
Heat shock protein 90: the cancer chaperone

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Heat shock protein 90 (Hsp90) is a molecular chaperone required for the stability and function of a number of conditionally activated and/or expressed signalling proteins, as well as multiple mutated, chimeric, and/or over-expressed signalling proteins, that promote cancer cell growth and/or survival. Hsp90 inhibitors are unique in that, although they are directed towards a specific molecular target, they simultaneously inhibit multiple cellular signalling pathways. By inhibiting nodal points in multiple overlapping survival pathways utilized by cancer cells, combination of an Hsp90 inhibitor with standard chemotherapeutic agents may dramatically 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. Further, because Hsp90 inhibitors also induce Hsf-1-dependent expression of Hsp70, and because certain mutated Hsp90 client proteins are neurotoxic, these drugs display ameliorative properties in several neurodegenerative disease models, suggesting a novel role for Hsp90 inhibitors in treating multiple pathologies involving neurodegeneration.

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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 the time of diagnosis (Hahn and Weinberg 2002; Stoler *et al* 1999). This genetic plasticity of cancer cells allows them to frequently escape the precise molecular targeting of a single signalling node or pathway, making them ultimately non-responsive 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 signalling pathways should be more likely to affect survival than would inhibition of one or even a few individual signalling 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>, as well as several excellent reviews (Nardai *et al* 2006; Sreedhar *et al* 2004; Zhao *et al* 2005)), 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 (i) self-sufficiency in growth signalling; (ii) insensitivity to anti-growth signalling; (iii) ability to evade apoptosis; (iv) sustained angiogenesis; (v) tissue invasion and metastasis; and (vi) limitless replicative potential. As is highlighted in figure 1, Hsp90 plays a pivotal role in acquisition and maintenance of each of these capabilities. Several excellent reviews provide an

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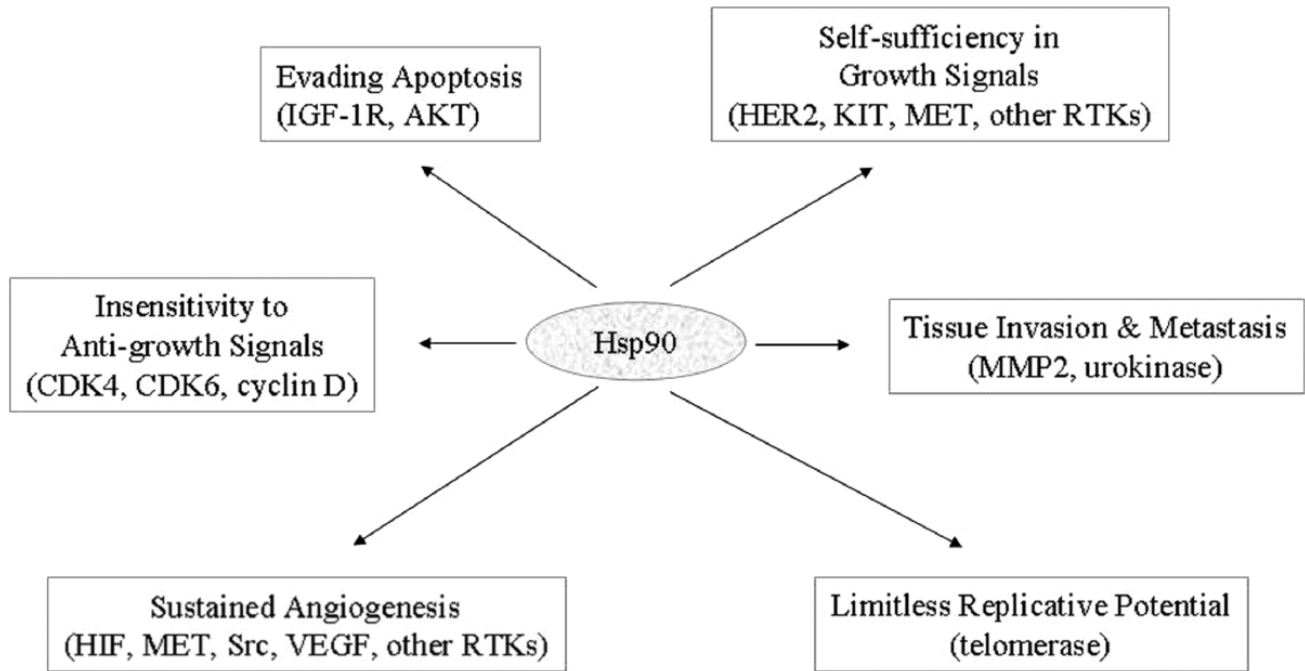


Figure 1. Hsp90 function is implicated in establishment of each of the hallmarks of cancer as first proposed by Hanahan and Weinberg (Hanahan and Weinberg 2000). Importantly, Hsp90 function may also permit the genetic instability on which acquisition of the six hallmarks depends.

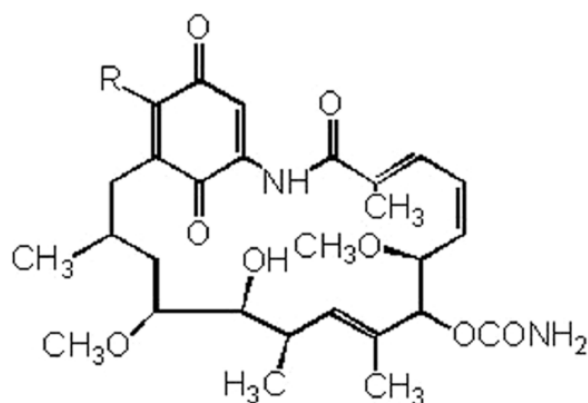
in depth description of the many signalling nodes regulated by Hsp90 (Goetz *et al* 2003; Isaacs *et al* 2003; Bagatell and Whitesell 2004; Chiosis *et al* 2004; Workman 2004; Zhang and Burrows 2004; Isaacs 2005).

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 tumour cells. However, Hsp90 may be elevated in tumour 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 (Queitsch *et al* 2002; Rutherford and Lindquist 1998). 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.

The benzoquinoid ansamycin antibiotics, first isolated from the actinomycete, *Streptomyces hygroscopicus var. geldanus var. nova* (DeBoer *et al* 1970) include geldanamycin (GA) and its semi-synthetic derivatives, 17-allylamino-17-demethoxygeldanamycin (17-AAG) and the more water-soluble 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) (*see figure 2*). These small molecules inhibit the chaperone function of the heat shock protein Hsp90 (Schulte and Neckers 1998) and are currently being evaluated in phase 1 and 2 clinical trials. The parent compound, GA, is broadly cytotoxic in the NCI 60-cell line screen (Supko *et al* 1995); its poor solubility and unacceptable liver toxicity in dogs precluded testing in humans. Because 17-AAG is less toxic than GA in rats (Page *et al* 1997) and caused growth inhibition in breast (Paine-Murrieta *et al* 1999), melanoma (Burger *et al* 1998), and ovarian mouse xenograft models, the National Cancer Institute (NCI) initiated phase 1 trials in 1999.

2. Hsp90: A chaperone of oncogenes

The mechanics of Hsp90 function have been reviewed in detail (Prodromou and Pearl 2003; Bagatell and Whitesell 2004; Chiosis *et al* 2004; Siligardi *et al* 2004; Wegele *et al*



Compound	R Group
17-Allylaminogeldanamycin (17-AAG)	$\text{CH}_2=\text{CHCH}_2\text{NH}-$
17-Aminogeldanamycin (17-AG)	NH_2
17-Dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG)	$(\text{CH}_2)_2\text{NCH}_2\text{CH}_2\text{NH}-$
Geldanamycin	$\text{CH}_3\text{O}-$

Figure 2. The chemical structures of geldanamycin, 17-AAG, its biologically active metabolite 17-AG, and 17-DMAG, highlighting the unique substitutions to the quinone moiety of the pharmacophore that characterize each molecule.

2004; Zhang and Burrows 2004). For the purposes of the current update on Hsp90-directed therapeutics, suffice 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. Indeed, identification of the GA binding site as a nucleotide pocket favoring purines led Chiosis and colleagues to design a series of highly potent purine scaffold Hsp90 inhibitors with markedly improved drug-like properties (Chiosis *et al* 2002; Chiosis *et al* 2003; Llauger *et al* 2005; He *et al* 2006). Workman and colleagues used a high-throughput screen based on inhibition of Hsp90 ATPase activity to identify 3,4-diarylpyrazoles as a novel class of Hsp90 inhibitors (Cheung *et al* 2005; Dymock *et al* 2005). Employing biochemical evaluation and crystallography, these investigators found

that pyrazole inhibitors of Hsp90 provide a platform for extensive derivatization and provide an attractive starting point for exploration.

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 *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 percent 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-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 that 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 signalling molecules AKT and STAT3 to be down-regulated 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, sub-types of acute myelogenous leukemia and testicular cancer.

More recently, several groups have reported that mutated B-Raf and mutated epidermal growth factor receptor (EGFR) develop strong dependence on Hsp90 and thus acquire marked sensitivity to Hsp90 inhibitors (da Rocha Dias *et al* 2005; Shimamura *et al* 2005; Grbovic *et al* 2006). Since B-Raf is mutated in approximately 60% of melanomas and to a lesser degree in other cancers (Rajagopalan *et al* 2002), and since cells expressing mutated B-Raf appear to be dependent on its activity for their survival, Hsp90 inhibitors may have wide applicability in melanoma. Indeed, results of a recent clinical trial support this hypothesis (Banerji *et al* 2005). Similarly, the EGFR mutations described in a small percentage of non-small cell lung cancer patients also confer Hsp90 dependence and sensitivity to Hsp90 inhibitors (Shimamura *et al* 2005). While these patients initially respond to EGFR inhibitor therapy, they almost invariably become refractory with time. However, even tumors refractory to EGFR inhibitors remain very sensitive to Hsp90 inhibitors, suggesting that Hsp90 inhibitor therapy may be an efficacious second-line therapy in these patients (Shimamura *et al* 2005).

2.2 *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 over-expression, 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 GA, as was the ability of the receptor to become transcriptionally active in the presence of synthetic androgen. In addition, Georget *et al* (2002) 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. 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.

2.3 Hsp90 inhibitors exert anti-angiogenic activity by promoting oxygen- and VHL-independent inactivation and degradation of hypoxia inducible factor 1 α (HIF-1 α) leading to inhibition of VEGF expression

Hypoxia inducible factor-1 α (HIF-1 α) 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-1 α for proteasome-dependent degradation (Maxwell *et al* 1999). Hypoxia normally impairs VHL function, thus allowing HIF to accumulate. HIF-1 α 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 hypermethylation, resulting in constitutive over-expression 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 up-regulation 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-1 α 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 cofactors. Likewise, Isaacs *et al* reported GA-dependent, transcriptional inhibition of VEGF. Additionally, GA down-regulated HIF-1 α protein expression by stimulating VHL-independent HIF-1 α proteasomal degradation (Isaacs *et al* 2002; Mabjeesh *et al* 2002).

HIF-1 α induction and VEGF expression has been associated with migration of glioblastoma cells *in vitro* and metastasis of glioblastoma *in vivo*. Zagzag *et al* (2003) in agreement with the findings described above, have reported that GA blocks HIF-1 α induction and VEGF expression in glioblastoma cell lines. Further, these investigators have shown that GA blocks glioblastoma cell migration, using an *in vitro* assay at non-toxic 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* (2002) 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. 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.

2.4 Hsp90 inhibitors target MET and RET receptor tyrosine kinases

The Met receptor tyrosine kinase is frequently over-expressed 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-1 α 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 over-expression, 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 signalling proteins.

Mutation of a related receptor tyrosine kinase, RET, is associated with human cancer and several human neuroendocrine diseases. Point mutations of RET are responsible for multiple endocrine neoplasia type 2 (MEN2A, MEN2B, and familial medullary thyroid carcinoma [FMTC]). Somatic gene rearrangements juxtaposing the TK domain of RET to heterologous gene partners are found in papillary carcinomas of the thyroid (PTC) (Jhiang 2000; Santoro *et al* 2002; Ichihara *et al* 2004).

Possible effects of 17-AAG on RET activity and cell growth of the TT MTC cell line have been examined (Cohen *et al* 2002). Following treatment with 17-AAG, RET tyrosine kinase activity was inhibited by nearly 80%, as was the rate of cell growth. Thus, 17-AAG should be considered as an attractive pharmacologic agent for use as systemic therapy in patients with recurrent metastatic MTC for which non-surgical therapy has been ineffective.

2.5 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 non-transformed 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.

2.6 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 to 48 hours after drug treatment. Importantly, at 17-AAG concentrations that were themselves non-toxic, Hsp90 inhibition enhanced cell kill in response to an otherwise ineffective radiation exposure (2 Gray) by more than one log. Even at moderately effective levels of radiation exposure (4-6 Gray), addition of non-toxic 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 multi-factorial, since several pro-survival Hsp90 client proteins were rapidly down-regulated 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 hours 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.

2.7 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 7 out of 8 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. Metabolism of 17-AAG and 17-DMAG *in vivo*

In human or murine hepatic microsome assays, 17-aminogeldanamycin (17-AG), a diol, and an epoxide are the

three major metabolites of 17-AAG (Egorin *et al* 1998). The 17-AAG diol was the major metabolite in human hepatic microsomes, followed by 17-AG; in contrast, 17-AG was the most abundant metabolite in murine microsomes. Acrolein, a nephrotoxin, is a potential by-product of the 17-AG metabolite. Finally, the epoxide is probably formed by addition of oxygen across the double bond of the allylamino side chain. CYP3A4 enzymatic metabolism is responsible for 17-AG and epoxide formation. Microsomal epoxide hydrolase catalyzes the conversion of the diol to 17-AG, which does not undergo further microsomal metabolism. 17-AAG metabolites are active and may have clinical significance. The biologically active epoxides and acrolein may induce toxic effects in humans (Egorin *et al* 1998). Pharmacodynamic studies show that the 17-AG metabolite (see figure 2) is as active as 17-AAG in decreasing cellular p185^{erbB2} in human breast cancer SKBr3 cells in culture (Schnur *et al* 1995). 17-AG causes growth-inhibition in six human colon cancer lines and three ovarian cancer cell lines (Kelland *et al* 1999).

In contrast to 17-AAG, 17-DMAG appears to be only minimally metabolized by CYP3A4 (Egorin *et al* 2002). Therefore, intestinal CYP3A4 should not impede 17-DMAG's oral activity. 17-AG does not appear to be a metabolite of 17-DMAG based on the lack of conversion at the 17 position of the compound. The marked metabolic differences between 17-AAG and 17-DMAG suggest that they may have distinct toxicity profiles and therapeutic indices.

4. Why are tumor cells uniquely sensitive to Hsp90 inhibition?

It is apparent, from both preclinical and clinical observations, that Hsp90 inhibitors can be administered *in vivo* at doses and schedules that significantly impact tumor growth but with minimal target related toxicity to normal tissues. This is the case for several small molecule inhibitors, including 17-AAG and 17-DMAG, the synthetic purine mimetic PU24FCl, and it even applies to a novel peptidomimetic inhibitor of the N-terminal Hsp90 nucleotide binding site, shepherdin (Xu *et al* 2003; Vilenchik *et al* 2004; Banerji *et al* 2005; Eiseman *et al* 2005; Plescia *et al* 2005). Since Hsp90 is highly expressed in most, if not all normal tissues, these findings require an explanation. Indeed, when murine model systems are examined *in vivo*, Hsp90 inhibitors are found to concentrate in tumor tissue, while being rapidly cleared from normal tissue with a half-life similar to that of drug in plasma (Xu *et al* 2003; Vilenchik *et al* 2004; Banerji *et al* 2005; Eiseman *et al* 2005). The Hsp90 inhibitor 17-AAG also has been reported to actively concentrate in tumor cells *in vitro* (Chiosis *et al* 2003).

Since preferential accumulation of these Hsp90 inhibitors in tumor vs. normal tissue may provide the observed

therapeutic (or at least biologic) index, it is important to understand the reason for this phenomenon. A possible explanation put forth by Kamal and colleagues suggests that enhanced drug binding to tumor cell Hsp90 reflects the activity state of the Hsp90 chaperone machine in tumor vs. normal cells (Kamal *et al* 2003). They proposed that enhanced the ATPase activity of the chaperone in tumor cells, which is dependent on preferential recruitment of Hsp90 to a multi-component chaperone complex, is responsible for the increased affinity of Hsp90 inhibitors in tumor cells.

Others have reported that expression of NAD(P)H: Quinone Oxidoreductase I (NQO1), also known as DT-diaphorase, dramatically enhances cellular sensitivity to 17-AAG (Kelland *et al* 1999; Banerji *et al* 2005). NQO1 generates the hydroquinone version of 17-AAG, which has recently been reported to bind more tightly to Hsp90 when compared to 17-AAG itself (Guo *et al* 2005). Further, the presence of NQO1 in a cell seems also to lead to increased total accumulation of intracellular ansamycin molecules, presumably reflecting the increased water solubility of the 17-AAG dihydroquinone and its decreased propensity to cross membranes. Thus, by this model NQO1 serves to trap 17-AAG in cells while simultaneously enhancing its Hsp90 binding affinity. Intriguingly, these investigators and others have shown that the presence of NQO1 in tumor cells dramatically affects cellular sensitivity to 17-AAG (Kelland *et al* 1999; Banerji *et al* 2005; Guo *et al* 2005). Since high levels of NQO1 have been observed in diverse tumor types (e.g. liver, lung, colon, breast) as compared to normal tissues of the same origin (Belinsky and Jaiswal 1993), these data suggest an explanation for the disparate sensitivity of tumor and normal tissue to 17-AAG. However, the similar preference of other Hsp90 inhibitors, such as the synthetic purine analog PU24FC1 and the peptidomimetic shepherdin, for tumor cells remains to be explained. Several groups are currently examining altered states of post-translational modification of Hsp90 in tumor vs. normal cells as a possible contributing factor to this phenomenon.

5. Clinical trial data

The Institute of Cancer Research (UK) phase 1 trial of 17-AAG in malignant melanoma used a once weekly administration schedule. The starting dose was 10 mg/m²/week administered intravenously once weekly in a cohort of three patients. Doses were doubled in each succeeding cohort (Banerji *et al* 2001). Adverse events included grade 1/2 nausea and grade 1/2 fatigue in 3 and 9 of the first 15 patients, respectively. One patient experienced grade 3 vomiting at the 80 mg/m²/week dose. Grade 3 nausea and vomiting occurred in two of six patients treated at the 320 mg/m²/week dose, following which the dose was escalated by 40% to 450 mg/m²/week (Banerji *et al* 2002). A total of

28 patients have been treated to date on this trial. Among the six patients treated at the 320-450 mg/m²/week dose range, two patients showed stable disease for 27 and 91 weeks, respectively.

Pharmacodynamic marker analysis of tumour biopsies done before and 24 h after treatment in nine patients showed depletion of the Hsp90 client c-Raf in four of seven samples (where the marker was expressed), and cdk4 (Hsp90 client) depletion and Hsp70 induction in eight of the nine samples (Banerji *et al* 2003). At the highest dose level, pharmacokinetic analysis indicated a $t_{1/2}$ of 5.8±1.9 h, V_{dss} of 274±108 L, clearance of 35.5±16.6 L/h, and C_{max} of 16.2±6.3 µM (Banerji *et al* 2003), which is above the levels of 375 nM to 10 µM reported to inhibit Hsp90 *in vitro* (Burger *et al* 2000). Although a maximal tolerated dose was not established in this trial, the dose/schedule that will be taken forward to phase II evaluation is likely to be 450 mg/m²/week, as there was evidence of tumor target inhibition at that dose level (Banerji *et al* 2003). Updated results of this phase I trial have recently been published (Banerji *et al* 2005).

Hsp90 inhibitors are a class of agents that affect a diverse group of client proteins involved in oncogenesis. Many of these clients are expressed in a disease-specific fashion. The development of these inhibitors as biomodulators is complex and not necessarily governed by standard approaches. The clinical approach taken with the Hsp90 inhibitors was to proceed simultaneously with single agent phase 2 studies as well as disease-specific combinations that would be used to evaluate the biomodulatory effects of 17-AAG and 17-DMAG. As these studies mature and reach completion, the utility of Hsp90 inhibitors for the treatment of cancer should be better defined with regard to their activity and molecularly targeted effects.

6. Hsp90 inhibitors in neurodegenerative diseases

Unfolded or misfolded proteins have exposed hydrophobic segments that render them prone to aggregation. Protein aggregates are toxic to the cell (Taylor *et al* 2002), and molecular chaperones, especially Hsp70, bind to hydrophobic surfaces of misfolded proteins to insure their continued solubility or to promote their degradation by the proteasome (Hershko and Ciechanover 1998). Under pathologic conditions, the level of misfolded proteins may exceed the ability of the cell to either maintain them in a soluble form or to degrade them, allowing aggregation to proceed (Cohen 1999; Zoghbi and Orr 2000). Protein aggregates have been found in most chronic neurodegenerative diseases (Kakizuka 1998; Taylor *et al* 2002), as well as in global and focal ischemia and in hypoglycemic coma (Hu *et al* 2000, Ouyang and Hu 2001). Thus, pharmacologic induction of molecular chaperones in general, and Hsp70 in particular, may be

ameliorative in these cases. The Hsp90 inhibitors currently in clinical trial, 17-AAG and 17-DMAG, have the property of inducing Hsp70 in normal cells and tissues, via disruption of Hsp90 sequestration of the heat shock transcription factor Hsf1 (Ali *et al* 1998, Kim *et al* 1999, Lu *et al* 2002), and therefore, they have become of interest in this regard.

Giffard and colleagues have reported that GA, an Hsp90 inhibitor structurally related to 17-AAG and 17-DMAG, via its ability to induce Hsp70, reduces protein aggregation in a rodent model of global ischemia and blocks apoptotic astrocyte death induced by glucose deprivation (Giffard *et al* 2004). These investigators showed that GA-treated astrocyte cultures were twice as viable as untreated cultures after 24 h of glucose deprivation, and they make the point that, because Hsp70 can block both apoptotic and necrotic cell death, it is an intriguing target for anti-ischemic therapy.

The progressive loss of dopaminergic neurons in the substantia nigra is the defining pathogenic feature of Parkinson disease (PD). α -Synuclein is mutated in rare familial forms of PD and it is a major component of the pathologic protein aggregates characteristic of the disease (Polymeropoulos *et al* 1997; Kruger 2004; Tofaris and Spillantini 2005). Expression of normal as well as mutant α -synuclein in *Drosophila melanogaster* causes selective loss of dopaminergic neurons (Feany and Bender 2000), and this can be completely prevented by raising the level of Hsp70 by transgenic expression (Auluck *et al* 2002a). Thus, dopaminergic neurons may be sensitive to compromised chaperone levels. A recent study demonstrated that pharmacologic enhancement of Hsp70, via GA administration to adult *Drosophila* during a 3-week period, completely protected their dopaminergic neurons from α -synuclein-induced toxicity (Auluck and Bonini 2002b). Moreover, in contrast to the findings of a previous study, which treated developing flies in a similar fashion, prolonged exposure of adult flies to effective doses of GA caused no noticeable deleterious effects (Rutherford and Lindquist 1998). Given the complete protection of dopaminergic neurons afforded by GA, the authors of this study propose that GA and its derivatives warrant a careful examination as cytoprotective agents for treating PD and other neurodegenerative diseases.

The 17-AAG has recently been shown to ameliorate polyglutamine-mediated motor neuron degeneration (Waza *et al* 2005). Because mutated androgen receptor (AR) is a pathogenic gene product in spinal and bulbar muscular atrophy (SBMA), Waza and colleagues examined whether 17-AAG could potentiate degradation of the polyglutamine-expanded mutant AR. These investigators found that administration of 17-AAG markedly ameliorated motor impairments in the SBMA transgenic mouse model without detectable toxicity and reduced the amount of detectable monomeric and aggregated mutated AR protein. As

expected, polyglutamine-expanded AR showed a higher affinity for Hsp90 as compared to wild-type AR, and it was preferentially degraded in the presence of 17-AAG in both cells and transgenic mice. 17-AAG also mildly induced Hsp70 in this model. These investigators suggest that 17-AAG thus provides a novel therapeutic approach to SBMA by promoting the degradation of a pathologic mutant Hsp90 client protein.

Finally, two groups have reported that, using both mouse and *Drosophila* models of Huntington's disease (HD), pharmacologic induction of Hsp70 with Hsp90 inhibitors provides a useful therapeutic strategy. Hay *et al* (2004) report that a progressive decrease in Hsp70 and other chaperones in brain tissue contributes to disease pathogenesis in the R6/2 mouse model of HD. Both radicicol and GA were able to maintain chaperone induction for at least three weeks and were able to improve the detergent-soluble properties of polyglutamine-containing aggregates over this time course. Meanwhile, Agrawal and colleagues have shown, using a *Drosophila* model of HD (flies transgenically express glutamine-expanded human huntingtin protein), that feeding affected flies GA alone or in combination with a histone deacetylase inhibitor (suberoylanilide hydroxamic acid, SAHA) strongly suppresses the degeneration of photoreceptor neurons while causing no overt toxicity in control flies (Agrawal *et al* 2005). Intriguingly, we and others have recently shown that several classes of histone deacetylase inhibitors, including SAHA, have the unexpected property of inhibiting Hsp90 by promoting its hyperacetylation (Yu *et al* 2002; Bali *et al* 2005). Thus, in the *Drosophila* HD model the observed beneficial activity of each agent alone as well as the synergistic activity of their combination suggests that Hsp90 inhibition (and/or the resultant increase in Hsp70) is the primary mechanism of action of both drugs.

The apparent *in vivo* safety and efficacy of these benzoquinone ansamycin Hsp90 inhibitors in several models of neurodegeneration considerably extend the therapeutic application of these drugs (and perhaps other Hsp90 inhibitors) beyond oncology. Whether the primary mechanism is degradation of an Hsp90-dependent polyglutamine-expanded mutant protein, the pharmacologic induction of Hsp70, or a combination of the two processes, Hsp90 inhibitors have a promising future in the treatment of neurodegenerative pathologies.

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