Targeting of multiple signalling pathways by heat shock protein 90 molecular chaperone inhibitors

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Abstract

The last decade has seen the molecular chaperone heat shock protein 90 (HSP90) emerge as an exciting target for cancer therapy. This is because HSP90 is involved in maintaining the conformation, stability, activity and cellular localisation of several key oncogenic client proteins. These include, amongst others, ERBB2, C-RAF, CDK4, AKT/PKB, steroid hormone receptors, mutant p53, HIF-1α, survivin and telomerase hTERT. Therefore, modulation of this single drug target offers the prospect of simultaneously inhibiting all the multiple signalling pathways and biological processes that have been implicated in the development of the malignant phenotype. The chaperone function of HSP90 requires the formation of a multichaperone complex, which is dependent on the hydrolysis of ATP and ADP/ATP exchange. Most current inhibitors of HSP90 act as nucleotide mimetics, which block the intrinsic ATPase activity of this molecular chaperone. The first-in-class inhibitor to enter and complete phase I clinical trials was the geldanamycin analogue, 17-allylamino-17-demethoxygeldanamycin. The results of these trials have demonstrated that HSP90 is a valid drug target. Evidence of clinical activity has been seen in patients with melanoma, breast and prostate cancer. This article provides a personal perspective of the present efforts to increase our understanding of the molecular and cellular consequences of HSP90 inhibition, with examples from work in our own laboratory. We also review the discovery and development of novel small-molecule inhibitors and discuss alternative approaches to inhibit HSP90 activity, both of which offer exciting prospects for the future.

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HSP90 as a cancer drug target

As our understanding of the genetic and molecular biology of cancer has increased, there has been a shift over the last decade in the approaches used in the discovery of novel cancer therapeutics (Workman 2005). In contrast to the earlier development of cytotoxic agents, focus has moved to the development of treatments that target the pathways responsible for malignancy. Validation of this approach has been provided by the clinical activity and approval of small-molecule kinase inhibitors such as imatinib (Gleevec), gefitinib (Iressa) and erlotinib (Tarceva) as well as the therapeutic antibodies trastuzumab (Herceptin), cetuximab (Erbitux) and bevacizumab (Avastin). However, despite the success that these agents have enjoyed, it is likely that modulation of a single molecular target will be insufficient for optimal therapy (Workman 2003). Even where malignancies are driven by single genes or pathways, the development of resistance is a major concern. For example, resistance to imatinib has been shown to arise by acquisition of mutations within the kinase domain of BCR–ABL (Gorre et al. 2001, Shah et al. 2002). Furthermore, the majority of cancers involve multiple molecular abnormalities that are likely to be involved in malignant progression. These observations have reinforced the suggestion that inhibition of multiple targets will be required to cure
most human cancers (Workman 2003). It is this concern which provides the foundation for the increasing amount of interest in targeting the heat shock protein 90 (HSP90) molecular chaperone (Workman 2004).

HSP90 exerts its chaperone function to ensure the correct conformation, activity, intracellular localisation and proteolytic turnover of a range of proteins that are involved in cell growth, differentiation and survival (Maloney & Workman 2002, Whitesell & Lindquist 2005). Of particular importance is that HSP90 is essential for the stability and the function of many oncogenic client proteins, which contribute to the hallmark traits of cancer (Fig. 1). These include ERBB2, BCR–ABL, AKT/PKB, C-RAF, CDK4, PLK-1, MET, mutant p53, HIF-1α, steroid hormone receptors (oestrogen and androgen), survivin and telomerase hTERT (Maloney & Workman 2002). Inhibition of HSP90 function has been shown to cause degradation of client proteins via the ubiquitin-proteasome pathway (Connell et al. 2001, Demand et al. 2001), which results in the simultaneous depletion of multiple oncoproteins, the combinatorial down-regulation of signals propagated through numerous oncogenic signalling pathways and modulation of all aspects of the malignant phenotype (Maloney & Workman 2002, Workman 2004). The ability to deliver a combinatorial effect through a single drug target may have promise in treating cancers driven by multiple molecular abnormalities and could also reduce the opportunity for resistance developing (Workman 2004, 2005).

In this paper, we will focus on our interest in developing inhibitors of the HSP90 molecular chaperone family and the progress we have made in understanding the effects of this modulation in both the preclinical and clinical settings. Examples will be taken mainly from the work of our own laboratory.

Presently, five isoforms of HSP90 have been identified, which differ in their cellular localisation. The two major cytoplasmic isoforms are HSP90α and HSP90β which share approximately 85% sequence identity at the protein level (Hickey et al. 1989, Gupta 1995). Other major isoforms are Grp94 in the endoplasmic reticulum (Argon & Simen 1999), TRAP1 in the mitochondrial matrix (Felts et al. 2000) and HSP90N, which has been suggested to be associated with cellular transformation via its association with RAF (Grammatikakis et al. 2002). It is currently believed that all the mammalian HSP90 isoforms described above share a similar overall structure, which comprises a C-terminal dimerisation domain, a middle domain that is implicated in client protein binding and also an N-terminal ATPase domain (reviewed in Pearl & Prodromou 2001, Prodromou & Pearl 2003), which is absent in HSP90N (Schweinfest et al. 1998).

The chaperone activity of HSP90 is dependent on its transient N-terminal dimerisation, which stimulates the intrinsic and essential ATPase activity (Prodromou et al. 2000). This process is controlled by an orchestrated set of interactions with a range of accessory proteins referred to as co-chaperones (Pratt et al. 2004, Riggs et al. 2004; Fig. 2). Initially, client proteins interact with an HSP70/HSP40/HIP complex. The HSP70 and HSP90 chaperone systems are then linked by the adaptor protein HOP/p60, which interacts with the C-terminals of both HSP90 and HSP70 via its tetracopeptide repeat domain (Scheufler et al. 2000). HOP/p60 can only bind to ADP-bound-HSP90, which has an open conformation and a high affinity for hydrophobic substrates (Pratt et al. 2004).

The present model for chaperone activity suggests that when HSP90 exchanges ADP for ATP, it undergoes a conformational change, which includes the transient dimerisation of the N-terminal domains (Prodromou et al. 2000). This leads to the dissociation of HSP70/HSP40/hip and HOP, allowing the ATP-dependent association of other co-chaperones (e.g. CDC37, p23 or immunophilins) to form the mature complex (reviewed in Whitesell & Lindquist 2005, Sharp & Workman 2006). CDC37 is involved specifically in the loading of kinase clients onto HSP90 (Roe et al. 2004) and p23 has recently been shown to stabilise HSP90 in the ATP-bound form, which extends the time in which HSP90 is in the

Figure 1 Schematic illustrating how inhibition of HSP90 may interfere with all of the six hallmark traits of cancer. Examples of client proteins involved in the various phenotypic aspects of malignancy are shown.
conformation required for client protein activation (Ali et al. 2006). It is while the HSP90 chaperone cycle is in the mature state that the associated client protein becomes activated to either bind ligand (steroid hormone receptor) or be phosphorylated during signal transduction (AKT/PKB).

Inhibition of ATP binding to HSP90 prevents the formation of the mature complex and results in the proteasome-dependent degradation of associated client proteins. This can occur by the recruitment of the E3-ubiquitin ligase, CHIP, which is a TPR protein that is able to interact with both HSP70 and HSP90 (Connell et al. 2001, Demand et al. 2001). Both of these molecular chaperones may be present in the immature complex, which has been stabilised by the presence of an inhibitor (Fig. 2).

At first sight, HSP90 would not appear to be an obvious drug candidate for the design of novel cancer therapeutics. This is because it is not, to our knowledge, subject to mutation or amplification in cancer. It is, however, well-documented as being over-expressed in a range of human malignancies (Maloney & Workman 2002, Sreedhar et al. 2004). This may be a consequence of the hostile conditions created in tumour cells by the effects of deregulated oncogenes and tumour suppressor genes (many of which are HSP90 client proteins), along with the stressful microenvironmental features of solid tumours, which include nutrient deprivation, hypoxia and acidosis (Whitesell et al. 2003, Mosser & Morimoto 2004). Collectively, these factors may lead tumour cells to become highly stressed and much more reliant on HSP90 than cells from normal, non-malignant tissue (Whitesell & Lindquist 2005). This will increase the opportunity for therapeutic selectivity when HSP90 inhibitors are used clinically. This ‘stress hypothesis’ has been explored by Kamal et al. (2003) who demonstrated that HSP90 extracted from tumour cells exists in a high-affinity, activated super-chaperone complex which is approximately 100-fold more sensitive to HSP90 inhibitors when compared with the uncomplexed HSP90 isolated from normal cells (Kamal et al. 2003). In addition to the stressed nature of cancer cells, HSP90 inhibitors could exert therapeutic selectivity by exploiting multiple oncogene addiction and via the preferential dependence of certain oncoproteins on chaperoning by HSP90 (see later).

It is known that the natural product geldanamycin exerts its antitumour effect by binding to the N-terminal ATPase domain of HSP90 to inhibit its chaperone function (Roe et al. 1999). The progress of geldanamycin into the clinic was stopped due to instability and the unacceptable hepatotoxicity seen at therapeutic doses during preclinical in vivo studies (Supko et al. 1995). Further analogues were developed for clinical use, which included 17-AAG (Schnur et al. 1995a,b).

Preclinical studies with 17-AAG

We have investigated the detailed molecular consequences of exposing cancer cells to 17-AAG in vitro in an attempt to identify genes and proteins that influence the sensitivity to HSP90 inhibitors. In addition, these studies have enabled us to identify and validate biomarkers of HSP90 inhibition, which could be of clinical use (Banerji et al. 2003, Maloney et al. 2003).

It has been well documented that inhibition of HSP90 function induces the expression of HSP72 and degradation of client proteins (reviewed in Workman 2005, Clarke et al. 2006). We have demonstrated the principle of combinatorial inhibition of multiple signal transduction pathways via targeting HSP90 using a panel of human colon cancer cell lines (Hostein et al. 2006).
In that study, we showed inhibition of both the RAS–RAF–MEK–ERK1/2 and the PI3K–AKT/PKB pathways following treatment with 17-AAG (Hostein et al. 2001). We showed that this was accompanied by cytostasis, cell-cycle arrest and cell-line-dependent apoptosis (Hostein et al. 2001). The extent of the apoptotic effect was hypothesised to be influenced by the expression of the proapoptotic BCL2 family member, BAX. This was based on the observation that the KM12 colon cancer cell line, which over-expressed BAG-1 and lacked expression of BAX, was unable to undergo apoptosis in response to 17-AAG (Hostein et al. 2001). We and other researchers have subsequently used an isogenic pair of HCT116 cells in which the BAX gene has been removed by homologous recombination (Zhang et al. 2000) to demonstrate that this proapoptotic protein is required for apoptosis to occur in response to 17-AAG treatment (Nimmanapalli et al. 2003). Interestingly, we have demonstrated that in the absence of BAX cell death still occurs but via a necrotic rather than apoptotic mechanism (MV Powers, PA Clarke & P Workman, unpublished observations).

The observed reduction of the RAS–RAF–MEK–ERK1/2 and PI3K–AKT/PKB pathways in the earlier-mentioned study occurs because of the pleiotropic role of HSP90 in modulating the activity of several components of these pathways (see Fig. 3). In addition, both of these oncogenic pathways are activated by the receptor tyrosine kinase ERBB2 (Yarden & Sliwkowski 2001, Holbro et al. 2003), which has been shown to be one of the most sensitive and potentially important HSP90 client proteins (Citri et al. 2004). We have examined the effects of ERBB2 overexpression on the cellular sensitivity to HSP90 inhibitors using an isogenic model that we created, in which ERBB2 was overexpressed and functionally active in ERBB2-negative CH1 cells (Smith et al. 2002). Using this approach, we showed that ERBB2 overexpression resulted in a fivefold increase in sensitivity to geldanamycin (Smith et al. 2002).

In addition, we have recently demonstrated that in human ovarian cancer cells, which can become resistant to conventional cytotoxics (e.g. paclitaxel) due to increased expression of ERBB2 and/or the

**Figure 3** Schematic of the PI3K–AKT/PKB and RAS–RAF–MEK–ERK1/2 pathways demonstrating the components which are reliant on HSP90 chaperone function. 90, HSP90; P, phosphate.
PI3K–AKT/PKB pathway, sensitisation to the effects of paclitaxel can be achieved when the taxane is used in combination with non-growth inhibitory concentrations of 17-AAG (Sain et al. 2006). These observations highlight the potential use of 17-AAG both as a single agent and as a potential sensitisier to current chemotherapeutic agents.

Consistent with the proposed combinatorial action of HSP90 inhibitors on the hallmarks of cancer, we and other researchers have recently demonstrated the profound effects of 17-AAG and geldanamycin on key aspects of tumour angiogenesis and potentially also of lymphangiogenesis (de Candia et al. 2003, Kaur et al. 2004, Sanderson et al. 2006). Effects included decreased production of VEGF and significantly reduced expression of several members of the VEGF receptor family including VEGFR-1 in human vascular endothelial cells and VEGFR-3 in lymphatic endothelial cells in vitro (Sanderson et al. 2006). These molecular changes were accompanied by reduced endothelial cell migration, tubular differentiation, invasion through Matrigel and secretion of urokinase-type plasminogen activator in human endothelial cells in response to treatment with geldanamycin and its derivatives (Sanderson et al. 2006). Collectively, our results suggest that effects on angiogenesis may play a considerable role in the response to HSP90 inhibitors in vivo.

To further our understanding of the molecular changes associated with HSP90 inhibition, we conducted the first cDNA expression profiling analysis of a human colon cancer cell line panel following 17-AAG treatment (Clarke et al. 2000). We showed that the expression of genes encoding client proteins was not altered at the mRNA level, whereas the expression of genes encoding HSP72, HSC70, keratins 8 and 18 and caveolin 1 was decreased by 17-AAG (Clarke et al. 2000).

The induction of HSP70 family members observed in this study and elsewhere (Hostein et al. 2001, Banerji et al. 2005b, Smith et al. 2005) was of particular interest since this group of molecular chaperones have a well-documented antiapoptotic function (Mosser & Morimoto 2004, Clarke et al. 2006). This generated the hypothesis that induction of these pro-survival genes/proteins may be reducing the apoptotic effect of 17-AAG. Using a short interfering RNA (siRNA) approach, we and other researchers have demonstrated in a range of human cancer cell lines that reducing the expression of HSP72 prior to 17-AAG significantly increases the cell death response to pharmacologically relevant concentrations of 17-AAG (MV Powers, PA Clarke & P Workman, unpublished observations, Gabai et al. 2005, Guo et al. 2005a). In our laboratory, we have also studied the effects of reducing the expression of HSC70 alongside HSP72 in the human HCT116 and ovarian A2780 cell lines prior to treatment with 17-AAG (MV Powers, PA Clarke & P Workman, unpublished observations). Interestingly, under these conditions, the apoptotic response to 17-AAG is dramatically enhanced to an extent far greater than is associated with this predominantly cytostatic agent (MV Powers, PA Clarke & P Workman, unpublished observations).

Along with induction of HSP70 family members, our gene expression profiling studies have also shown a cell-line-dependent modulation of HSP90β, but not HSP90α, expression levels (Clarke et al. 2000). The direction of this modulation correlated with the cellular sensitivity to 17-AAG. For example, the more sensitive HT29 cells exhibited depletion of HSP90β, whereas the more resistant HCT116 cells exhibited induction of the drug target (Clarke et al. 2000). HSP90 is itself antiapoptotic and we have, therefore, hypothesised that the alteration of target expression by the drug may be a factor influencing the cellular sensitivity to 17-AAG.

We have gained further understanding of the molecular responses to 17-AAG treatment by using proteomic analysis alongside cDNA microarray profiling in the human ovarian cancer cell line A2780 (A Maloney, PA Clarke, S Naaby-Hansen, R Stein, JO Koopman, A Akpan, A Yang, M Zvelebil, R Cramer, L Stimson, W Ahene, U Banerji, I Judson, S Sharp, J Salmons, M Walton, A Burlingame, M Waterfield & P Workman, unpublished observations). The expected changes described above were shown to be reproducible in this cell line, but at an even greater level of detail than was demonstrated previously. However, a novel and important observation was the altered expression of a group of proteins involved in chromatin regulation, acetylation and methylation in response to 17-AAG (A Maloney et al. unpublished observations). These observations, along with the decrease in total cell acetylation observed during these studies in response to 17-AAG suggest that there is an interaction between protein acetylation and HSP90 function. This is reinforced by other studies, which have suggested that HSP90 function may be inhibited by histone deacetylase inhibitors via a direct increase in acetylation of the chaperone (Yu et al. 2002). In addition, it is well established that there is interplay between HSP90, chromatin regulation and gene transcription in morphological evolution (Whitesell & Lindquist 2005).

Collectively, these examples highlight the power of using an approach that analyses global changes in gene expression following drug treatment to identify factors which may influence the cellular sensitivity/response to treatment and also to discover changes in gene/protein
expression that could be used clinically as biomarkers to demonstrate inhibition of the target (see below).

**Clinical evaluation of 17-AAG**

Based on the promising preclinical activity of 17-AAG, we carried out one of the initial phase I clinical trials, which provided the first convincing proof-of-concept for HSP90 inhibition in human patients (Banerji et al. 2005a). Molecular biomarkers that we and other researchers selected for use during the clinical trial included the induction of HSP72 and depletion of HSP90 client proteins C-RAF and CDK4 in the tumour tissue and peripheral blood lymphocytes of treated patients (Banerji et al. 2005a, Goetz et al. 2005, Grem et al. 2005). These biomarkers were validated in studies of pharmacodynamic–pharmacokinetic relationships carried out in a human ovarian xenograft model (Banerji et al. 2005b).

We were able to confirm, using pharmacokinetic analysis, that 17-AAG was present at therapeutic plasma concentrations after i.v. infusion (Banerji et al. 2005a). The analysis of molecular biomarkers in tumour biopsies obtained 24 h after exposure to 17-AAG at doses of 320 and 450 mg/m² per week demonstrated that HSP72 was induced in eight out of nine patients, CDK4 was depleted in eight out of nine patients and C-RAF expression was reduced in four of the six informative patient samples (Banerji et al. 2005a). Encouragingly, we observed prolonged stable disease in two patients with advanced, metastatic malignant melanoma, which led to a phase II clinical trial being initiated at our institution and the Royal Marsden Hospital in collaboration with the Royal Free Hospital, London. In addition to melanoma, evidence of clinical activity has also been reported by others in breast and prostate cancer (Pacey et al. 2006).

To understand the sensitivity of melanoma cells to 17-AAG, mechanistic studies were carried out, in particular to investigate the hypothesis that melanoma responsiveness may be related to the high incidence of B-RAF mutation in this disease (Davies et al. 2002). In collaboration with Professor Richard Marais, we have shown that a number of B-RAF mutants, including the most common V600E form, are HSP90 client proteins which are highly dependent on HSP90 function for stability and activity as compared with wild-type B-RAF (da Rocha Dias et al. 2005). As a result, B-RAF mutants are very sensitive to 17-AAG-mediated proteasomal degradation (da Rocha Dias et al. 2005), which may suggest a possible mechanism for the clinical activity of 17-AAG in melanoma. However, it should be noted that we saw no relationship between B-RAF status and sensitivity in melanoma cell lines. This may be because C-RAF is also a sensitive client protein. Thus, the RAS–RAF–MEK–ERK1/2 pathway will be inhibited by 17-AAG regardless of the mechanism of its activation in melanoma cells (da Rocha Dias et al. 2005). Similar results have been obtained in an independent study (Grbovic et al. 2006).

Overall, our phase I clinical trial demonstrated that it was possible to deliver a dose of 17-AAG, using a once weekly schedule, which achieved potentially therapeutic plasma concentrations, HSP90 target inhibition in tumour tissue and possible antitumour activity (Banerji et al. 2005a). However, the cumbersome formulation of 17-AAG prevented the true evaluation of its maximum tolerated dose. Nevertheless, phase II studies are underway and combination studies are in progress; some of these involve alternative formulations (Pacey et al. 2006).

**Limitations of 17-AAG and future directions for HSP90 inhibitors**

17-AAG continues to show promise as the first-in-class HSP90 inhibitor. However, in addition to its poor solubility and cumbersome formulation, this compound does have additional limitations, which have provided a strong case for the development of improved second generation inhibitors. These include relatively weak target potency, reduced activity in the presence of P-glycoprotein (Kelland et al. 1999) and low bioavailability and metabolism by polymorphic cytochrome P450 CYP3A4 (Egorin et al. 1998). In addition, we have shown that the antiproliferative activity of 17-AAG can be increased by overexpression of the polymorphic oxidoreductase enzyme NQO1/DT-diaphorase (Kelland et al. 1999). We proposed that this was due to metabolism to a more active HSP90 inhibitor. Recent studies have confirmed that this increase in potency is indeed due to the metabolism of 17-AAG to the more active hydroquinone form (Guo et al. 2005b). By studying different analogues of 17-AAG, we were able to show that the NQO1 potentiation effect was restricted to alkyl-substituted 17-amino analogues of geldanamycin (Kelland et al. 1999). In the case of 17-DMAG in which the 17-allylamino moiety is replaced by 17-diethylaminoethylamino, the effect of NQO1/DT-diaphorase was determined to be dramatically reduced using an NQO1± isogenic pair that we developed (Sharp et al. 2000). Analogues that lack the NQO1 effect may have the advantage over 17-AAG of reducing the risk of resistance developing. However, there is a
concern that the lack of NQO1 effect on 17-DMAG may also reduce the potential therapeutic advantage in high NQO1-expressing cancers. In addition to metabolism by NQO1, as mentioned earlier, 17-AAG also undergoes extensive metabolism in vivo by cytochrome P450 CYP3A4 to the 17-amino compound, which becomes insensitive to the effects of NQO1/DT-diaphorase (Kelland et al. 1999), suggesting that the conversion of 17-AAG to the dihydroquinone form may not be a critical factor for activity in vivo. On the other hand, metabolism by polymorphic CYP3A4 and NQO1 is likely to be a cause of variability in pharmacokinetics.

The analogue 17-DMAG is more water soluble than 17-AAG, but exhibits equal or greater activity (Hollingshead et al. 2005). Therefore, it may be possible to circumvent the solubility and formulation issues seen with 17-AAG. Clinical trials by ourselves and other researchers have been initiated using this novel derivative (Hollingshead et al. 2005). In addition, the hydroquinone form of 17-AAG, IPI-504 is very water soluble and has now entered clinical evaluation (Infinity Pharmaceuticals; http://www.ipi.com).

The wide array of HSP90 inhibitors currently in the preclinical pipeline has been reviewed extensively elsewhere (Chiosis et al. 2006, Pacey et al. 2006, Sharp & Workman 2006). The first generation of synthetic small-molecule HSP90 inhibitors were purine analogues (Chiosis et al. 2002) that were designed to mimic the unusual ‘C-shape’ adopted by ADP/ATP when bound to the N-terminal domain of HSP90 (Prodromou et al. 1997, Stebbins et al. 1997). In collaborative studies with Vernalis, the X-ray co-crystal structure of human HSP90α and HSP90β complexed with a lead purine inhibitor PU3 was determined (Wright et al. 2004). Unexpectedly, we showed that although the purine does indeed mimic ATP/ADP, it also induces a conformational change in the loop region, which caps the ATP-binding site, to create a new binding domain (Wright et al. 2004). From this, more potent analogues were designed, which demonstrated at least 50 times more activity than PU3 (Wright et al. 2004).

Our progress in developing novel HSP90 inhibitors was furthered by a high-throughput screen of our (then) 53,000 compound library using a malachite green assay to measure the ATPase activity of the full-length recombinant yeast HSP90 (Aherne et al. 2003, Rowlands et al. 2004). Using this screen, we identified the diaryl pyrazole resorcinol series of HSP90 inhibitors exemplified by the initial micromolar hit CCT018159 (Cheung et al. 2005). Yeast HSP90 was used in the initial screen due to its high ATPase activity; however, the compounds were confirmed as active against human HSP90 activated by AHA1 (see later). In collaboration with Professor Laurence Pearl's laboratory, X-ray co-crystal structures were obtained for CCT018159 and related analogues and structure–activity relationship studies quickly identified optimisation opportunities (Cheung et al. 2005). As part of a productive collaboration with Vernalis, structure-guided introduction of the 5-amide substitution increased hydrogen bond interaction with Gly-97 of human HSP90 and led to the generation of the more potent analogues. This is exemplified by VER49009 (CCT129397), which compares favourably with 17-AAG, has nanomolar activity and has the required potency and potential to become a clinical candidate (Dymock et al. 2005).

We have shown that the 3,4 diaryl pyrazole CCT018159 described above was able to inhibit human HSP90β with a similar potency to 17-AAG and with a very high degree of selectivity towards HSP90 compared with topoisomerase II, HSP72 and a representative panel of kinases (SY Sharp, K Boxall, M Rowlands, C Prodromou, SM Roe, A Maloney, M Powers, PA Clarke, G Box, S Sanderson, L Patterson, TP Matthews, KMJ Cheung, K Ball, A Hayes, F Raynaud, R Morais, L Pearl, S Eccles, W Aherne, E McDonald & P Workman, unpublished observations). Unlike 17-AAG, we have also demonstrated that the cellular sensitivity to CCT018159 was not affected by NQO1/DT-diaphorase or P-glycoprotein (SY Sharp et al. unpublished observations). We have demonstrated that CCT018159 inhibits a range of different cancer cell lines in vitro at micromolar concentrations, which caused degradation of client proteins and induction of HSP72. This molecular biomarker signature of HSP90 inhibition was accompanied by cell cytostasis, G1 cell-cycle arrest and apoptosis (SY Sharp et al. unpublished observations). In agreement with our earlier studies using geldanamycin and its derivatives, CCT018159 was observed to reduce tumour cell invasion and exhibit antiangiogenic activity (Sharp et al. unpublished observations). It was concluded from these studies that the diaryl pyrazole resorcinol series of novel HSP90 inhibitors have similar cellular properties to 17-AAG, but have several possible advantages (e.g. aqueous solubility, independence from NQO1 and P-glycoprotein), which may provide the basis for the future development of clinically superior HSP90 modulators.

These studies demonstrate that high-throughput screening combined with X-ray crystallography and structure-based design can provide a powerful
approach for the discovery of HSP90 ATPase inhibitors.

**Novel approaches to inhibit the HSP90 molecular chaperone**

The results from our laboratory and others discussed so far have focused on the effect of inhibiting the ATPase activity of HSP90 activity directly. However, as described earlier, HSP90 functions in a multi-protein complex comprised of client proteins and co-chaperones. One of these co-chaperones, AHA1 (activator of HSP90 ATPase) (Panaretou et al. 2002) was identified as being a stress-regulated protein, which activates the essential, intrinsic ATPase of HSP90 (Panaretou et al. 2002). During the above-mentioned cDNA microarray and proteomic studies, we were able to identify that AHA1 was induced at both the mRNA and protein levels by 17-AAG in human cancer cells (Panaretou et al. 2002). We have since used an siRNA approach to specifically reduce the expression of AHA1 prior to treatment with 17-AAG. This has been shown to increase the sensitivity of human cancer cell lines to 17-AAG in vitro (JL Holmes, SY Sharp, S Hobbs & P Workman, unpublished observations). In addition, we have demonstrated that reducing the expression of AHA1 can directly affect the activity of HSP90, as demonstrated by reduced signalling via the RAS–RAF–MEK–ERK1/2 and PI3K–AKT/PKB pathways (JL Holmes, SY Sharp, S Hobbs & P Workman, unpublished observations). These data have led us to propose that AHA1 may be an interesting drug target with potential inhibitors being used either alone or alongside existing HSP90 modulators to improve their therapeutic efficacy.

As discussed earlier, induction of HSP70 isoforms has been shown by our laboratory and others to occur in response to 17-AAG both in vitro and in vivo (Hostein et al. 2001, Banerji et al. 2005b, Smith et al. 2005). HSP70 has been well documented as a co-chaperone involved in substrate-loading onto HSP90 (Whitesell & Lindquist 2005, Sharp & Workman 2006). We have pursued this using siRNA to simultaneously reduce the expression of the two major isoforms of the HSP70 family, HSP72 and HSC70. We have shown that this reduces the activity of HSP90, as determined by decreased expression of C-RAF, CDK4 and ERBB2 in human colon and ovarian cell lines (MV Powers, PA Clarke & P Workman, unpublished observations). This was accompanied by inhibition of cell growth and induction of cell death, the extent of which was far greater than is observed with 17-AAG.

AHA1 and HSP70 isoforms are just two examples of the array of co-chaperones that have been shown to interact with and modulate the activity of HSP90. We are currently investigating whether targeting other co-chaperones including p50/CDC37, p23 and CHIP could have benefits as therapeutic targets in their own right or could be modulated alongside the use of existing HSP90 inhibitors.

**Concluding remarks**

HSP90 is an exciting new therapeutic target, inhibition of which delivers a combinatorial attack on multiple oncogenic targets and pathways and on all of the hallmark traits of malignancy. The development of HSP90 inhibitors has moved forward rapidly alongside our growing understanding of the role of the chaperone in normal and malignant cells. The first and second generations of HSP90 inhibitors act by blocking its intrinsic ATPase activity. Following on from the natural product-based agents, exemplified by 17-AAG and related analogues that have entered clinical trials, a variety of HSP90-inhibitory chemotypes are now under development. It is also possible that new classes of inhibitor could be developed which act upon the co-chaperones of HSP90.

Clinical activity has been seen with 17-AAG in melanoma, breast and prostate cancer. Although a strength of HSP90 inhibitors is their combinatorial action in depleting multiple client proteins, this can, at the same time, obscure the precise mechanism of action that may predominate in a particular cancer. Clearly, there is potential for activity in endocrine-related cancers through effects on steroid hormone receptors, receptor tyrosine kinases and downstream signalling proteins, including members of both the RAS–RAF–MEK–ERK1/2 and PI3K–AKT–mTOR pathways. Action on these and other client proteins, such as mutant B-RAF, provides the potential for activity in a wide range of cancers.

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