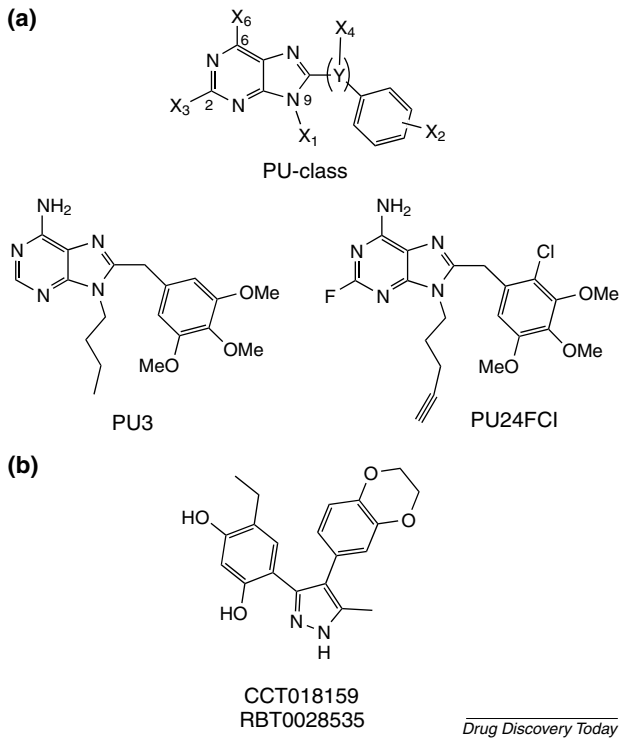

Figure 4. Synthetic Hsp90 ATPase inhibitors.

addition, the mitochondrial Hsp90 member, Trap-1, is insensitive to pharmacological doses of PU24FCl [Chiosis, G., pers. commun.].

Crystal structures of PU3 and PU24FCl [42] in complex with the N-terminal region of human Hsp90 α, reveal that these compounds induce a conformational change in the top 'lid' of the Hsp90 ATP pocket. Upon binding, PU24FCl adopts the bent shape characteristic of ligands specific to this pocket.

Unlike ansamycins and RD, PU24FCl does not seem to be preferentially active against a small panel of tumors cells such as those overexpressing Her2. By contrast, the agent has wide-ranging anti-cancer activities that occur at similar doses (~3–6 μM) in all tested tumor cell lines, including those resistant to 17AAG cells (i.e. SCLC). PU24FCl equipotently affects multiple tumor-specific aspects of oncogenesis regulated by the chaperone. In concordance with its higher affinity for tumor Hsp90, PU24FCl accumulates *in vivo* in tumors while being rapidly cleared from normal tissues [5]. In MCF-7 tumor xenografts, one dose of PU24FCl causes a significant depletion of receptor tyrosine kinases (Her2, Her3 and Her4) as well as degradation and inactivation of Akt and Raf-1, with a close correlation between its tumor accumulation profile and its effect on Hsp90 client proteins [5,40,41]. The magnitude and durability of Raf-1 and Akt protein depletion by PU24FCl *in vivo* seems to be greater than that observed with 17AAG. Due to their broad antitumor activity, the PU-class of Hsp90 inhibitors could have clinical use in a wide range of tumor types, both alone and in combination with cytotoxics, radiation or other biological agents.

Pyrazoles – CCT018159 (RBT0028535): Another novel scaffold Hsp90 inhibitor was reported by Workman and colleagues (Figure 4b) [43]. This agent, the pyrazole derivative CCT018159, was identified by HTS against a library of 60,000 compounds. The assay was designed to identify inhibitors of the intrinsic and biologically essential ATPase activity of yeast Hsp90. CCT018159 inhibits the growth of human colon, ovarian and melanoma tumor cells at concentrations similar to those that inhibit human Hsp90 ATPase activity (~8 μM). The addition of CCT018159 to cancer cells results in depletion of Hsp90 client proteins and induction of Hsp70 [44]. Recently, this group has reported a series of CCT018159 analogues with improved activity [45]. The cellular signature of Hsp90 inhibition has been confirmed for these agents with a reported increase in Hsp70 and Aha1 and depletion of Raf-1, Her2 and phospho-ERK1/2. The i*n vivo* activity and toxicity of these agents have yet to be reported.

Binders to the C-terminal domain of Hsp90

In contrast to the well-studied N-terminus, the crystal structure of the C-terminal region has yet to be solved. This region has been implicated in the binding of a second ATP molecule. Studies suggest that the site becomes available to ATP only after the N-terminal ATP pocket is occupied by either ATP or an inhibitor such as GM. Although the contribution of this site to Hsp90 function remains unknown, it has been hypothesized that it might regulate the ATPase activity of the N-terminal region and therefore the cycling of the Hsp90 machinery [46]. It is, however, clear that compounds that interact with this region of the chaperone might also impair Hsp90 function and result in anti-cancer effects.

Novobiocin: Marcu and colleagues have reported that novobiocin, an antibiotic known as an inhibitor of DNA gyrase subunit B, interacts with Hsp90 [47]. Cells exposed to high micromolar concentrations of novobiocin demonstrate destabilization of various Hsp90 client proteins, including Her2, Raf-1, mutant p53 and v-src [48]. Reduction in Raf-1 levels in murine splenocytes *in vivo* was seen following novobiocin treatments. Novobiocin also interferes with association of the co-chaperones Hsp70 and p23 with Hsp90. The binding site of novobiocin has been mapped to a region in the C-terminus of the chaperone [49].

Cisplatin: Itoh *et al.* found that cisplatin binds to Hsp90 and induces a conformational change in the structure of the chaperone [50]. Later studies have shown that cisplatin

interacts with the C-terminal region of Hsp90 and interferes with nucleotide binding in the region [49]. Recently, Rosenhagen *et al.* reported that cisplatin dose-dependently inhibited the transcriptional activity of the androgen and glucocorticoid receptors by disrupting their binding to Hsp90. Cisplatin treatment of neuroblastoma cells leads to steroid receptor inhibition followed by its proteasome-dependent degradation. Other Hsp90-regulated proteins, such as Raf-1, lck and c-src, were not affected, nor did cisplatin elicit a stress response [51]. This is an interesting observation especially in light of a recent finding by McCollum *et al.*, who revealed that cisplatin treatment during or before, but not after, GM treatment, decreases the levels of inducible Hsp70 and HSF-1 in a dose-dependent manner [52]. Two other alkylating agents, melphalan and oxaliplatin, had similar effects. These two findings may well be linked. Previously, Nardai *et al.* have suggested that the oxidation state of the C-terminal cysteines Cys-521 and Cys-589/590 is important for Hsp90 chaperone function [53]. It is thus possible that these nucleophilic cysteines are the target sites of the alkylating agents on Hsp90. If true, these amino acids might hold the clue to the differential regulation of steroid receptors versus kinases (and possibly HSF-1) by Hsp90. These findings also raise the possibility that if the cysteines are involved in the dimerization of the chaperone, the dimer might not be the only form in which the chaperone participates in the activated superchaperone complexes.

Inactivators of Hsp90 function by posttranslational modifications

The importance of posttranslational modifications in regulating Hsp90 function has not been entirely elucidated. Previous research has found that Hsp90 phosphorylation leads to the release of the chaperone from the target protein, a process that can be inhibited by GM [54]. Recently, acetylation and ubiquitinylation of Hsp90 have also been demonstrated to alter its activity although the sites responsible for such posttranslational regulation have yet to be mapped.

Acetylation: HDAC inhibitors, such as depsipeptide FR901228 (FK228), induce growth arrest and apoptosis in a variety of human cancer cells by mechanisms that cannot be attributed solely to histone acetylation. Studies have found that Hsp90 is a downstream target of the HDAC inhibitors; these agents induce acetylation of Hsp90, resulting in inhibition of its binding to ATP which, in turn, impairs association of Hsp90 with its client proteins [55]. As such, FK228 treatment of NSCLC cells results in reduced expression of mutant, but not wild-type, p53, depletion of Her1, Her2, and Raf-1 proteins, and lower ERK1/2 activity; these effects are similar to inhibiting Hsp90 ATPase activity by direct binders of its N-terminal ATP-pocket. Similar effects and induction of Hsp70 were obtained with another HDAC inhibitor, LAQ824, in Her2 overexpressing breast cancer cells and in lymphocytes from patients treated with this agent [56].

Ubiquitinylation: Hsp90 function might also be altered by ubiquitinylation [57]. Treatment of cells with hypericin enhances chaperone ubiquitinylation resulting in the proteasome-independent degradation of Hsp90-client proteins, such as mutant p53, Cdk4, Raf-1 and Plk. Treated cells exhibit retardation at the G2/M checkpoint, increased cell volume and multinucleation, all of which are hallmarks of mitotic cell death.

Collectively, the above findings suggest that, together with co-chaperones and ATP hydrolysis, the posttranslational status of Hsp90 could be responsible for regulating the activation state of the Hsp90 super-chaperone complexes.

Unknown mechanism

Using a forward chemical genetics screen for the identification of Akt pathway inhibitors, Cellular Genomics (http://www.cellulargenomics.com) has selected cgi242 as a compound that demonstrates cellular inhibition profiles similar to that of GM [58]. Treatment of HCT-15 colon cancer cells with micromolar concentrations of cgi242 causes the degradation of Hsp90 client proteins such as Her2, Her3, Raf-1, Akt and RIP and an increase of Hsp70 levels. The structure of cgi242 or its mode of interaction with Hsp90 has yet to be disclosed.

Perspectives

Early clinical results with 17AAG, the first Hsp90 inhibitor to have entered clinical trials, suggest that doses of drug that are sufficient to inhibit Hsp90 function can be administered alone and in combination with cytotoxic chemotherapy with surprisingly little target-associated toxicity. Such observations have prompted a search for novel classes of chaperone inhibitors including natural products and synthetic small-molecule inhibitors of Hsp90 and, in addition, regulators of Hsp90 acetylation and ubiquitinylation. These structurally dissimilar inhibitors might have differential selectivity and improved pharmacologic properties over ansamycins. Many of these agents are in late preclinical development or are already in the clinic and we will soon learn if they live up to the promise of their target and produce sustained clinical anti-tumor responses.

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