Hsp90: an emerging target for breast cancer therapy

Jason Beliakoff[a] and Luke Whitesell[b]

Rapidly evolving insights into the specific molecular genetic abnormalities that drive the growth and metastasis of breast cancer have led to the development of targeted therapeutics that do not rely on the generalized disruption of DNA metabolism and cell division for activity. Of particular interest are inhibitors of cellular signal transduction pathways involving tyrosine kinases as well as selective modulators of steroid hormone signaling, histone acetylation, angiogenesis and tumor cell apoptosis. Unique within this array of promising new agents, however, are compounds that target heat shock protein 90 (Hsp90). This molecular chaperone associates with a distinct, but surprisingly diverse, set of proteins that are referred to as Hsp90 client proteins. Hsp90 binds to these clients, and plays a key role in regulating their stability and function. Many of the proteins chaperoned by Hsp90 are involved in breast cancer progression and resistance to therapy, including the estrogen receptor, receptor tyrosine kinases of the erbB family, Akt, and mutant p53. Several small molecule inhibitors of Hsp90 have been identified that can deplete cellular levels of multiple oncogenic client proteins simultaneously by enhancing their ubiquitination and proteasome-mediated degradation. The activity of Hsp90 inhibitors has been well validated in preclinical breast cancer models, both in single-agent studies and in combination with conventional chemotherapy. One of these inhibitors, 17-allylamino, 17-demethoxygeldanamycin (17-AAG, NSC 330507) has recently completed phase I testing. The agent was well tolerated at drug exposures that were shown to cause modulation of Hsp90 client protein levels. Given the redundancy and complexity of the molecular abnormalities present in most breast cancers, the ability of Hsp90 inhibitors to alter the activity of multiple oncogenic targets may prove of unique therapeutic benefit. *Anti-Cancer Drugs* 15:651–662 © 2004 Lippincott Williams & Wilkins.

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[a]Department of Urology, Stanford University School of Medicine, Stanford, CA, USA and [b]Steele Memorial Children's Research Center and Arizona Cancer Center, Tucson, AZ, USA.

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Correspondence to L. Whitesell, Department of Pediatrics, Hematology/Oncology, Room 5341, Arizona Health Sciences Center, 1501 N. Campbell Avenue, Tucson, AZ 85724, USA.
Tel: +1 520 626-4851; fax: +1 520 626-6986;
e-mail: whitelj@peds.arizona.edu

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Introduction

Breast cancer is the most frequently diagnosed non-skin cancer in American women. Mortality rates due to the disease are second only to lung cancer. In women aged 35–54, breast cancer is the leading cause of all deaths [1]. Over the last decade, improvements in early detection through mammographic screening have correlated with decreased breast cancer-associated mortality and increased detection of localized breast cancer (breast cancer *in situ*). Importantly, patients with breast cancer *in situ* have a 97% 5-year survival rate following treatment. Unfortunately, survival rates decrease rapidly for patients with more advanced disease. Despite the best currently available treatment, five-year survival rates for patients with regional disease and metastatic/disseminated breast cancer drop to 78 and 23%, respectively [2]. Clearly, more effective medical therapies are greatly needed.

Recent advances in understanding the molecular genetic pathology underlying the growth and metastasis of breast cancers have made possible the development of targeted therapeutics that do not rely on the generalized disruption of DNA metabolism and cell division. Because of its well-recognized role in breast cancer biology, one of the first molecular targets identified for therapeutic intervention using non-cytotoxic drugs was the estrogen receptor (ER) [3]. Although most patients initially respond to ER-targeted therapy, the approach is severely limited by the development of acquired resistance [4]. Much of this resistance appears to be mediated by critical crosstalk between the estrogen receptor and other signaling pathways [5,6]. In particular, members of the erbB and Akt family of kinases have been shown to cooperate with the ER in mediating breast tumor cell proliferation/survival, and have been identified as potential therapeutic targets in their own right [7]. Unfortunately, genomic instability and the heterogeneity of molecular defects present in most breast cancers are likely to make emergence of resistance a problem for inhibitors of these pathways as well as for ER-targeted therapeutics. Indeed, clinical experience with imatinib mesylate, a potent and selective inhibitor of the fusion kinase Bcr–Abl in chronic myeloid leukemia suggests that

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such resistance may prove a common limitation to molecularly targeted anticancer drugs in general [8–10].

An intriguing approach to addressing the problem of resistance to inhibitors of specific signal transducers could be provided by agents that disrupt heat shock protein 90 (Hsp90) function. This molecular chaperone associates with a distinct, but surprisingly diverse, set of proteins that are referred to as Hsp90 client proteins. Hsp90 binds to these clients, and plays a key role in regulating their stability and function [11]. Importantly, many of these Hsp90 client proteins are kinases and transcription factors involved in the malignant biology of breast cancers. In particular, the steroid hormone receptors (SHRs) are exquisitely sensitive to alterations in Hsp90 function [12], suggesting that inhibitors of this chaperone may prove quite useful in the management of hormonally regulated breast tumors. For the interested reader, detailed reviews are available addressing the molecular and cellular biology of heat shock proteins, in general [13–15], and Hsp90, in particular [16,17]. Recent phase I trials of 17-allylamino, 17-demethoxygeldanamycin (17-AAG, NSC 330507), the first Hsp90 inhibitor to undergo clinical development, have demonstrated that Hsp90 function can be modulated pharmacologically without excessive toxicity in humans. A number of second-generation compounds are now in late preclinical development. Recent reviews are available that detail the rationale for targeting Hsp90 in cancer and summarize the clinical experience with 17-AAG to date [18–20]. In this paper, we will focus on the role of Hsp90 in regulating several of the key signaling proteins involved in breast cancer progression and discuss how Hsp90 inhibitors may provide a useful new approach for the treatment of this disease, either alone or in combination with other therapies.

Chaperone biology and Hsp90 function

The molecular chaperones are ubiquitous proteins that act to maintain proper protein folding within the cell. They are required for several critical cellular processes, including folding of nascent polypeptide chains, prevention of protein aggregation and protein transport across cell membranes [21]. They were first identified as proteins that were highly expressed under conditions of elevated temperature and were named 'heat shock proteins' (Hsps) [22]. Subsequently, it was appreciated that expression of these proteins was induced not just by heat, but by a variety of cellular stresses including exposure to heavy metals, oxidative stress and inflammation [15]. There are six major Hsp families, grouped according to their molecular size: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and the small Hsps (20–25 kDa in size). Members of these Hsp families are localized to the cytosol, the endoplasmic reticulum and the mitochondria, where they carry out their functions. Although there is significant overlap in function between members of the molecular chaperone superfamily, Hsp90 is unique in its ability to stabilize a number of signaling proteins involved in cancer progression. The functions of other Hsp family members are beyond the scope of this review and will only be discussed as they relate to Hsp90.

Hsp90 is one of the most abundant proteins in the unstressed cell, comprising about 1–2% of the total soluble cytosolic protein [23]. Hsp90 is highly conserved from bacteria to animals and knockout is lethal in all eukaryotic organisms, indicating that its function is important for vital cellular processes [15,24]. Unlike many other Hsp family members, which are involved in the general maintenance of protein folding, Hsp90 has a unique set of proteins with which it interacts. These substrate proteins have been termed Hsp90 'client proteins' and depend on Hsp90 interactions for their activity. Over 100 Hsp90 clients have been documented, many of which are involved in regulating growth and survival (see below). A frequently updated list is maintained by the lab of D. Picard and can be accessed at: www.picard.ch/DP/downloads/Hsp90interactors.pdf.

There are two isoforms of Hsp90 in humans: Hsp90α and Hsp90β. In most tissues, Hsp90α levels are elevated under conditions of cellular stress and Hsp90β is constitutively expressed [16]. The proteins are 76% identical and are the likely result of gene duplication 500 million years ago [25]. Hsp90α has been mapped to chromosome 14 and the Hsp90β gene is located on chromosome 6 [26–29]. It is unclear what functional differences exist between Hsp90α and Hsp90β, and in most cases the two isoforms are not distinguished from one another. However, the proteins are probably not entirely redundant, since a knockout mouse strain for Hsp90β displays embryonic lethality [30]. The Hsp90 genes are regulated, at least in part, by a family of cell stress-inducible transcription factors called the heat shock factors (HSFs). Both Hsp90 genes contain heat shock-responsive elements (HSEs) in their promoters [31–33], which are bound by the HSFs to initiate transcription.

The Hsp90 protein contains three functional domains: an ~25-kDa N-terminal domain, a 'charged linker' domain and an ~55-kDa C-terminal domain [34]. The crystal structure of the N-terminal domain has been elucidated, and contains a unique ATP-binding pocket similar to that of DNA gyrase and the histidine kinase Mut L [35–37]. The N-terminal domain has weak ATPase activity, which is enhanced by binding of client proteins [38–40]. The highly charged linker region bridges the N- and C-termini. It is specific for eukaryotic Hsp90, and may facilitate binding to some client proteins through alternating lysine and glutamic acid residues (so-called

'KEKE motifs') [41]. However, this region is dispensable for most Hsp90 functions [42]. The C-terminal portion of Hsp90 contains an essential constitutive dimerization domain [43]. Recent studies with cisplatin and novobiocin have shown that these drugs can bind, albeit with very low affinity, within the C-terminus of Hsp90. The findings suggest that this region of the chaperone may harbor a second, cryptic ATP-binding domain that is revealed by nucleotide occupancy of its N-terminal site [44,45]. A better understanding of this site's role in regulating Hsp90 function and its potential as an anticancer drug target awaits the discovery of more potent, site-specific inhibitors than the compounds identified so far. The far end of the C-terminus contains the amino acid sequence MEEVD, which is the binding site for the tetratricopeptide repeat (TPR) region that is present on most Hsp90 co-chaperones [46]. Based on recent information about the structural domains of Hsp90, an overall model for Hsp90 chaperoning function has emerged. This model suggests that Hsp90 is constitutively dimerized at the C-terminus, with the ADP-bound N-terminal domains pointing in opposing directions. Upon ATP binding, the N-terminal regions transiently dimerize, forming a molecular clamp around the client protein [47,48]. Hsp90 co-chaperones appear to regulate this clamp by altering the ability of Hsp90 to bind/hydrolyze ATP [40,49,50].

In 1992, it was discovered that geldanamycin (GA) and other benzoquinone ansamycin antibiotics possessed potent antitumor activity that was unrelated to their proposed mechanism of action as inhibitors of the tyrosine kinase Src [51]. Two years later, the target for these agents was demonstrated to be Hsp90 [52]. Subsequently, the GA binding site on Hsp90 was identified as an ATP-binding pocket in the N-terminus of the chaperone [35,53,54]. This pocket is also the binding site of the structurally unrelated natural product radicicol. These drugs act as ADP mimetics, but bind to Hsp90 with higher affinity than the natural nucleotides [55]. Drug binding locks Hsp90 in its ADP-bound conformation and prevents formation of mature Hsp90-containing multi-chaperone complexes with subsequent effects on client protein stability and function in biochemical and cell culture systems. Due to extensive hepatic metabolism, however, neither GA nor radicicol retain activity in whole animals. *In vivo* activity has been demonstrated for the GA derivative 17-AAG which is now undergoing clinical testing, but it too suffers from significant problems due to metabolism and poor solubility. As a result, considerable effort is being directed at developing new inhibitors with better pharmacological and toxicity profiles (Table 1). A variety of approaches are being pursued by academic labs as well as the pharmaceutical industry. These include structure-based screens targeting the Hsp90 nucleotide-binding pocket [56], a colorimetric screen for inhibitors of Hsp90 ATPase activity [57], a stress response-guided natural product screen (L. Whitesell, unpublished), a forward chemical genetic approach [58], directed synthesis of radicicol oxime derivatives [59] and the synthesis of modified purine scaffolds [60]. In attempts to achieve more specific targeting of GA to individual client proteins relevant to breast cancer, conjugation of the drug to estradiol [61], the phosphatidyl inositol-3 kinase (PI3K) inhibitor LY294002 [62] and the anti-erbB2 monoclonal antibody Herceptin [63] has also been reported. Most of this new agent development work is at the stages of lead optimization and preclinical validation, but next generation compounds are expected to become available for clinical trial shortly. For example, a water-soluble, orally bioavailable GA derivative, 17-dimethylaminoethylamino, 17-demethoxygeldanamycin (17-DMAG; NSC 707545) is now available to begin NCI-sponsored phase I testing.

Table 1 **Inhibitors of Hsp90's N-terminal ATPase activity**

Class	Members	Clinical status
Benzoquinone	geldanamycin	NA
Ansamycin	herbimycin A	NA
	macbecins I and II	NA
	17-allylamino, 17-demethoxygeldanamycin (17-AAG)	phase II [156]
	17-dimethylaminoethylamino, 17-demethoxy-geldanamycin (17-DMAG)	phase I [157]
Macrolide	radicicol	NA
	monocillin I	NA
Macrolide	KF58333 (radicicol oxime derivative)	preclinical [117]
Purine scaffold	PU3	NA
	PU24FC1	preclinical [158]
Pyrazole	CCT018159	preclinical [159]

NA: not applicable, compound suitable for laboratory use only.

Hsp90 clients with particular relevance to breast cancer

SHRs

The ER is a member of the SHR superfamily of nuclear receptors, which includes receptors for androgens, glucocorticoids, progestins, thyroid hormone and retinoids. A shared characteristic between all members of the family is the ability to directly activate gene transcription upon ligand binding. The ER is a particularly attractive target for the treatment of breast cancer because protein levels are elevated in many premalignant and malignant lesions, and constitutive ER transcriptional activation is seen in over 50% of diagnosed breast cancers [64,65]. The mechanism for increased ER protein and transcriptional activity is not due to mutations, since alterations in the ER gene are rarely observed in breast carcinomas [65,66]. Indeed, most hormone-refractory tumors retain a functional ER and many are responsive to second-line hormonal therapy [67]. Therefore, it appears that the ER remains a relevant target for the treatment of hormone-insensitive tumors.

Early studies with SHRs utilized a cell-free rabbit reticulocyte lysate system to demonstrate that chaperone proteins are required for assembly of mature receptor complexes that are capable of binding hormone with high affinity. It was initially shown that the *in vitro* glucocorticoid receptor (GR) translation product from reticulocyte lysate was indistinguishable from the native receptor in terms of steroid binding and association with DNA [68]. It was subsequently shown that salt-stripped progesterone receptor (PR) could assemble into a functional complex when added to reticulocyte lysate, indicating that protein translation was not a requirement for receptor maturation [69]. Further studies identified Hsp70, p60/Hop, p23 and the large immunophilins (such as cyclophilin 40 in the case of the ER) as additional components that bind to SHRs [12]. The immunophillins clearly participate in mature Hsp90–co-chaperone–client protein complexes, but their function is not yet well understood. They are not absolutely required for the formation of functional SHR complexes [70,71]. Instead, they appear to modulate the affinity of such complexes for ligand [72,73]. There is also some evidence to suggest that they may link Hsp90 to a dyenin motor protein to facilitate glucocorticoid receptor translocation to the nucleus [17]. Based on findings in reticulocyte lysate and purified protein reconstitution systems, the minimal requirements for mature complex assembly have now been identified as: Hsp90, Hsp70, p60/Hop, Hsp40 and p23 [70,71].

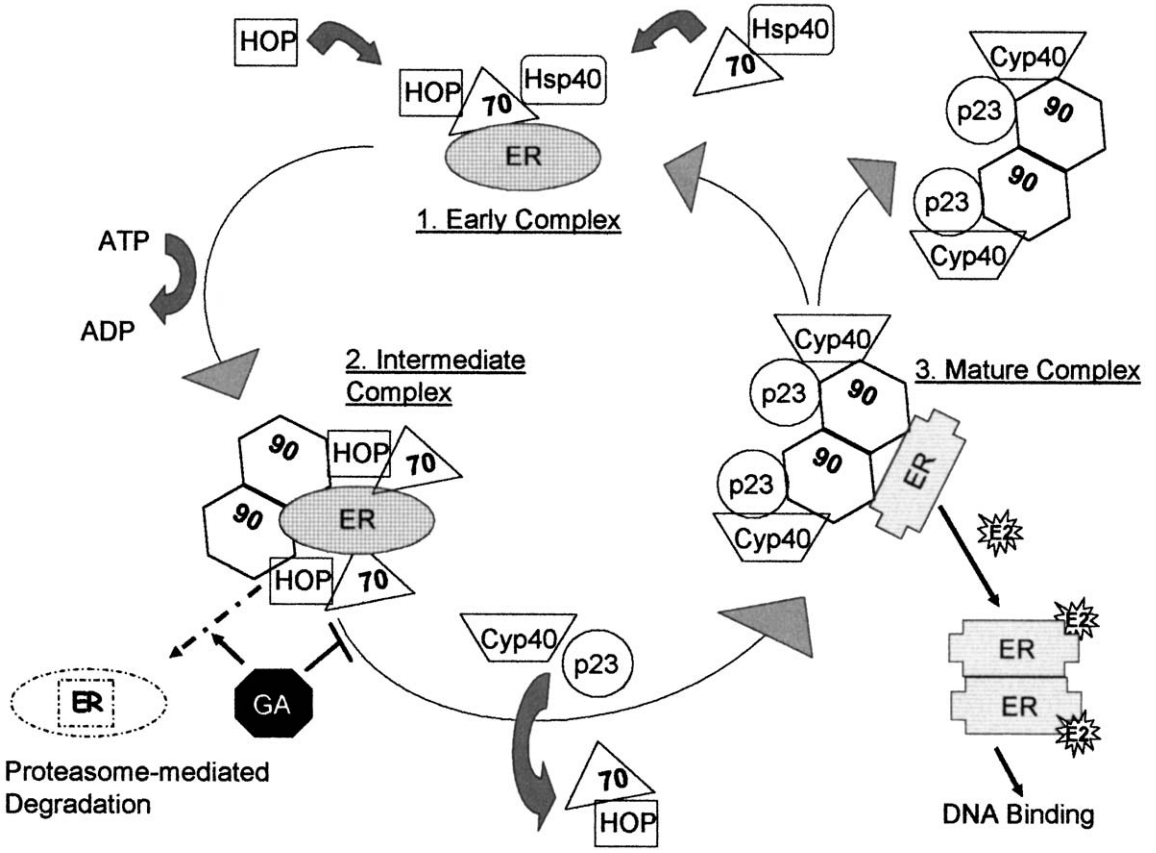
Hsp90 interacts with the ligand-binding domain (LBD) of SHRs. The relative affinity of this association is not uniform across the SHRs, as the interaction between Hsp90 and the ER LBD is much less stable than that of the GR or PR [74]. It has recently been shown that the Hsp90 N-terminal ATP-binding domain (amino acids 1–224) is required for its interaction with the ER [75]. Hsp90 is critical for maintaining the LBD of SHRs in a conformation that is capable of high-affinity ligand binding [76] and SHRs cannot be activated by ligand in the absence of Hsp90 [77]. Hsp70 also binds the LBD of SHRs and deletion of the LBD abrogates the Hsp70 interaction [74,78]. Recent studies have also shown that Hsp70 binds directly to Hsp90 and that these two chaperones cooperate to maintain the LBD in a state capable of activation [79,80]. Another mechanism for Hsp90–Hsp70 association during SHR maturation is through interactions with the co-chaperone p60/Hop. p60/Hop acts as a physical linker by binding to Hsp70 via a TPR domain at the N-terminus and to Hsp90 through a separate TPR domain at the center of the protein [81,82]. Further, the binding of p60/Hop to Hsp90 inhibits ATP binding and its ATPase activity [40,49]. This suggests that p60/Hop influences Hsp90's function as well as its association with Hsp70. p23 associates only with the ATP-bound form of Hsp90 [83] and appears to stabilize mature Hsp90–SHR complexes [84]. The Hsp90 binding site for p23 is localized to the N-terminus, and requires an Hsp90 dimer for association [85]. Hsp40 is not essential for the formation of a ligand binding-competent SHR, although it greatly enhances ligand binding activity [70,71,86]. This is likely due to the ability of Hsp40 to bind to Hsp70 and increase its ATPase activity. Although chaperone involvement has been studied most extensively in the case of SHR maturation and function, similar mechanisms appear to exist for many other Hsp90 clients [87]. While the core machinery remains constant, however, the restricted recruitment of specific co-chaperones to certain classes of client has been demonstrated, most notably p50/cdc37 in the case of kinases [88].

Chaperone interactions with their clients are not static. In the case of SHRs, they appear to occur in a dynamic, iterative fashion to stabilize the receptors in a conformation capable of binding hormone with high affinity. A cartoon summarizing the key chaperone interactions thought to be important in functional maturation of the ER is provided in Figure 1. As described above and depicted in Fig. 1, Hsp90 inhibitors such as GA act as nucleotide mimetics that lock Hsp90 in an ADP-bound conformation. A major consequence of this interaction is inhibition of p23–Hsp90 association, effectively blocking formation of the mature ER–multi-chaperone complex. Instead, the ER accumulates in an intermediate complex that is directed toward the ubiquitin–proteasome pathway by E3 ligases such as CHIP. This results in marked depletion of ER levels and loss of ER function [89,90]. Similar effects have been observed for the androgen receptor (AR) [91,92], PR [93] and GR [94,95], indicating that Hsp90 binding agents may also be useful in the treatment of other hormone-associated malignancies.

Tumor suppressor p53

Mutation of the p53 gene is among the most commonly detected genetic alterations in cancer [96,97] and occurs in approximately 30% of breast tumors [65]. Furthermore, several studies have correlated p53 mutation with poor prognosis and decreased survival [98–100]. The p53 protein regulates an array of important cellular functions, including regulation of the cell cycle and apoptosis and DNA repair. p53 levels are tightly regulated at the post-translational level by the ubiquitin–proteasome pathway, mainly by the MDM2 E3 ubiquitin ligase [101,102]. Wild-type p53 has been shown to interact with Hsp90 and other components of the chaperone machinery [103,104]. However, more stable association is observed with many p53 mutants as a result of their misfolded conformations [13,105,106]. The extended interaction of p53 with the chaperone machinery appears to protect the protein from normal proteolytic turnover, leading to the

Fig. 1



The role of molecular chaperones in ER function. Schematic representation of the steps involved in the chaperone-mediated maturation of the ER. The newly synthesized ER associates with Hsp70, Hsp40 and the co-chaperone p60/Hop to form an early complex (1). The hydrophobic hormone-binding domain is partially exposed in this complex and Hsp90 binds to this region and displaces Hsp40 to form an intermediate complex (2). In an ATP-dependent step, the ATP-bound form of Hsp90 fully exposes the hormone-binding domain and the co-chaperone p23 stabilizes the ATP-bound Hsp90. Cyclophillin 40 (Cyp40) fills the open TPR acceptor site on Hsp90 to complete a mature complex (3). In the absence of ligand, the ER is eventually released from the mature complex to undergo another cycle of ATP-dependent chaperone interactions. Estrogen binding, however, results in a conformational change in the ER, which leads to release of chaperone components, tight binding of the receptor protein to ER response elements and recruitment of the co-activators needed to drive transcription. GA binding to Hsp90 locks the chaperone in an ADP-bound conformation that prevents formation of mature complexes. As a result, the ER accumulates in an intermediate chaperone complex that targets it for ubiquitination and proteasome-mediated degradation.

prolonged half-life and increased cellular levels observed for many mutant p53 proteins [107]. Treatment of cells with GA restores the proteasome-mediated degradation of mutant p53 proteins and decreases their cellular levels [108]. This may diminish the dominant negative effect mutant p53 exerts on the function of wild-type p53 in heterozygous cells, but it does not restore wild-type function to the mutant protein [106].

Angiogenesis transcription factor Hif-1α

Hif-1α is a member of the hypoxia-inducible family of transcription factors, which are upregulated under low oxygen conditions [109]. As with p53, the cellular levels of Hif-1α protein appear to be primarily regulated at the posttranslational level. In the presence of oxygen, Hif-1α associates with the Von Hippel-Lindau (VHL) tumor suppressor protein, an E3 ubiquitin ligase, and is degraded through the ubiquitin–proteasome pathway [110]. Hypoxia inhibits the Hif-1α–VHL interaction, allowing Hif-1α to accumulate and translocate to the nucleus. Hif-1α then dimerizes with Hif-1β, forming an active transcriptional complex. The Hif-1α/Hif-1β heterodimer associates with hypoxia-response elements (HREs) to activate the transcription of Hif-1 target genes, such as vascular endothelial growth factor (VEGF). Importantly, increased Hif-1α levels are associated with poor prognosis and increased metastatic disease in breast cancer patients [111,112], and decreased responsiveness to hormonal therapy [113]. Recent evidence has shown that Hif-1α cellular levels are depleted in several different cancer cell types following exposure to GA, implicating Hsp90 in the regulation of Hif-1α stability

[114]. This effect was observed under both normoxic and hypoxic conditions, and resulted in inhibition of Hif-1α transcriptional activity. Furthermore, co-immunoprecipitation studies have determined that Hsp90 physically interacts with Hif-1α [115]. Interestingly, the degradative effects of GA on Hif-1α appear to be through a VHL-independent mechanism, indicating that a novel Hif-1α degradation pathway exists [116]. Taken together, these data suggest a potential application for Hsp90 binding agents in the clinic as inhibitors of tumor angiogenesis [117].

Antiapoptotic kinase Akt

The Akt serine/threonine kinase is a downstream effector of the PI3K pathway and plays a key role in propagating pro-survival signals within the cell [118]. Co-immunoprecipitation experiments have determined that Akt forms a complex with Hsp90 and the co-chaperone Cdc37. Exposure to Hsp90 inhibitors reduces Akt half-life, resulting in an 80% decrease in cellular Akt levels [119]. Depletion of Akt protein following exposure to Hsp90 inhibitors is also observed in breast and prostate tumor xenografts, and correlates with decreased tumor growth [89,92]. Furthermore, depletion of Akt by 17-AAG also appears to provide enhanced tumor sensitivity to taxol administration [120], providing a rationale for the use of Hsp90 inhibitors in combination with conventional chemotherapy drugs. Interestingly, treatment with Hsp90 binding agents results in rapid loss of Akt phosphorylation (and Akt activity) well before depletion of protein levels are observed [121]. This is likely due to depletion of other Hsp90 clients that regulate Akt phosphorylation, such as the erbB2 receptor tyrosine kinase.

Raf-1 kinase

The serine/threonine kinase Raf-1 is a member of the highly conserved mitogen-activated protein (MAP) kinase signaling pathway, which plays key roles in cell differentiation, proliferation, and survival. The GTP-binding protein Ras regulates Raf-1 activity and is mutated in as many as 30% of human cancers [122]. Upon growth factor stimulation, GTP-bound Ras recruits Raf-1 to the plasma membrane where it is phosphorylated on serine and threonine residues. Raf-1 then phosphorylates MEK, which leads to activation of the extracellular-regulated kinases (ERKs) 1 and 2. The ERKs in turn activate a number of transcription factors, including c-*myc*, Elk-1 and Jun [123]. Raf-1 is known to form a complex with Hsp90 and the p50 chaperone. Studies have shown that Raf-1 levels are depleted in cells exposed to GA, while other members of the MAP kinase cascade are unaffected. However, this reduction is sufficient for decreased MEK phosphorylation, as well as reduced Raf-1-stimulated cell proliferation and transcriptional activity [124]. Importantly, the depletion of Raf-1 by Hsp90 inhibitors is also observed *in vivo*. Treatment with 17-AAG has been shown to cause reproducible depletion of Raf-1 levels in mouse tumor xenograft models of breast cancer [89] and in patients treated on phase 1 clinical trials [19].

Receptor tyrosine kinases

ErbB2 and EGFR are members of the erbB family of transmembrane receptor tyrosine kinases. Activation of these family members leads to downstream signaling through the PI3K/Akt and MAP kinase pathways, resulting in enhanced cell proliferation and survival [125]. Increased erbB2 levels are one of the most common alterations in breast cancer, occurring in about 30% of cases. Importantly, erbB2 overexpression is associated with poor prognosis and resistance to therapy [125,126]. Given its important role in breast cancer progression, erbB2 has become a major target for therapeutic intervention. Several EGFR/erbB2 inhibitors are in various stages of clinical development, and have displayed antiproliferative activity in erbB2 overexpressing cells, as well as enhanced sensitization to radiation therapy [7,127]. Studies have shown that both EGFR and erbB2 physically interact with Hsp90 in whole cells, and that exposure to GA results in rapid depletion of these proteins [128,129]. The intracellular kinase domain of erbB2 appears to be the Hsp90-interaction site, as deletion of this region renders the receptor insensitive to GA-induced degradation. Interestingly, recent evidence implicates the ubiquitin ligase CHIP in GA-induced degradation of the erbB2 protein, indicating that the mechanism of erbB2 depletion may be similar to that of other Hsp90 client proteins [130]. The type 1 insulin-like growth factor receptor (IGF-1R) is another receptor tyrosine kinase that is sensitive to the degradative effects of GA (L. Whitesell, unpublished observations). Importantly, the IGF-1R pathway provides potent mitogenic and survival signals in breast cells that can promote oncogenesis, and inhibition of this pathway sensitizes breast cancer cells to radiation therapy [131,132]. Although the contribution of GA-mediated IGF-1R depletion to the anti-breast cancer activity of the compound is unclear at present, it is tempting to speculate that disruption of IGF-1R signaling could play an important role in the antitumor effects of Hsp90 inhibitors.

Hsp90 inhibitors in breast cancer treatment

Conceptually, Hsp90 function provides an attractive therapeutic target, but the efficacy of this approach has yet to be demonstrated in breast cancer patients. Both cytotoxic and cytostatic anticancer activities have been reported for Hsp90 inhibitors in cell culture and animal tumor models. Information regarding clinical responses in patients with cancer, however, is limited because the first in class Hsp90 inhibitor, 17-AAG, has only been studied in the phase I setting so far. To date, the only responses observed have been disease stabilization consistent with a

cytostatic effect [20]. Given what is known regarding Hsp90's role in malignant transformation, however, this is not terribly surprising. The ability of Hsp90 inhibitors to disrupt multiple oncogenic clients simultaneously is an attractive feature therapeutically, but the pleiotropic effects of targeting Hsp90 make it very difficult to identify predictive indicators of activity and which patients are likely to benefit most from the drug. Despite this basic problem, some molecular genetic and pharmacological determinants of drug sensitivity with relevance to the clinical setting are beginning to emerge and are summarized in [133]. Additional insights are likely to be gained through upcoming NCI-sponsored phase II trials of 17-AAG that will focus on specific malignancies, including breast cancer, in which Hsp90 clients are known to play an important role.

An obvious concern regarding the use of agents that target an essential and ubiquitously expressed protein like Hsp90 is that disruption of the function of the target might be expected to have prohibitive side-effects in normal tissues. Based on phase I studies of the Hsp90 inhibitor 17-AAG, however, this does not appear to be the case. Potential explanations for the minimal toxicity observed in patients treated with the first in class of the Hsp90 inhibitors have recently emerged. It appears likely that tumor cells have a higher requirement for Hsp90 function than normal cells, perhaps due to their markedly increased load of mutated, misfolded proteins [134]. Since it has not been feasible to measure directly the extent of Hsp90 inhibition achieved in whole cells, it is possible that the level of drug-mediated inhibition achieved *in vivo* is sufficient to deplete mutant client protein levels in tumor cells, but is not sufficient to lethally compromise normal cells. Such appears to be the case with inhibitors of the proteasome, another essential multi-protein complex that has been successfully targeted for cancer therapy [135]. Very recently, another potential mechanism for the antitumor selectivity of 17-AAG was reported by Kamal *et al.* This group found that most of the Hsp90 in tumor cells is engaged in multi-chaperone complexes, while the Hsp90 in normal cells is not. The extensively complexed Hsp90 in tumors demonstrated a 100-fold greater affinity for 17-AAG in competitive binding assays than the mostly non-complexed Hsp90 isolated from non-malignant cells [136]. Additional studies designed to shed further light on the differential sensitivity of normal cells and tumor cells to Hsp90 inhibitors are ongoing.

Because 17-AAG has been well tolerated in phase I trials, studies of its use in combination with other therapies are also proceeding. The ability to disrupt signaling pathways that promote tumor cell survival suggests that Hsp90 inhibitors may well prove most effective in the clinic as sensitizers to existing therapeutic regimens. Preclinical data generated *in vitro* and *in vivo* indicate that 17-AAG does indeed enhance the induction of tumor cell death by radiation [137,138]. Studies have also appeared demonstrating supra-additive cell killing by the combination of 17-AAG with cytotoxic agents in breast cancer [140,141], chronic myeloid leukemia [139] and non-small cell lung cancer [142,143]. Of note, significant schedule dependence was observed in some breast cancer cell lines when the Hsp90 inhibitor was combined with taxol [140]. Such dependence is probably related to the profound cell cycle arrest induced by Hsp90 inhibitors. The exact mechanism responsible for this arrest is not clear. It appears to be p53-independent [144], but may involve the tumor suppressor Rb [145] and/or the critical cell cycle protein Chk1 [146]. Conflicting findings regarding synergy versus antagonism have been reported for the combination of 17-AAG with the cell cycle non-specific agent cisplatin [147,148]. This may be due to differences in the cell lines and techniques used to perform the studies. Nevertheless, the combination of these two drugs remains quite intriguing in light of the evidence that cisplatin itself is an Hsp90-binding drug (see above). Occupancy of Hsp90's N-terminal ATP-binding site by GA clearly enhances the ability of cisplatin to bind the chaperone [45], but the extent to which this contributes to its anticancer activity is not known.

While the use of Hsp90 inhibitors in combination with conventional cytotoxic agents may be clinically beneficial with several specific agents, recent work in our laboratory suggests that agents to be used in combination with Hsp90 inhibitors must be selected carefully. We chose to evaluate the effects of Hsp90 inhibitors, which modulate ER function in a non-ligand dependent manner, in combination with the ER antagonist tamoxifen. The ER is a very well validated molecular target for breast cancer therapy, and drugs such as tamoxifen that antagonize ER function are in widespread clinical use. Unfortunately, the frequent emergence of resistance severely limits their ability to cure patients. Several mechanisms for tamoxifen resistance have been proposed, including increased ligand-independent ER activation and alterations in ER cofactor availability [149,150]. Regardless of the mechanism, however, it appears that the ER protein itself is still required for the progression of tamoxifen-resistant tumors [151]. Therefore, the ER remains an important target for the treatment of hormone refractory disease. Because the mechanism of Hsp90 inhibitors involves degradation of client proteins, they present an intriguing alternative for the inhibition of molecular targets that have become resistant to small molecule antagonists. Indeed, we have observed that ER levels are depleted comparably in both tamoxifen-sensitive and tamoxifen-resistant breast cancer cell lines, and that tamoxifen-resistant tumors remain sensitive to 17-AAG [89]. Interestingly, tamoxifen inhibits the degradative effects

of GA and 17-AAG on the ER *in vitro* and *in vivo*. This effect appears to be ER-specific, as the expected depletion of other Hsp90 clients was still observed. The mechanisms responsible for tamoxifen-mediated inhibition of ER degradation are not completely defined, but we have found in MCF-7 cells that tamoxifen disrupts normal and GA-enhanced proteolytic processing of the ER by prolonging its association with DNA at estrogen response elements (Beliakoff and Whitesell, unpublished observations). The clinical significance of this finding is not yet clear, but caution appears appropriate as use of Hsp90 inhibitors in combination with tamoxifen is considered.

Additive antiproliferative activity against MCF-7 breast cancer cells has been reported for the combination of GA with proteasome inhibitors such as Bortezomib (PS-341). At a molecular level, this effect may result from increased protein misfolding induced by GA coupled to impaired clearance of proteins by the ubiquitin proteasome pathway [152,153]. Lastly, synergistic anticancer activity has also been reported for Hsp90 inhibitors combined with histone deacetylase (HDAC) inhibitors [154]. The precise mechanisms underlying this effect are still not clear, but it is intriguing that increased acetylation of Hsp90 has been demonstrated following exposure of cells to HDAC inhibitors, and hyper-acetylation of the protein appears to inhibit its ATP binding and chaperone activities. Some of the cellular effects of HDAC inhibitor exposure, including client protein depletion, are reminiscent of the effects of classical Hsp90 inhibitors such as 17-AAG [155]. The extent to which the effects of HDAC inhibitors on Hsp90 contribute to their anticancer activity or their ability to alter chromatin structure are just beginning to be explored.

Summary and conclusions

It is an exciting time in the development of Hsp90 inhibitors as novel agents for cancer chemotherapy. These drugs appear to provide an alternative strategy to treat breast cancer that has become resistant to currently available therapies, but their efficacy remains to be demonstrated in the clinic. Much of the enthusiasm for their development is derived from their ability to simultaneously deplete cellular levels of proteins such as erbB2, Akt, Raf-1 and mutant p53 that have been associated with resistance to hormonal therapies, conventional chemotherapeutics and radiation.

Studies in cell culture and animal breast cancer models have shown that Hsp90 inhibitors possess antitumor activity as single agents or as sensitizers to conventional treatments. 17-AAG, the first Hsp90-binding agent to enter the clinic, has completed phase I trials and is well tolerated. Importantly, modulation of Hsp90 client proteins has been demonstrated in post-therapy breast tumor tissue at doses below the maximum tolerated dose and additional studies of molecular target modulation in patients treated on subsequent trials will be performed in the near future. Next generation compounds are currently under development that will hopefully provide a better therapeutic index and greater ease in formulation than 17-AAG. The best ways to use Hsp90 inhibitors in patients remain to be defined, but they clearly represent an emerging class of anticancer drug with a unique mechanism of action. As such they appear to hold considerable promise for the treatment of refractory breast cancer, either alone or in combination with other agents.

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