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Chaperoning Oncogenes: Hsp90 as a Target of Geldanamycin

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Abstract Heat shock protein 90 (Hsp90) is a molecular chaperone required for the stability and function of a number of conditionally activated and/or expressed signaling proteins, as well as multiple mutated, chimeric, and/or over-expressed signaling proteins, that promote cancer cell growth and/or survival. Hsp90 inhibitors, by interacting specifically with a single molecular target, cause the inactivation, destabilization, and eventual degradation of Hsp90 client proteins, and they have shown promising anti-tumor activity in preclinical model systems. One Hsp90 inhibitor, 17-AAG, has completed Phase I clinical trial and several Phase II trials of this agent are in progress. Hsp90 inhibitors are unique in that, although they are directed toward a specific molecular target, they simultaneously inhibit multiple signaling pathways that frequently interact to promote cancer cell survival. Further, by inhibiting nodal points in multiple overlapping survival pathways utilized by cancer cells, a combination of an Hsp90 inhibitor with standard chemotherapeutic agents may dramati-

cally increase the in vivo efficacy of the standard agent. Hsp90 inhibitors may circumvent the characteristic genetic plasticity that has allowed cancer cells to eventually evade the toxic effects of most molecularly targeted agents. The mechanism-based use of Hsp90 inhibitors, both alone and in combination with other drugs, should be effective toward multiple forms of cancer.

Keywords Heat shock protein 90 · Cancer · Molecular chaperone · Molecularly targeted therapeutics · Genetic plasticity · Oncogene · Geldanamycin

1 Introduction

Cancer is a disease of genetic instability. Although only a few specific alterations seem to be required for generation of the malignant phenotype, at least in colon carcinoma there are approximately 10,000 estimated mutations at time of diagnosis (Stoler et al. 1999; Hahn and Weinberg 2002). This genetic plasticity of cancer cells allows them to frequently escape the precise molecular targeting of a single signaling node or pathway, making them ultimately nonresponsive to molecularly targeted therapeutics. Even Gleevec (Novartis Pharmaceuticals Corp.), a well-recognized clinically active Bcr-Abl tyrosine kinase inhibitor, can eventually lose its effectiveness under intense, drug-dependent selective pressure, due to either mutation of the drug interaction site or expansion of a previously existing resistant clone (La Rosee et al. 2002). Most solid tumors at the time of detection are already sufficiently genetically diverse to resist single-agent molecularly targeted therapy (Kitano 2003). Thus, a simultaneous attack on multiple nodes of a cancer cell's web of overlapping signaling pathways should be more likely to affect survival than would inhibition of one or even a few individual signaling nodes. Given the number of key nodal proteins that are Hsp90 clients (see the website maintained by D. Picard, http://www.picard.ch/DP/downloads/Hsp90interactors.pdf), inhibition of Hsp90 may serve the purpose of collapsing, or significantly weakening, a cancer cell's safety net. Indeed, following a hypothesis first proposed by Hanahan and Weinberg several years ago (Hanahan and Weinberg 2000), genetic instability allows a cell to eventually acquire six capabilities that are characteristic of most if not all cancers. These are:

- Self-sufficiency in growth signaling
- Insensitivity to anti-growth signaling
- Ability to evade apoptosis
- Sustained angiogenesis
- Tissue invasion and metastasis
- Limitless replicative potential

As is highlighted in Fig. 1, Hsp90 plays a pivotal role in acquisition and maintenance of each of these capabilities. Several excellent reviews provide an in-depth description of the many signaling nodes regulated by Hsp90 (Goetz et al. 2003; Isaacs et al. 2003; Bagatell and Whitesell 2004; Chiosis et al. 2004; Workman 2004; Zhang and Burrows 2004).

Cancer cells survive in the face of frequently extreme environmental stress, such as hypoxia and acidosis, as well as in the face of the exogenously applied environmental stresses of chemotherapy or radiation. These stresses tend to generate free radicals that can cause significant physical damage to cellular proteins. Given the combined protective role of molecular chaperones toward damaged proteins and the dependence of multiple signal transduction pathways on Hsp90, it is therefore not surprising that molecular chaperones in general, and Hsp90 in particular, are highly expressed in most tumor cells. However, Hsp90 may be elevated in tumor cells and may provide a unique molecular target therein for an additional reason. Using *Drosophila* and *Arabidopsis* as model systems, Lindquist and colleagues have shown that an ancient function of Hsp90 may be to permit accumulation at the protein level of inherent genetic mutations, and thus the chaperone may play a pivotal role in the evolutionary process itself (Rutherford and Lindquist 1998; Queitsch et al. 2002). Extrapolating this hypothesis to genetically unstable cancer cells, it is not a great leap to think that Hsp90 may be critical to their ability to survive in the presence of an aberrantly high mutation rate.

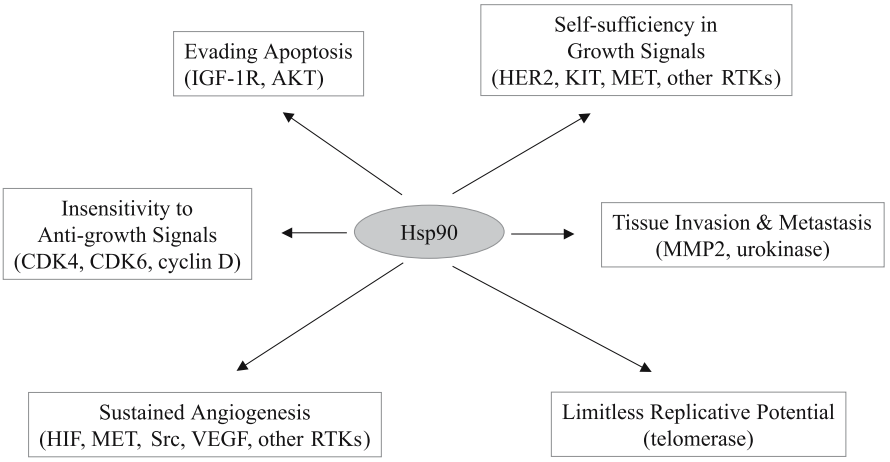

Fig. 1 Hsp90 function is implicated in establishment of each of the hallmarks of cancer as first proposed by Hanahan and Weinberg (2000). Importantly, Hsp90 function may also permit the genetic instability on which acquisition of the six hallmarks depends

2
Hsp90: A Chaperone of Oncogenes

Several recent, excellently detailed reviews of the mechanics of Hsp90 function are in the scientific literature (Prodromou and Pearl 2003; Bagatell and Whitesell 2004; Chiosis et al. 2004; Siligardi et al. 2004; Wegele et al. 2004; Zhang and Burrows 2004; Sangster and Queitsch 2005). The reader is also directed to other chapters in the current volume. For the purposes of the current update on Hsp90-directed therapeutics, suffice it to say that Hsp90 is a conformationally flexible protein that associates with a distinct set of co-chaperones in dependence on nucleotide (ATP or ADP) occupancy of an amino-terminal binding pocket in Hsp90. Nucleotide exchange and ATP hydrolysis (by Hsp90 itself, with the assistance of co-chaperones) drive the so-called Hsp90 chaperone machine to bind, chaperone, and release client proteins. The Hsp90 inhibitors currently in clinical trial (17-AAG and 17-DMAG), as well as those under development, all share the property of displacing nucleotide from the amino terminal pocket in Hsp90, and therefore short-circuiting the Hsp90 chaperone machine, much as one would stop the rotation of a bicycle wheel by inserting a stick between the spokes. Cycling of the chaperone machine is critical to its function. The Hsp90 inhibitors, by preventing nucleotide-dependent cycling, interfere with the chaperone activity of Hsp90, resulting in targeting of client proteins to the proteasome, the cell's garbage disposal, where they are degraded (Neckers 2002). Even if the proteasome is inhibited, client proteins are not rescued from Hsp90 inhibition, but instead accumulate in a misfolded, inactive form in detergent-insoluble subcellular complexes (An et al. 2000).

2.1
Can Hsp90 Inhibitors Distinguish Between Cancer Cell and Normal Cell Hsp90?

An initial concern prior to the initiation of phase I testing of 17-AAG was that inhibition of Hsp90 would be as harmful to normal cells as it seemed to be to cancer cells. This concern has turned out to be unfounded, as no Hsp90-dependent toxicities have been reported from any of the clinical trials. There are two possible explanations for this fact: first, perhaps Hsp90 in normal cells and tissues is not vital to their survival and function. Experiments with yeast suggest that eukaryotic cells can survive with markedly depleted Hsp90 levels, although they do not respond well to nutrient deprivation (Xu et al. 1999). Experiments in nontransformed mammalian cells to test the importance of Hsp90 (e.g., using siRNA techniques) have not been reported.

A second explanation for the lack of in vivo Hsp90-dependent toxicity during administration of Hsp90 inhibitors relates to drug clearance characteristics and differential binding affinity to normal and cancer cell Hsp90. Thus, 17-AAG is cleared rapidly from blood and normal tissues in a number of animal studies (and in humans), while its retention in tumors is prolonged (Eiseman et al.

2004) (to date, information is available for animal studies only). Although the mechanism for drug retention in tumors is not known, it is not unreasonable to speculate that enhanced binding to Hsp90 in tumors as compared to normal tissues may contribute to this phenomenon (Chiosis et al. 2003). This is especially the case, given the recent report that the affinity of tumor cell Hsp90 for 17-AAG and other benzoquinone ansamycins is 20- to 50-fold stronger than is the affinity of Hsp90 isolated from normal tissues (Kamal et al. 2003). An explanation for this differential affinity remains to be uncovered, but it is intriguing to speculate that either post-translational modification (e.g., phosphorylation, acetylation, or other) of Hsp90 is different in transformed as compared to nontransformed cells, resulting in altered affinity for these drugs, or perhaps that co-chaperone complexes associating with Hsp90 are distinct in tumor cells and the nature of these complexes enhance drug binding affinity to Hsp90.

While these possibilities are highly speculative, the fact that 17-AAG and other benzoquinone ansamycins bind to tumor cell Hsp90 with markedly enhanced affinity as compared to normal cell Hsp90 has been confirmed by at least one other laboratory. Whether this will prove to be the case with non-benzoquinone ansamycin Hsp90 inhibitors, or whether this is a unique property of this structural class remains to be determined.

2.2 Hsp90 Inhibitors Target Mutated and Chimeric Proteins Uniquely Expressed in Certain Cancers

Hsp90 characteristically chaperones a number of mutated or chimeric kinases that are key mediators of disease. Thus, anaplastic large cell lymphomas are characterized by expression of the chimeric protein NPM-ALK, which originates from a fusion of the nucleophosmin (*NPM*) and the membrane receptor anaplastic lymphoma kinase (*ALK*) genes. The chimeric kinase is constitutively active and capable of causing malignant transformation (Fujimoto et al. 1996). Bonvini and colleagues have shown that NPM-ALK kinase is an Hsp90 client protein, and that GA and 17-AAG destabilize the kinase and promote its proteasome-mediated degradation in several anaplastic large cell lymphoma cell lines (Bonvini et al. 2002).

FLT3 is a receptor tyrosine kinase that regulates proliferation, differentiation, and survival of hematopoietic cells. FLT3 is frequently expressed in acute myeloid leukemia, and in 20% of patients with this cancer the tumor cells express a FLT3 protein harboring an internal tandem duplication in the juxtamembrane domain. This mutation is correlated with leukocytosis and a poor prognosis (Naoe et al. 2001). Minami and colleagues have reported that Hsp90 inhibitors cause selective apoptosis of leukemia cells expressing tandemly duplicated FLT3. Further, these investigators reported that mutated FLT3 was an Hsp90 client protein and that brief treatment with multiple Hsp90 inhibitors

resulted in the rapid dissociation of Hsp90 from the kinase, accompanied by the rapid loss of kinase activity together with loss of activity of several downstream FLT3 targets including MAP kinase, Akt, and Stat5a (Minami et al. 2002). Minami et al. propose that Hsp90 inhibitors should be considered as promising compounds for the treatment of acute myeloid leukemia characterized by tandemly duplicated FLT3 expression.

BCR-ABL ($p210^{Bcr\text{-}Abl}$) is an Hsp90 client protein that is also effectively inhibited by the novel tyrosine kinase inhibitor imatinib (Druker et al. 1996; An et al. 2000; Shiotsu et al. 2000). While imatinib has proven very effective in initial treatment of patients with chronic myelogenous leukemia, a majority of patients who are treated when their disease is in blast crisis stage (e.g., advanced) eventually relapse despite continued therapy (Sawyers et al. 2002). Relapse is correlated with loss of BCR-ABL inhibition by imatinib, due either to gene amplification or to specific point mutations in the kinase domain that preclude association of imatinib with the kinase (Shah et al. 2002). Gorre and colleagues have reported the very exciting finding that BCR-ABL protein, which was resistant to imatinib, remained dependent on Hsp90 chaperoning activity and thus retained sensitivity to Hsp90 inhibitors, including GA and 17-AAG. Both compounds induced the degradation of wild type and mutant BCR-ABL, with a trend indicating more potent activity toward mutated imatinib-resistant forms of the kinase (Gorre et al. 2002). These findings were recently confirmed by other investigators (Nimmanapalli et al. 2002), thus providing a rationale for the use of 17-AAG in treatment of imatinib-resistant chronic myelogenous leukemia.

Mutations in the proto-oncogene c-*kit* cause constitutive kinase activity of its product, KIT protein, and are associated with human mastocytosis and gastrointestinal stromal tumors (GIST). Although currently available tyrosine kinase inhibitors are effective in the treatment of GIST, there has been limited success in the treatment of mastocytosis. Treatment with 17-AAG of the mast cell line HMC-1.2, harboring the Asp816Val and Val560Gly KIT mutations, and the cell line HMC-1.1, harboring a single Val560Gly mutation, causes both the level and activity of KIT and downstream signaling molecules AKT and STAT3 to be downregulated following drug exposure (Fumo et al. 2004). These data were validated using Cos-7 cells transfected with wild type and mutated KIT. 17-AAG promotes cell death of both HMC mast cell lines. In addition, neoplastic mast cells isolated from patients with mastocytosis and incubated with 17-AAG ex vivo are selectively sensitive to Hsp90 inhibition as compared to the mononuclear fraction as a whole. These data provide compelling evidence that 17-AAG may be effective in the treatment of c-*kit*-related diseases, including mastocytosis, GIST, mast cell leukemia, subtypes of acute myelogenous leukemia, and testicular cancer.

2.3
Hsp90 Inhibitors Target the Androgen Receptor in Prostate Cancer

Androgen receptor continues to be expressed in the majority of hormone-independent prostate cancers, suggesting that it remains important for tumor growth and survival. Receptor overexpression, mutation, and/or post-translational modification may all be mechanisms by which androgen receptor can remain responsive either to low levels of circulating androgen or to anti-androgens. Vanaja et al. have shown that Hsp90 association is essential for the function and stability of the androgen receptor in prostate cancer cells (Vanaja et al. 2002). These investigators reported that androgen receptor levels in LNCaP cells were markedly reduced by the Hsp90 inhibitor geldanamycin (GA), as was the ability of the receptor to become transcriptionally active in the presence of synthetic androgen. In addition, Georget et al. have shown that GA preferentially destabilized androgen receptor bound to anti-androgen, thus suggesting that the clinical efficacy of anti-androgens may be enhanced by combination with an Hsp90 inhibitor (Georget et al. 2002). These investigators also reported that GA prevented the nuclear translocation of ligand-bound androgen receptor, and inhibited the transcriptional activity of nuclear-targeted receptors, implicating Hsp90 in multiple facets of androgen receptor activity. Finally, Solit and colleagues have reported that 17-AAG caused degradation of both wild type and mutant androgen receptors and inhibited both androgen-dependent and androgen-independent prostate tumor growth in nude mice (Solit et al. 2002). Importantly, these investigators also demonstrated the loss of Her2 and Akt proteins, two Hsp90 clients that are upstream post-translational activators of the androgen receptor, in the tumor xenografts taken from 17-AAG-treated animals.

2.4
Hsp90 Inhibitors Exert Anti-angiogenic Activity by Promoting Oxygen- and VHL-Independent Inactivation and Degradation of HIF-1α Leading to Inhibition of VEGF Expression

Hypoxia inducible factor-1α (HIF-1α) is a nuclear transcription factor involved in the transactivation of numerous target genes, many of which are implicated in the promotion of angiogenesis and adaptation to hypoxia (for a review, see Harris 2002). Although these proteins are normally labile and expressed at low levels in normoxic cells, their stability and activation increase several-fold in hypoxia. The molecular basis for the instability of these proteins in normoxia depends upon VHL, the substrate recognition component of an E3 ubiquitin ligase complex that targets HIF-1α for proteasome-dependent degradation (Maxwell et al. 1999). Hypoxia normally impairs VHL function, thus allowing HIF to accumulate. HIF-1α expression has been documented in

diverse epithelial cancers and most certainly supports survival in the oxygen-depleted environment inhabited by most solid tumors.

VHL can also be directly inactivated by mutation or hyper-methylation, resulting in constitutive overexpression of HIF in normoxic cells. In hereditary von Hippel-Lindau disease, there is a genetic loss of VHL, and affected individuals are predisposed to an increased risk of developing highly vascular tumors in a number of organs. This is due, in large part, to deregulated HIF expression and the corresponding upregulation of the HIF target gene vascular endothelial growth factor (VEGF). A common manifestation of VHL disease is the development of clear cell renal cell carcinoma (CC-RCC) (Seizinger et al. 1988). VHL inactivation also occurs in nonhereditary, sporadic CC-RCC.

HIF-1α interacts with Hsp90 (Gradin et al. 1996), and both GA and another Hsp90 inhibitor, radicicol, reduce HIF-dependent transcriptional activity (Hur et al. 2002; Isaacs et al. 2002). Hur et al. demonstrated that HIF protein from radicicol-treated cells was unable to bind DNA, suggesting that Hsp90 is necessary for mediating the proper conformation of HIF and/or recruiting additional co-factors. Likewise, Isaacs et al. reported GA-dependent, transcriptional inhibition of VEGF. Additionally, GA downregulated HIF-1α protein expression by stimulating VHL-independent HIF-1α proteasomal degradation (Isaacs et al. 2002; Mabjeesh et al. 2002).

HIF-1α induction and VEGF expression has been associated with migration of glioblastoma cells *i*n vitro and metastasis of glioblastoma in vivo. Zagzag et al., in agreement with the findings described above, have reported that GA blocks HIF-1α induction and VEGF expression in glioblastoma cell lines (Zagzag et al. 2003). Further, these investigators have shown that GA blocks glioblastoma cell migration, using an in vitro, assay at nontoxic concentrations. This effect on tumor cell motility was independent of p53 and PTEN status, which makes Hsp90 inhibition an attractive modality in glioblastoma, where mutations in p53 and PTEN genes are common and where tumor invasiveness is a major therapeutic challenge.

Dias et al. have recently reported that VEGF promotes elevated Bcl2 protein levels and inhibits activity of the pro-apoptotic caspase-activating protein Apaf in normal endothelial cells and in leukemia cells bearing receptors for VEGF (Dias et al. 2002). Intriguingly, these investigators show that both phenomena require VEGF-stimulated Hsp90 association (e.g., with Bcl2 and Apaf), and that GA reverses both processes. Thus, GA blocked the pro-survival effects of VEGF by both preventing accumulation of anti-apoptotic Bcl2 and blocking the inhibition of pro-apoptotic Apaf.

2.5
Hsp90 Inhibitors Target MET Receptor Tyrosine Kinase

The Met receptor tyrosine kinase is frequently overexpressed in cancer, and is involved in angiogenesis, as well as in the survival and invasive ability of

cancer cells. A recent report by Maulik et al. has demonstrated a role for Met in migration and survival of small cell lung cancer (Maulik et al. 2002). Met is an Hsp90 client protein, and these investigators went on to show that GA antagonized Met activity, reduced the Met protein level, and promoted apoptosis in several small cell lung cancer cell lines, even in the presence of excess Met ligand.

Hypoxia potentiates the invasive and metastatic potential of tumor cells. In an important recent study, Pennacchietti and colleagues reported that hypoxia (via two HIF-1α response elements) transcriptionally activated the Met gene, and synergized with Met ligand in promoting tumor invasion. Further, they showed that the pro-invasive effects of hypoxia were mimicked by Met overexpression, and that inhibition of Met expression prevented hypoxia-induced tumor invasion (Pennacchietti et al. 2003). Coupled with an earlier report describing induction of HIF-1 transcriptional activity by Met ligand (Tacchini et al. 2001), these data identify the HIF-VEGF-Met axis as a critical target for intervention using Hsp90 inhibitors, either alone or in conjunction with other inhibitors of angiogenesis. As Bottaro and Liotta recently pointed out (Bottaro and Liotta 2003), the sole use of angiogenesis inhibitors to deprive tumors of oxygen might produce an unexpectedly aggressive phenotype in those cells that survived the treatment. These authors speculated that combination of Met inhibitors with anti-angiogenesis agents should therefore be beneficial. We would suggest that combination of an anti-angiogenesis drug with an Hsp90 inhibitor would not only potentiate the anti-tumor effects obtained by inhibiting angiogenesis, but would also break the HIF-Met axis by simultaneously targeting both Hsp90-dependent signaling proteins.

2.6
Combined Inhibition of Hsp90 and the Proteasome Disrupt the Endoplasmic Reticulum and Demonstrate Enhanced Toxicity Toward Cancer Cells

Proteasome-mediated degradation is the common fate of Hsp90 client proteins in cells treated with Hsp90 inhibitors (Mimnaugh et al. 1996; Schneider et al. 1996). Proteasome inhibition does not protect Hsp90 clients in the face of chaperone inhibition—instead client proteins become insoluble (An et al. 2000; Basso et al. 2002). Since the deposition of insoluble proteins can be toxic to cells (French et al. 2001; Waelter et al. 2001), interest has arisen in combining proteasome inhibition with inhibition of Hsp90, the idea being that dual treatment will lead to enhanced accumulation of insoluble proteins and trigger apoptosis. This hypothesis is particularly appealing since a small molecule proteasome inhibitor has demonstrated efficacy in early clinical trials (Aghajanian et al. 2002; L'Allemain 2002). Initial experimental support for such a hypothesis was provided by Mitsiades et al. (2002), who reported that Hsp90 inhibitors enhanced multiple myeloma cell sensitivity to proteasome inhibition. Importantly, transformed cells are more sensitive to the cytotoxic

effects of this drug combination than are nontransformed cells. Thus, 3T3 fibroblasts are fully resistant to combined administration of 17-AAG and Velcade at concentrations that prove cytotoxic to 3T3 cells transformed by *HPV16* virus encoding viral proteins E6 and E7 (Mimnaugh et al. 2004). In the same study, Mimnaugh et al. demonstrated that the endoplasmic reticulum is one of the main targets of this drug combination. In the presence of combined doses of both agents that show synergistic cytotoxicity, these investigators noted a nearly complete disruption of the architecture of the endoplasmic reticulum. Since all secreted and transmembrane proteins must pass through this organelle on their route to the extracellular space, it is not surprising that a highly secretory cancer such as multiple myeloma would be particularly sensitive to combined inhibition of Hsp90 and the proteasome. One might speculate that other highly secretory cancers, including hepatocellular carcinoma and pancreatic carcinoma, would also respond favorably to this drug combination.

2.7 Hsp90 Inhibitors Sensitize Cancer Cells to Radiation

Gius and colleagues have reported that 17-AAG potentiates both the in vitro and in vivo radiation response of cervical carcinoma cells (Bisht et al. 2003). An enhanced radiation response was noted when cells were exposed to radiation within 6–48 h after drug treatment. Importantly, at 17-AAG concentrations that were themselves nontoxic, Hsp90 inhibition enhanced cell kill in response to an otherwise ineffective radiation exposure (2 Gy) by more than one log. Even at moderately effective levels of radiation exposure (4–6 Gy), addition of nontoxic amounts of 17-AAG enhanced cell kill by more than one log. Importantly, the sensitizing effects of 17-AAG observed in the cervical carcinoma cells were not seen in 3T3 cells, but were observed in *HPV16-E6* and *-E7* transformed 3T3 cells. The authors demonstrated convincingly that the effect of 17-AAG was multifactorial, since several pro-survival Hsp90 client proteins were rapidly downregulated upon drug treatment. In vitro findings were confirmed by a murine xenograft study in which the anti-tumor activity of both single and fractionated radiation exposure was dramatically enhanced by treatment with 17-AAG, either 16 h prior to single radiation exposure or on days 1 and 4 of a 6-day period during which the animals received fractionated radiation exposure. Machida and colleagues reported similar findings for lung carcinoma and colon adenocarcinoma cells in vitro (Machida et al. 2003). Thus, 17-AAG has been validated as a potential therapeutic agent that can be used at clinically relevant doses to enhance cancer cell sensitivity to radiation. It is reasonable to expect that other Hsp90 inhibitors will have a similar utility.

2.8
Targeting Hsp90 on the Cancer Cell Surface

Recently, Becker and colleagues reported that Hsp90 expression is dramatically upregulated in malignant melanoma cells as compared to benign melanocytic lesions, and that Hsp90 is expressed on the surface of seven out of eight melanoma metastases (Becker et al. 2004). Eustace et al. have identified cell surface Hsp90 to be crucial for the invasiveness of HT-1080 fibrosarcoma cells in vitro (Eustace and Jay 2004; Eustace et al. 2004). Taken together, these data implicate Hsp90 as an important determinant of tumor cell invasion and metastasis. Indeed, in the Eustace et al. study, the investigators demonstrated that GA covalently affixed to cell impermeable beads was able to significantly impair cell invasion across a Matrigel-coated membrane. These findings have been confirmed using a polar (and thus cell impermeable) derivative of 17-DMAG in place of GA-beads (Neckers et al., unpublished observations). Coincident with its inhibitory effects on cell invasiveness, cell impermeable GA also antagonized the maturation, via proteolytic self-processing, of the metalloproteinase MMP2, a cell surface enzyme whose activity has been previously demonstrated as essential to cell invasion. Further, these investigators demonstrated that Hsp90 could be found in association with MMP2 in the culture medium bathing the HT-1080 cells. It is intriguing to speculate that association with Hsp90 on the cell surface is necessary for the self-proteolysis of MMP2. Thus, a possible chaperone function for cell surface Hsp90 may be directly implicated in tumor cell invasiveness and metastasis. As such, cell surface Hsp90 may represent a novel, perhaps cancer-specific target for cell-impermeant Hsp90 inhibitors.

3
Hsp90 Inhibitors that Do not Target the Amino Terminal Nucleotide Binding Site

In the last several years, it has become apparent that Hsp90 activity can be impacted by pharmacologic attack at other sites on the protein. However, these other inhibitors are not specific for Hsp90 and thus cannot be considered to be "clean" drugs. Several years ago, we identified the coumarin antibiotics (e.g., novobiocin) as capable of binding to a C-terminal ATP-binding domain on Hsp90, with resultant inhibition of Hsp90 activity (Marcu et al. 2000a, 2000b). These results have since been confirmed and extended by others (Soti et al. 2002), but the affinity of novobiocin for Hsp90 is poor (several hundred micromolar), and it is a much better inhibitor of topoisomerase than it is of Hsp90.

Cisplatin, which exerts anti-tumor activity, at least in part, by forming DNA adducts and thus interfering in transcription, has recently been shown to also

bind to the C-terminus of Hsp90, and to interfere with nucleotide binding at this site (Soti et al. 2002). However, cisplatin interacts with other thiol-containing proteins and its interaction with Hsp90 occurs at concentrations that are too high to be pharmacologically relevant. Nonetheless, the fact that both the coumarin antibiotics and cisplatin share an Hsp90 binding domain in the C-terminus of the chaperone highlights the potential importance of this domain to Hsp90 function, as well as places emphasis on the pharmacologic accessibility of this site (for review see Marcu and Neckers 2003). Nevertheless, little progress has been made in identifying high-affinity Hsp90-specific agents that target this region.

A 3rd class of agent that has been shown to affect Hsp90 are the inhibitors of histone deacetylases (HDAC inhibitors). Schrump and colleagues first demonstrated that the HDAC inhibitor depsipeptide (FR901228) was able to promote degradation of several Hsp90 client proteins, including mutated p53, c-raf-1, and ErbB2 (Yu et al. 2002). These investigators also showed that depsipeptide promoted Hsp90 acetylation and disrupted its ability to bind to ATP-Sepharose. Similar results have since been reported by Bhalla et al. (Fuino et al. 2003) using the HDAC inhibitor LAQ824, a molecule structurally different from depsipeptide. Thus, acetylation of Hsp90, which clearly occurs in response to administration of diverse HDAC inhibitors, inhibits Hsp90 function, most likely by interfering with nucleotide-dependent cycling of the chaperone complex. Certainly, Hsp90 inhibition must be considered as a component of the anti-tumor activity of these drugs, but protein hyperacetylation is now a commonly observed property of these agents, and Hsp90 is probably one of many hyperacetylated proteins. It will be of interest to determine the specific HDAC (there are several classes and multiple members of each class) or HDACs that deacetylate Hsp90 under physiologic conditions, and then to test a specific inhibitor of this HDAC, but even in the best case scenario it is unlikely that Hsp90 would be the only substrate affected. In summary, the only pure Hsp90 inhibitors identified to date are those that bind to the N-terminal nucleotide pocket of the chaperone.

4
Issues Relating to Further Development of Hsp90 Inhibitors

The toxicity, if any, produced as a result of inhibiting Hsp90 in normal tissues remains to be determined. The dose-limiting toxicity of 17-AAG (hepatic) may be due primarily to the chemical structure of the drug (e.g., presence of a quinone moiety) and not to any Hsp90-dependent effect. Ultimately, this question will be addressed when nonansamycin Hsp90 inhibitors reach the clinic. However, it is important to remember that the differential affinity of ansamycin-based Hsp90 inhibitors for tumor cell vs normal cell Hsp90 may play a role in the apparent lack of target-based toxicity of 17-AAG in normal

tissues. Some of the nonansamycin Hsp90 inhibitors in development do not possess this discriminatory property, and therefore careful evaluation of their in vivo toxicities will help determine the importance of this phenomenon to overall drug efficacy. The ideal Hsp90 inhibitor should be an orally bioavailable agent that favors inhibition of tumor cell Hsp90.

Hsp90 inhibition leads to activation of the heat shock transcription factor Hsf1 and therefore promotes induction of Hsp70 and other chaperones as part of a classical heat shock response (Ali et al. 1998; Kim et al. 1999; Xiao et al. 1999; Guo et al. 2001; Lu et al. 2002; Matthews et al. 2003). Indeed, induction of Hsp70 in vivo following 17-AAG administration has proven to be the most robust pharmacodynamic indicator of drug activity in patients. However, Hsp70 is generally considered to be cytoprotective for tumor cells, and Whitesell and colleagues have reported that loss of Hsf1 expression potentiates the cytotoxicity of GA (Bagatell et al. 2000). Very recently, Gabai et al. have confirmed this hypothesis by showing that siRNA-dependent depletion of Hsp70 promotes the cytotoxicity, in vitro, of Hsp90 targeting agents (Gabai et al. 2005). Therefore, it is conceivable that the effectiveness of prolonged treatment with 17-AAG or similar Hsp90 inhibitor may be self-limiting, making selection of the appropriate schedule of drug administration a possibly critical component of its success.

Although the focus to date has been on identification of small molecule Hsp90 inhibitors, it is now clear that proper function of Hsp90 requires that it associate in a regulated manner with a host of co-chaperone proteins. Thus, investigators in the field have begun to examine whether small molecule inhibitors of specific co-chaperone–Hsp90 interactions can be designed, and if so, what effect they might have on Hsp90 function. However, this approach requires identification of inhibitors of protein–protein surface interactions, generally a more difficult undertaking than designing competitive inhibitors of the nucleotide pocket.

Another approach to pharmacologically inhibiting Hsp90 lies in understanding the role of various post-translational modifications in the function of the chaperone. For example, two studies have demonstrated that Hsp90 phosphorylation is coupled to the release of the chaperone from its client protein (Mimnaugh et al. 1995; Zhao et al. 2001). GA inhibits Hsp90 phosphorylation, suggesting that this post-translational modification is conformation-dependent, while the phosphatase inhibitor okadaic acid leads to dramatic hyperphosphorylation of Hsp90, suggesting that cycles of phosphorylation and dephosphorylation of the chaperone are continuously occurring in a regulated manner. Further, Hsp90 acetylation, as discussed earlier, has also been shown to modulate its activity, and several HDAC inhibitors have been reported to inhibit the chaperone, with consequences similar to those observed following exposure to pure Hsp90 inhibitors (Yu et al. 2002; Fuino et al. 2003).

5 Conclusion

By their very nature, cancer cells are genetically unstable. Indeed, it is this property that is the key to their etiology. Thus, in most cases, cancers display the heterogeneity and redundancy of signaling pathways that make them resistant to many environmental insults, including those imposed by chemotherapeutics and radiation. Molecular chaperones in general, and Hsp90 in particular, clearly play a major role in promoting this robustness, and therefore Hsp90 inhibitors, by targeting the very property upon which cancer cells depend for their survival, comprise a truly unique class of anti-cancer agent. Only one class of small molecule Hsp90 inhibitor has reached the clinic to date. More are certainly needed. Nonetheless, the preference of 17-AAG for tumor cell Hsp90, and the demonstration that Hsp90 can be pharmacologically modulated in vivo without severe toxicity are both very encouraging stimuli that should spur the development of second- and third-generation Hsp90 inhibitors with better pharmacologic properties. The preceding discussion of some of the latest developments in Hsp90 biology make it clear that ongoing multidisciplinary efforts of basic and clinical investigators are dramatically expanding our appreciation of the possible ways in which inhibition of Hsp90 can be exploited to mount a multifaceted attack on cancer. Further development of additional Hsp90 inhibitors is needed to most effectively take advantage of our rapidly evolving knowledge.

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