Chapter 7
HSP90 Inhibition as an Anticancer Strategy: Novel Approaches and Future Directions

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Contents

Abstract Heat shock protein 90 is an ATP-dependent molecular chaperone involved in the maturation and stabilisation of a wide-range of proteins in both the presence and absence of cellular stress. Within the ever expanding list of HSP90 client proteins is a broad range of *bona fide* oncoproteins. This has thrust HSP90 into the spotlight as an exciting anticancer drug target. Several natural product and semi-synthetic derivatives have been described which inhibit the activity of HSP90 by preventing the association of the N-terminal domain with ATP. This approach is exemplified by 17-AAG which is the first-in-class HSP90 inhibitor to complete phase I clinical trial and provide proof-of-concept for this approach with the observation of responses in patients with malignant melanoma, multiple myeloma, prostate and breast carcinoma. Research is now focused on the design of more potent and drug-like synthetic small-molecule inhibitors. This article provides a personal

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perspective of the advances made in the development of novel HSP90 inhibitors with particular emphasis on work from our own laboratory. We will also review alternative approaches to inhibit HSP90 which are currently being evaluated. These include selectively inhibiting particular HSP90 isoforms, blocking co-chaperone interactions, designing substrate mimetics and modulating the post-translational modifications of HSP90.

Keywords 17-AAG · Heat shock protein inhibitors · HSP90 · siRNA

7.1 The Therapeutic Potential of HSP90 Inhibition

Heat shock protein 90 (HSP90) is a highly abundant molecular chaperone induced in response to stress to prevent the misfolding and aggregation of unfolded proteins (Young et al., 2004). When this is not possible, HSP90 directs irreparably damaged proteins for degradation via the ubiquitin-proteasome pathway (Connell et al., 2001; Demand et al., 2001). Furthermore, in the absence of stress HSP90 is important in managing the conformation, localisation and functional maturation of a wide range of so-called 'client proteins'. These include steroid hormone receptors, receptor tyrosine kinases and other proteins involved in a variety of cellular processes (Wegele et al., 2004; Sreedhar et al., 2004b).

The function of HSP90 is critically linked to the cycle of N-terminal ATP/ADP exchange and ATP hydrolysis which is controlled by a conformational change in its structure (reviewed in Pearl et al., 2008). HSP90 exists as a dimer with each monomer constitutively associated with its partner via the C-terminal domain. In the ADP-bound state the N-terminal ATP binding domains are not closely associated and the chaperone is considered to be in an immature, inactive, 'open' state (Fig. 7.1). However, binding of ATP induces a conformational change in the chaperone to bring the N-terminal domains into close proximity with one another in a 'closed' conformation (Prodromou et al., 2000). This process has been referred to as a 'molecular clamp' mechanism (Prodromou et al., 2000). The rate of ATP hydrolysis by HSP90 is closely linked to the association of a number of co-chaperone proteins including HSP70, HOP, CDC37, p23 and AHA1 which act in concert with HSP90 to fine tune its activity (Fig. 7.1). Description of the function of each of these accessory proteins is beyond the scope of this article and the reader is referred to more specific reviews (Riggs et al., 2004; Pearl et al., 2008).

Because of the wide range of cellular processes with which HSP90 is associated, this molecular chaperone may not initially appear as an obvious target for therapeutic intervention. However, HSP90 has been implicated in oncogenesis and malignant progression due to its overexpression in many cancers and its association with poor prognosis (Jameel et al., 1992; Gress et al., 1994; Sreedhar et al., 2004a; Pick et al., 2007; Gallegos Ruiz et al., 2008). Furthermore, within the ever expanding list of substrates known to associate with HSP90 (see http://www.picard.ch/downloads/Hsp90interactors.pdf) there is a plethora of *bona fide* oncoproteins, including kinases

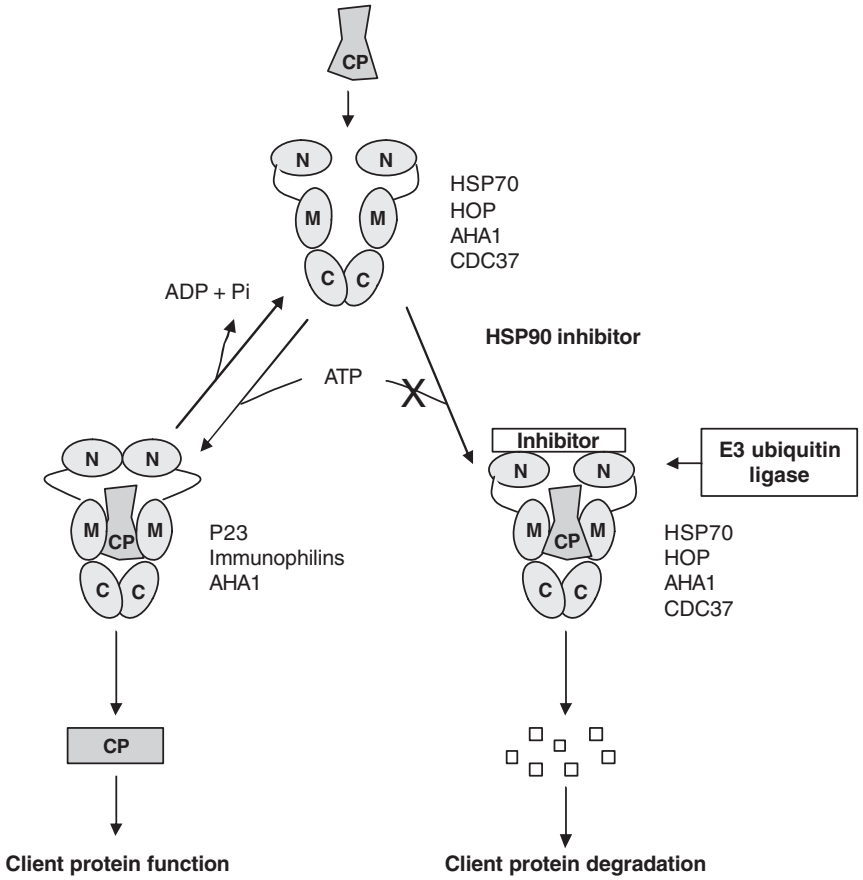

Fig. 7.1 Schematic of the proposed chaperone cycle for HSP90 showing the conformational changes associated with the activity of HSP90. Substrates are initially recruited to HSP90 by HSP70. During this phase HSP90 adopts an open conformation and also associates with the co-chaperones HOP, AHA1 and CDC37 to form the immature complex. Upon ATP binding a conformational change is induced which results in the transient dimerisation of the N-terminal ATP-binding domains and exchange of co-chaperones. ATP hydrolysis is coupled with substrate maturation and release. In the presence of an inhibitor, ATP is unable to bind and client proteins are retained within the immature complex. An E3 ubiquitin ligase is recruited resulting in client protein ubiquitination and degradation via the proteasome

such as BRAF, CRAF, AKT/PKB, ERBB2 and EGFR, together with oestrogen and androgen receptors, mutant p53, HIF1α and telomerase hTERT, all of which are involved in the six hallmarks cancer (Hanahan and Weinberg, 2000). HSP90 inhibition leads to the recruitment of an E3 ubiquitin ligase which ubiquitinates the associated client proteins resulting in their degradation via the ubiquitin-proteasome pathway (Connell et al., 2001; Demand et al., 2001). Therefore, inhibition of HSP90 function offers the opportunity to degrade a large number of oncogenic client proteins

and hence to simultaneously antagonise all of the hallmarks traits of malignancy, including uncontrolled proliferation, avoidance of apoptosis, immortalisation, invasion, angiogenesis and metastasis (Hanahan and Weinberg, 2000). This combinatorial attack on multiple oncogenic pathways should also reduce the opportunity for resistance developing to HSP90 inhibition when compared to more conventional therapies.

The ubiquitous involvement of HSP90 in regulating multiple cellular functions led to initial concerns about potential toxicity. However, there are several reasons why therapeutic selectivity for cancer versus healthy cells may be expected. First, increased expression of HSP90 has been widely reported in a range of human malignancies (reviewed in Sreedhar et al., 2004a). This could be a consequence of the stressful microenvironment of the solid tumour which may possibly increase the cancer cell's dependence on molecular chaperones. Consistent with this is the observation that HSP90 extracted from healthy cells exists in an uncomplexed, inactive state, whereas HSP90 from tumour cells is present in a large multi-chaperone complex which is more sensitive to inhibition (Kamal et al., 2003). Secondly, cancer cells become 'addicted' to survival pathways which dictate malignancy. Therefore, cancer cells are much more sensitive to the depletion of critical oncoproteins that drive these pathways than normal cells. Finally, oncoproteins which are involved in maintaining malignancy are often expressed in mutated, activated forms that have a greater dependence on HSP90 activity than their normal counterparts. An example of this is mutant BRAF which we and others have shown to be reliant on HSP90 function for folding and stability and to be much more sensitive to degradation following 17-AAG treatment than the wild type form (da Rocha Dias et al., 2005; Grbovic et al., 2006).

In this review, we provide an update of our previous studies (Powers and Workman, 2006) and describe our recent work aimed at developing inhibitors of the HSP90 molecular chaperone family and at understanding the consequences of inhibition in both the preclinical and clinical setting. We will discuss some of the latest developments with HSP90 N-terminal ATP site inhibitors. We will also describe novel approaches which are being evaluated to block HSP90 molecular chaperone function. These include selective inhibition of particular HSP90 isoforms, modulation of co-chaperone protein interactions, design of substrate mimetics and alteration of the post-translational modifications of HSP90. Once again examples will be taken mainly from the work in our own laboratory.

7.2 HSP90 ATP Site Inhibitors: Natural Products and Semi-Synthetic Derivatives

The first HSP90 inhibitors to be described were natural products which included radicicol and the benzoquinone ansamycin, geldanamycin (Fig. 7.2). Both bind to the N-terminal nucleotide binding domain of HSP90 and block the ATPase-coupled chaperone cycle leading to client protein degradation by the proteasome (Schulte

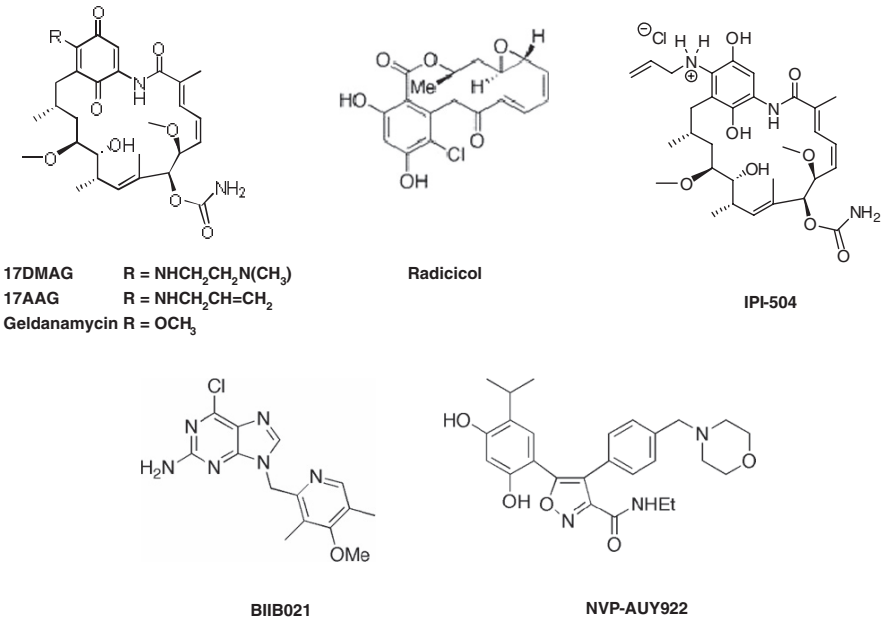

Fig. 7.2 Examples of different classes of HSP90 inhibitors

et al., 1998; Smith et al., 1995; Roe et al., 1999). However, whilst both natural products played a critical role in elucidating the biology of the HSP90 chaperone cycle and the consequences of its inhibition, neither was suitable for clinical development. Radicicol displayed little in vivo activity in animal models due to its chemical reactivity and instability (Soga et al., 1999) whereas development of geldanamycin was restricted by unacceptable levels of toxicity (Supko et al., 1995). However, a semi-synthetic analogue of geldanamycin, 17-AAG (17-allylamino-17-demethoxygeldanamycin; tanespimycin; Fig. 7.2), is better tolerated and exhibits a higher therapeutic index than its parent compound. Phase I clinical studies with 17-AAG carried out by ourselves (Banerji et al., 2005a) and others (e.g. Goetz et al., 2005; Grem et al., 2005) have provided the first convincing proof-of-concept for HSP90 inhibition in human patients. This was demonstrated by depletion of client proteins and induction of HSP70 expression (Banerji et al., 2005a; Goetz et al., 2005; Grem et al., 2005) which collectively represent the validated molecular signature of HSP90 inhibition (Banerji et al., 2005b). Early signs of therapeutic activity were seen in melanoma (Banerji et al., 2005a), breast and prostate cancers (see below and Pacey et al., 2006; Solit and Rosen, 2006; Modi et al., 2007).

We have recently extended our preclinical studies (da Rocha Dias et al., 2005) to investigate possible mechanisms underlying the clinical response to 17-AAG in patients with malignant melanoma observed during our own phase I trial. BRAF and NRAS mutations are extremely common in melanoma with most patients having either BRAF or NRAS mutations but not both (Davies et al., 2002; Edlundh-Rose

et al., 2006; Goel et al., 2006; Reifenberger et al., 2004). As mentioned earlier, mutated BRAF has a much greater dependence on HSP90 function than the wild type counterpart, making it necessary to understand the relationship between BRAF and NRAS mutation status and the response of patients with melanoma to 17-AAG (Banerji et al., 2008a). In collaborative studies with Professor Richard Marais, we have investigated the effect of the NRAS and BRAF mutation status in six melanoma patients, all of whom had been treated with pharmacologically active doses of 17-AAG (Banerji et al., 2008a). One patient with disease stabilisation for 49 months had a ^{G13D}NRAS mutation and ^{WT}BRAF whereas a second patient who had stable disease for 15 months had a ^{V600E}BRAF mutation and ^{WT}NRAS. Patients who had melanomas with ^{WT}BRAF/^{WT}NRAS all progressed within 1 to 1.5 months while receiving 17-AAG (Banerji et al., 2008a). These novel observations, though based on small patient numbers and therefore very preliminary, suggest that BRAF and NRAS mutation status should be considered during future phase II clinical trials of HSP90 inhibitors in melanoma (Banerji et al., 2008a).

In addition to the encouraging results being produced from the clinical trials using 17-AAG as a single agent, evidence of activity with 17-AAG has also been reported in combination with trastuzumab in trastuzumab-refractory ERBB2-positive breast cancer (Modi et al., 2007), with the proteasome inhibitor bortezomib (Mimnaugh et al., 2004) which may offer a therapeutic strategy for the treatment of multiple myeloma, and with cytotoxics such as paclitaxel as an effective therapy in lung adenocarcinoma patients (Sawai et al., 2008). The combination of 17-AAG with cytotoxic agents such as paclitaxel, cisplatin and oxalipatin (Munster et al., 2001; Rakitina et al., 2003; Vasilevskaya et al., 2003; Vasilevskaya et al., 2004), tyrosine kinase inhibitors like imatinib (Radujkovic et al., 2005) and radiation treatment (Enmon et al., 2003; Bisht et al., 2003) have been studied with positive results being observed. In collaborative studies with Professor Ann Jackman and colleagues, we have demonstrated a beneficial interaction between 17-AAG and paclitaxel in those human ovarian cancer cell lines that have PI3 kinase pathway activation (Sain et al., 2006) and have also recently reported, using both in vitro cell culture and in vivo human tumour xenograft models, a therapeutic benefit for the combination of 17-AAG with carboplatin for the treatment of human ovarian cancer (Banerji et al., 2008b).

Although no HSP90 inhibitor has yet been approved for cancer treatment, the early clinical results with 17-AAG have begun to validate the potential of inhibiting HSP90 as a therapeutic approach to treat cancer. However, 17-AAG is not without its limitations, which include hepatotoxicity which may be caused by the redox active benzoquinone moiety, poor solubility necessitating the use of cumbersome formulations, and variable reduction by the polymorphic oxidoreductase enzyme NQO1/DT-diaphorase to the more potent hydroquinone form (Kelland et al., 1999). In addition, 17-AAG demonstrates reduced activity in the presence of P-glycoprotein (Kelland et al., 1999) and is metabolised by the polymorphic cytochrome P450 CYP3A4 (Egorin et al., 1998) giving rise to the potential for variable pharmacokinetics and drug-drug interactions. A number of these issues have been circumvented by the clinical introduction of more soluble derivatives such

as 17-DMAG (17-dimethylaminoethylamino-17-demethoxygeldanamycin; alvespimycin; Fig. 7.2) and IPI-504 (17-allylamino-17-demethoxygeldanamycin hydroquinone hydrochloride; retaspimycin Fig. 7.2) which is the more potent hydroquinone form of 17-AAG (Sydor et al., 2006).

Macbecin is another example of a benzoquinone ansamycin which has antitumour activity. This natural product has been shown recently to inhibit HSP90 function by binding to the N-terminal ATPase domain of HSP90 with a higher affinity than geldanamycin (Martin et al., 2008). Reflecting this, macbecin inhibits the ATPase activity of HSP90 with greater potency than geldanamycin to induce depletion of HSP90 client proteins including CRAF and ERBB2 and growth arrest of prostate cancer cells in vitro and in vivo using tumour xenograft models (Martin et al., 2008). Structural studies, comparing macbecin to geldanamycn, revealed significant differences in HSP90 binding characteristics (Martin et al., 2008). These differences offer the opportunity to develop novel HSP90 inhibitors using a proven structural scaffold.

A number of radicicol analogues have been developed which include in particular oxime derivatives (KF25706 and KF58333) that retain the capacity to inhibit HSP90 function but also demonstrate therapeutic activity in human tumour xenograft models (Soga et al., 1999; Soga et al., 2001). However, these have not yet progressed into clinical evaluation possibly due to reported toxicity to the eye in animals (Janin, 2005).

7.3 Synthetic Small-Molecule HSP90 Inhibitors

Natural products are intrinsically complex and inherently offer the potential for off-target effects. In addition, their synthesis is not easily adaptable to the processes required in a drug development programme. As a consequence, focus has shifted to the development of lower molecular mass inhibitors of HSP90. The first class to be described was based on a purine scaffold (reviewed in detail in Chiosis, 2006) designed to mimic the unusual 'C-shape' adopted by ADP when bound to the N-terminal domain nucleotide binding site of HSP90 (Prodromou et al., 1997; Stebbins et al., 1997). In collaboration with Vernalis, we reported X-ray co-crystal structures for the lead purine inhibitor PU3 which showed that this agent did indeed mimic ADP (Wright et al., 2004). However, the compound also induced an unexpected conformational change in the ATP-binding site to open up a novel lipophilic pocket (Wright et al., 2004). More potent and soluble analogues with activity in human tumour xenografts have been generated (He et al., 2006; Kasibhatla et al., 2007) including the optimised purine-base drug BIIB021 (Fig. 7.2) which has recently entered clinical trials.

High-throughput screening was used in our own studies to identify a novel group of water soluble HSP90 inhibitors containing a pyrazole unit, a benzodioxan core and a resorcinol ring which is the binding mode anchor of this family (Cheung et al., 2005) and is also found in radicicol (Roe et al., 1999). The 3,4-diarylpyrazole resorcinol

lead, CCT018159, was subsequently shown to inhibit human HSP90β with a similar potency but greater selectivity than 17-AAG (Sharp et al., 2007a). Consistent with our earlier studies using 17-AAG (Clarke et al., 2000; Hostein et al., 2001; Maloney et al., 2007), CCT018159 induced the molecular and cellular changes associated with HSP90 inhibition such as client protein depletion, induction of heat shock proteins such as HSP70, growth arrest and apoptosis, as well as reducing tumour cell invasion and angiogenesis (Sharp et al., 2007a). In addition, unlike 17-AAG, cellular sensitivity to CCT018159 was not affected by NQO1/DT-diaphorase expression nor was it a substrate for P-glycoprotein (Sharp et al., 2007a).

In a collaboration with Vernalis, structure-based design using X-ray crystallography resulted in the introduction of a 5-amide substitution which generated the more potent pyrazole amide CCT0129397/VER-49009 (Dymock et al., 2005) and the corresponding isoxazole CCT0130024/VER-50589 (Sharp et al., 2007b). The pyrazole to isoxazole switch did not affect the critical hydrogen bonding network, including essential water molecules, which we have previously shown to be vital to anchor the pyrazole resorcinol unit of these compounds to the N-terminal ATP binding site of HSP90 (Cheung et al., 2005; Dymock et al., 2005; Sharp et al., 2007a). As with 17-AAG and CCT018159, both compounds caused depletion of client proteins, induction of heat shock proteins, cell cycle arrest and apoptosis (Sharp et al., 2007b). Unlike 17-AAG but consistent with results for CCT018159, the cellular potency of both VER-49009 and VER-50589 was not affected by DT-diaphorase or P-glycoprotein expression (Sharp et al., 2007b).

In collaboration with Professor Laurence Pearl and Dr Chris Prodromou, we have used isothermal titration calorimetry (ITC) to show that the isoxazole had a greater binding affinity than the corresponding pyrazole with a dissociation constant (K_d) of 4.5 ± 2.2 nmol/L for VER-50589 compared to 78.0 ± 10.4 nmol/L for VER-49009 (Sharp et al., 2007b). In addition, the cellular uptake of the isoxazole was far greater than the pyrazole, resulting in more potent HSP90 inhibition and antiproliferative activity (Sharp et al., 2007b). Mean antiproliferative GI_{50} values for both the pyrazole and isoxazole were in the nanomolar range but the switch to the isoxazole resulted in an approximate nine-fold gain in potency (Sharp et al., 2007b). Based on our previous experience with cassette and individual compound dosing pharmacokinetic studies with CCT018159 and other early pyrazole compounds in mice (Smith et al., 2006), we investigated the pharmacokinetic properties of VER-49009 and VER-50589. Plasma clearance of both compounds was rapid; however, in vivo tumour cell uptake and HSP90 inhibition were confirmed by depletion of ERBB2 in an orthotopic human ovarian OVCAR3 carcinoma ascites model following treatment with either VER-49009 or VER-50589 (Sharp et al., 2007b). Extent and duration of pharmacodynamic changes using this in vivo model confirmed the superiority of VER-50589 over VER-49009 (Sharp et al., 2007b). Further studies using VER-50589 revealed that the good cellular uptake properties of the isoxazole resulted in tumour levels in HCT116 human colon tumour xenograft being above the in vitro GI_{50} for 24 h, resulting in approximately 30% growth inhibition (Sharp et al., 2007b).

Subsequent optimisation of the isoxazole series focused on maintaining or increasing potency while improving physiochemical, pharmacokinetic and pharma-

codynamic properties. This led to the identification of the resorcinylic isoxazole amide NVP-AUY922/VER-52296 (Brough et al., 2008; Fig. 2). X-ray co-crystal structures of NVP-AUY922 bound to the N-terminal domain of recombinant human HSP90α confirmed that this novel compound binds deep into the ATP pocket in a manner similar to CCT018159, VER-49009 and VER-50589 (Dymock et al., 2005; Sharp et al., 2007a; Sharp et al., 2007b). Replacement of the chlorine in the resorcinol ring present in VER-49009 or VER-50589 with an isopropyl group in NVP-AUY922 resulted in an additional hydrophobic interaction with Leu^{107} in the flexible lipophilic pocket of HSP90 (Eccles et al., 2008). In addition, replacement of the methoxy group of VER-49009 or VER-50589 with a morpholino side chain in NVP-AUY922 resulted in improved solubility whilst also providing additional hydrophobic interactions with Thr^{109} and Gly^{135} (Eccles et al., 2008).

NVP-AUY922 has excellent potency against HSP90 in a fluorescence polarisation binding assay with an IC_{50} of 21 nmol/L against the β isoform (Brough et al., 2008) and of 7.8 ± 1.8 nmol/L for the α isoform (Eccles et al., 2008). ITC demonstrated a very high binding affinity to HSP90β with a K_d of 1.7 ± 0.5 nmol/L which is three-fold lower than VER-50589 (Eccles et al., 2008). To our knowledge, NVP-AUY922 exhibits the tightest binding of any small molecule synthetic inhibitor yet reported. This can be explained in part by the improved bonding interactions described above along with superior entropy and enthalpy factors (Eccles et al., 2008). Studies with an analogue of NVP-AUY922 revealed a slow off-rate for binding to HSP90 (Brough et al., 2008). Profiling NVP-AUY922 against other ATPases, kinases, and a large panel of other enzymes and receptors showed a very high degree of selectivity towards HSP90 (Eccles et al., 2008).

Consistent with the other diaryl-pyrazoles and diary-isoxazoles described above, the cellular activity of NVP-AUY922 is independent of NQO1/DT-diaphorase and P-glycoprotein expression (Eccles et al., 2008). NVP-AUY922 inhibited in vitro proliferation of a panel of human cancer lines with nanomolar potency (Brough et al., 2008; Eccles et al., 2008). Inhibition of cell proliferation was accompanied by a G_1 or G_1 plus G_2-M phase cell cycle arrest in most cell lines, cell-line dependent apoptosis, HSP90 client protein depletion and heat shock protein induction, all of which are consistent with the molecular signature of HSP90 inhibition (Brough et al., 2008; Eccles et al., 2008). In addition, NVP-AUY922 potently inhibits tumour cell invasion, endothelial cell function associated with in vitro angiogenesis which include proliferation, motility, matrix invasion and tubular differentiation (Eccles et al., 2008). It also has good pharmacokinetic properties, with accumulation in tissues and especially tumour tissue (Eccles et al., 2008). Furthermore, NVP-AUY922 exhibits antitumour and antiangiogenic activity in a range of subcutaneous, orthotopic and metastatic human tumour xenograft models including colon (Brough et al., 2008), melanoma, glioblastoma, and breast, ovarian and prostate carcinomas (Eccles et al., 2008; Jensen et al., 2008). Figure 7.3 shows the activity of NVP-AUY922 against the BT474 human breast cancer xenograft that expresses both ERα and ERBB2 (Eccles et al., 2008). A prolonged growth inhibition and a significant number of regressions were observed in this model, consistent with depletion of these client proteins by the drug. Based on these promising preclinical studies, the optimised analogue NVP-AUY922 has now entered phase I clinical trial.

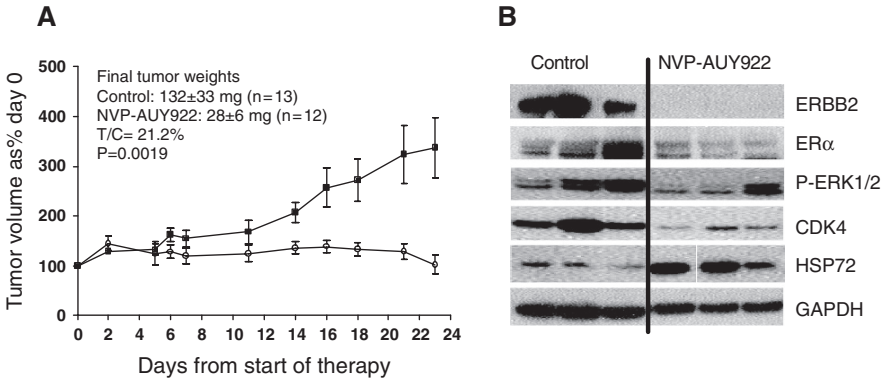

Fig. 7.3 Response of BT474 human breast cancer xenografts to NVP-AUY922. Tumour xenografts were established for ERBB2 + /ERα+ BT474 human breast carcinoma cells. Dosing with 50mg/kg of NVP-AUY922 or vehicle commenced 15 days after cell injection and continued daily for 23 days. (**A**) BT474 tumour xenograft growth curves, with (insert) final weights. *Solid squares*: vehicle controls; *open circles* NVP-AUY922 treated. (**B**) western blots showing biomarkers of HSP90 inhibition from representative control and treated BT474 xenografts. Reproduced with permission from Eccles et al., 2008

The design of NVP-AUY922 highlights the value of X-ray crystallography and structure-based design as a powerful approach to create novel HSP90 inhibitors. Also important in selecting NVP-AUY922 was the simultaneous optimisation of pharmacokinetic and pharmacodynamic properties, featuring a novel approach of determining tumour uptake in cassette dosing studies, together with pharmacodynamic biomarker determinations (Brough et al., 2008).

7.3.1 Agents That Inhibit HSP90 Function by Alternative Methods

Novobiocin is a member of the coumarin family of antibiotics which are known to bind to and inhibit the bacterial DNA gyrase B ATP binding site. However, it has also been shown to inhibit HSP90 function and induce client protein degradation (Marcu et al., 2000b). However, unlike the HSP90 inhibitors described so far, novobiocin is different in that it does not bind to the chaperone's N-terminal ATP-binding site (Marcu et al., 2000b). Instead novobiocin has been shown to interrupt HSP90 function by interacting with the C-terminal domain of HSP90 (Marcu et al., 2000b) and disrupting the interaction between HSP90 and its co-chaperones HSC70 and P23, both of which have been shown to be critical for the chaperone activity of HSP90 and both of which interact with the C-terminal domain of the chaperone (Marcu et al., 2000a). Novobiocin has also been proposed to inhibit HSP90 activity via an interaction with a proposed cryptic ATPase domain within the C-terminal domain of HSP90 (Marcu et al., 2000a). However, this domain has not yet been identified despite crystal structures for this region of the chaperone now being available (Dollins et al., 2005; Ali et al., 2006; Shiau et al., 2006).

Celastrol is a complex natural compound which been shown to inhibit the proteasome (Yang et al., 2006) and to restrict the growth of human prostate carcinoma (Yang et al., 2006), melanoma (Abbas et al., 2007) and glioma (Huang et al., 2008) xenograft models. Similar to HSP90 inhibitors, celastrol causes client protein depletion and induction of several heat shock proteins (Zhang et al., 2008; Hieronymus et al., 2006). Large scale gene expression studies revealed similarities between the molecular response to well documented HSP90 inhibitors and celastrol (Hieronymus et al., 2006). However, celastrol does not affect ATP or geldanamycin binding to HSP90, indicating that it does not associate with the N-terminal domain (Hieronymus et al., 2006). It has been suggested recently that celastrol inhibits HSP90 function by disrupting the association of CDC37 (Zhang et al., 2008), a co-chaperone which is required for loading of kinase clients onto HSP90 (Roe et al., 2004).

We have recently used a duplexed cell-based phenotypic assay (see below and Hardcastle et al., 2007) to screen our compound library and thereby to simultaneously identify compounds that inhibit HSP90 function and/or cellular acetylation in human colon carcinoma cells (Hardcastle et al., 2007). Using this approach we discovered CC002151 which induced the characteristic pattern of client protein depletion, heat shock induction and cell growth inhibition but did not inhibit HSP90 ATPase activity (Hardcastle et al., 2007). Further work is required to elucidate the mechanism of action of this compound.

7.4 Novel Approaches to Inhibit HSP90 Function

Inhibiting the ATPase domain of HSP90 has yielded significant information regarding the biological function of this chaperone, in addition to offering the most direct route to therapeutic manipulation. However, there are a number of alternative strategies to inhibit the function of this molecular chaperone which may broaden the therapeutic potential of chaperone modulation.

7.4.1 Targeting Individual HSP90 Isoforms

At present five isoforms of human HSP90 have been identified which differ in their cellular localisation (reviewed in Argon and Simen, 1999; Sreedhar et al., 2004a; Neckers et al., 2007). Evidence is also emerging of differences in specificity for particular client proteins and/or function, an aspect which could be potentially manipulated therapeutically to enhance the selectivity and reduce toxicity of HSP90 inhibitors. The two predominant cytoplasmic isoforms are HSP90α and HSP90β. HSP90β is constitutively expressed and considered to be important during cell differentiation and embryonic development (Sreedhar et al., 2004a). On the other hand, HSP90α basal expression is significantly lower than that of HSP90β, but its expression is significantly increased in response to stress and therefore it is considered

to more important than HSP90β for cytoprotection (Chen et al., 2005). Expression of either HSP90α or β as the sole isoform in yeast is sufficient to confer viability and to ensure stability of a number of, but not all, client proteins such as VSRC which more is reliant on HSP90α than HSP90β expression (Millson et al., 2007). In addition, activation of the heat shock factor-1 transcription factor (HSF1), which is known to be repressed by association with HSP90 (Shi et al., 1998), was more dependent on HSP90α expression than HSP90β (Millson et al., 2007). Interestingly, sensitivity to HSP90 inhibitors in yeast can be influenced by the expression levels of HSP90β. Expression of HSP90β as the sole isoform rendered yeast highly sensitive to radicicol, whereas sole expression of HSP90α did not (Millson et al., 2007). A further function which has been assigned solely to HSP90α is its unique ability to occupy a cell surface position and interact with the extracellular matrix protein, matrix metalloprotease-2, suggesting a potential role in cancer cell metastasis (Eustace et al., 2004).

Another cytoplasmic isoform is HSP90N which differs from HSP90α by the deletion of the N-terminal domain that is the site of the functional ATPase site (Grammatikakis et al., 2002). This isoform has been linked to cellular transformation via its association with CRAF (Grammatikakis et al., 2002). However, the details of its biological function remain to be defined.

The other major HSP90 isoforms are GRP94 (glucose regulate protein-1) in the endoplasmic reticulum (Argon and Simen, 1999) and TRAP1 (tumour necrosis factor receptor associated protein-1) in the mitochondrial matrix (Felts et al., 2000). There is limited literature regarding the specific functions of these isoforms. Although both GRP94 and TRAP1 share a similar overall structure to HSP90α and HSP90β, there is very little information regarding their interaction with or dependency on co-chaperones. GRP94 has been shown to play a role in the immune system by delivering peptides to MHC class I molecules for antigen presentation (Suto and Srivastava, 1995), ensuring immunoglobulin light chain formation and targeting unassembled subunits to the proteasome (Melnick et al., 1992; Melnick et al., 1994). GRP94 has also been associated with the maturation of receptor tyrosine kinases such as ERRB2 (Chavany et al., 1996) and the truncated EGFRvIII (Lavictoire et al., 2003) and also with the secretion of insulin-like growth factors (Wanderling et al., 2007). Finally, overexpression of GRP94 correlates with decreased sensitivity of cervical cancer cell lines to X-rays (Kubota et al., 2005) whereas reducing its expression increases the sensitivity of Jurkat cells to etoposide (Reddy et al., 1999). There is even less information on the biological function of the mitochondrial homologue TRAP1. Similar to HSP90α and HSP90β, TRAP1 has a functional ATPase domain; however, it does not associate with the co-chaperones P23 and HOP, potentially signifying a distinct mechanism of regulation (Felts et al., 2000). Despite its mitochondrial localisation, TRAP1 is implicated in the maturation of retinoblastoma protein (Felts et al., 2000). This interaction is unique to TRAP1 and is mediated by a LxCxE binding motif which is exclusive to this isoform (Felts et al., 2000). TRAP1 has also been linked to regulating mitochondrial function and protecting cells from mitochondrial-mediated apoptotic cell death induced by oxidative stress (Masuda et al., 2004; Pridgeon et al., 2007; Kang et al., 2007).

The ATPase cycles of both GRP94 and TRAP1 have recently been determined and compared to the well documented ATPase cycle of the cytosolic HSP90 isoforms (Frey et al., 2007; Leskovar et al., 2008). Subtle differences have been observed in the ATPase cycles of all four isoforms which, along with differences in 3-dimensional structure and in the affinities of each isoform for nucleotide (Leskovar et al., 2008), may offer the potential for the development of isoform-specific inhibitors. The geldanamycin derivative 17-AAG exhibits moderate selectivity over GRP94 and is highly selective against TRAP1 (Eccles et al., 2008). A degree of isoform specificity has been observed with the diaryl-isoxazole resorcinol HSP90 inhibitor NVP-AUY922. The IC_{50} values for NVP-AUY922 against the HSP90 family members were 535 ± 51 nmol/L and 85 ± 8 nmol/L for GRP94 and TRAP1 respectively compared to 7.8 ± 1.8 nmol/L and 21 ± 16 nmol/L for HSP90α and HSP90β, respectively, indicating significantly reduced potency against the non-cytosolic isoforms (Eccles et al., 2008). These data reinforce the possibility of achieving inhibitors that are more specific for a particular isoform of the HSP90 family. Further work is required to elucidate the consequences of this for the treatment of cancer and for effects on normal tissues.

7.4.2 Modulating the Association of HSP90 Co-Chaperones

As shown in Fig. 7.1, HSP90 function is supported by a number of co-chaperones which are involved in substrate recruitment and/or regulation of ATPase activity (Pearl, 2005; Pearl et al., 2008). Targeting co-chaperone interactions may enable a particular subset of client proteins to be inhibited which would lead to a more selective and, as a consequence, potentially less toxic inhibitor, the nature of which could be tailored for individual tumour types. We have provided evidence to support this concept using a small-interfering RNA (siRNA) approach to selectively knockdown the expression of AHA1, a co-chaperone which studies at our institution have shown to stimulate the relatively weak intrinsic ATPase activity of human HSP90 (Panaretou et al., 2002). Reduction of AHA1 expression resulted in decreased CRAF activity and reduced phosphorylation of the downstream kinases MEK1/2 and ERK1/2 (Holmes et al., 2008). Interestingly, total levels of the HSP90 client protein CRAF were unaffected by AHA1 knockdown, importantly suggesting that reduced AHA1 association with HSP90 prevented CRAF activation rather than reducing its stability (Holmes et al., 2008). Results of overexpression of AHA1 have provided further evidence that AHA1 recruitment is required for client protein activation rather than stabilisation. Thus higher AHA1 levels and HSP90 binding resulted in increased AKT phosphorylation and immunoprecipitated AKT catalytic activity (Holmes et al., 2008). Also of interest was the observation that, as with CRAF, the expression of CDK4 or ERBB2 was unaffected following the knockdown of AHA1 (Holmes et al., 2008), highlighting the potential for molecular specificity using this approach which might be translated into differential effects on tumours with distinct molecular pathologies.

The observations described above have been attributed to altering the ATPase activity of HSP90 by reducing AHA1 association (Holmes et al., 2008). Another obvious strategy is to target the co-chaperones involved in recruiting the client proteins to the HSP90 complex. HSP70 has a well documented role during the early stages of substrate loading onto HSP90 (Wegele et al., 2004). It is also implicated in malignant transformation due to its antiapoptotic role (Mosser and Morimoto, 2004; Calderwood et al., 2006). We have used an siRNA approach to selectively and simultaneously reduce the expression of the major constitutive and inducible isoforms of the HSP70 family, HSC70 and HSP72, respectively. We have shown that simultaneous knockdown of both isoforms inhibits the activity of HSP90 to induce degradation of CRAF, CDK4 and ERBB2 in human colon and ovarian cell lines (Powers MV, Clarke PA and Workman P, unpublished observations). This was accompanied by inhibition of cell growth and induction of cell death, the extent of which was greater than that seen with 17-AAG. Importantly, the effect of the combinatorial knockdown was significantly less in a number of non-tumorigenic cell lines, providing the first evidence of tumour selectivity and potentially reduced toxicity using this approach (Powers MV, Clarke PA and Workman P, unpublished observations). Another way of inhibiting substrate recruitment by HSP70 is to target HOP, an adaptor protein which links the HSP70 and HSP90 chaperone cycles (Chen and Smith, 1998). An engineered HSP90-tetracopeptide repeat (TPR) binding module has been designed which disrupts the interaction between the HOP TPR domain and the C-terminal of HSP90 (Cortajarena et al., 2008). Preliminary evidence of activity has been demonstrated by depletion of the ERBB2 client protein and inhibition of cell proliferation in the BT474 breast cancer cell line (Cortajarena et al., 2008). In addition, inhibition of HSP90 function using this approach was not associated with the undesirable increase in HSP70 expression associated with conventional ATPase HSP90 inhibitors (Cortajarena et al., 2008).

CDC37 is also emerging as an interesting target for modulation based on its selective recruitment of protein kinase client proteins to the chaperone complex (Roe et al., 2004) and its possible role in malignant transformation (reviewed in Pearl, 2005). Agents which prevent CDC37 interaction with HSP90 may have a particular advantage in the treatment of tumours driven by overexpressed or mutated kinases. Compared to approaches that target all HSP90 functions, this approach could also have less toxicity to normal cells since inhibiting CDC37 function would not be expected to affect the activity of the large number of non-kinase HSP90 client proteins, which include the steroid hormone receptors. We have used an siRNA approach to selectively reduce the expression of CDC37 in human colon cancer cells. Knockdown of CDC37 resulted in reduced association of protein kinase clients with HSP90 and decreased expression of several of these including CDK4, CDK6, AKT, ERBB2 and CRAF (Smith JR, Clarke PA and Workman P unpublished observations). This resulted in decreased cell signalling through the kinase clients, as demonstrated by reduced phosphorylation of downstream substrates and a subsequent G1/S phase cell cycle arrest (Smith JR, Clarke PA and Workman P unpublished observations). Similar observations have been reported in prostate cancer cell lines which undergo irreversible growth arrest following the molecular silencing of CDC37 (Gray et al., 2007).

The evidence presented above reinforces the view that HSP90 co-chaperones represent potential targets in their own right. However, we and others have also investigated the consequences of combining co-chaperone interference with classical pharmacological HSP90 inhibitors such as 17-AAG. For AHA1, HSP70 and CDC37, we and others have demonstrated in each case that the response to 17-AAG, including depletion of client proteins, inhibition of cell growth and induction of cell death, can be dramatically enhanced by the combinatorial silencing of co-chaperone expression (Gabai et al., 2005; Guo et al., 2005; Gray et al., 2007; Holmes et al., 2008, Powers MV et al, unpublished observations, Smith et al, unpublished observations). These observations suggest a method to potentially increase the therapeutic benefit of existing HSP90 inhibitors. Since combining co-chaperone targeting with pharmacologic HSP90 inhibition increases apoptosis, this approach represents a promising form of synthetic lethality, with potential for greater effects in cancer versus normal cells. However, it is necessary, when considering the targeted disruption of co-chaperone interactions, to think about the potential complexities of this approach. It is technically more difficult to design compounds to disrupt protein-protein interactions than to inhibit ATP binding. However, crystal structures of HSP90 and its co-chaperones are now available which offer the opportunity for lead identification using techniques such as fragment-binding and virtual-screening.

7.4.3 Post-Translational Modification of HSP90

It has emerged in recent years that, alongside co-chaperone interaction, HSP90 function may be regulated by a series of post-translational modifications. Several studies have demonstrated client protein depletion and HSP70 induction following the inactivation of histone deacetylase (HDAC) enzymes (Kovacs et al., 2005; Bali et al., 2005). This is due to hyperacetylation of HSP90 which disrupts its molecular function, possibly via dissociation of the co-chaperone P23 (Bali et al., 2005; Kovacs et al., 2005). Acetylation of HSP90 is mediated by inhibition of HDAC6 (Kovacs et al., 2005). A critical residue involved in this modification is Lys^{294} which, when hyperacetylated, decreases the function of yeast Hsp90 by reducing the interaction with its client proteins and co-chaperones (Scroggins et al., 2007). It is currently not understood if acetylation is required for the normal regulatory function of HSP90, nor is it known which acetyltransferase is responsible for the modification.

As mentioned earlier, we have utilised a high-throughput screen to identify agents which inhibit cellular acetylation or HSP90 function (Hardcastle et al., 2007). We developed a high-throughput, duplexed, cell-based phenotypic screen which utilised a multiplexed time-resolved fluorescence cell immunosorbent assay (TRF-Cellisas) to simultaneously detect compounds which induce HSP70, as a mark of HSP90 inhibition, together with agents that modulate cellular acetylation (Hardcastle et al., 2007). Using this approach it may be possible to discover compounds which interrupt HSP90 function by modulating cellular acetylation (Hardcastle et al., 2007). Several hits were identified in both arms of the screen. The precise mechanism of action of these compounds remains to be further defined.

Alongside acetylation, HSP90 function can also be regulated by phosphorylation (Zhao et al., 2001). Serine and threonine phosphorylation have been reported to have a negative effect on the activity of HSP90 although the exact residues involved are unknown (Zhao et al., 2001). CDC37 also requires phosphorylation for its activity (Miyata and Nishida, 2005), indicating an alternative approach to inhibiting HSP90 function. A kinase-based strategy is reinforced by the observation of inhibition of HSP90 activity following blockade of phosphatase activity (Wandinger et al., 2006). Some initial success with this approach has already been achieved with the identification of compound IC101 which induces HSP90 dephosphorylation, client protein depletion and apoptosis (Fujiwara et al., 2004).

7.4.4 Substrate Mimetics

Inhibiting the association of a single client protein substrate with HSP90 would be an efficient approach to achieving a very high level of selectivity, particularly in the treatment of cancers driven by a single, dominant oncogenic protein, or perhaps a group of closely related clients. An example of an attempt to target the interaction of HSP90 with a particular client is the peptidometic shepherdin which was designed to interfere with the interaction between HSP90 and its client protein survivin (Plescia et al., 2005). Exposure of cancer cells to shepherdin caused depletion of client proteins, including survivin, induced apoptotic and non-apoptotic cell death, and brought about the eradication of acute myeloid leukaemia xenografts (Plescia et al., 2005; Gyurkocza et al., 2006). Subsequently, a non-peptidic small molecule inhibitor, AICAR, was developed which retained a similar profile of HSP90 inhibition (Meli et al., 2006). Although general client protein depletion was observed following treatment with shepherdin and AICAR, the molecular profile of HSP90 inhibition following their use was distinct from other HSP90 inhibitors in that it did not include the induction of HSP70. As described above, we and others have previously demonstrated that HSP70 induction dramatically reduces the cell death effects of 17-AAG (reviewed in Powers and Workman, 2007). Therefore, the lack of induction of HSP70 observed following shepherdin treatment may be of therapeutic benefit.

The design of inhibitors that very specifically block the interaction of individual client proteins with HSP90 will await the solving of what is probably the most outstanding and important problem in HSP90 biology: How does HSP90 recognise its client proteins (Pearl et al., 2008)?

7.5 Targeting HSP90 Function in Endocrine-Related Cancers

There is a growing body of evidence validating the potential of HSP90 inhibitors in the treatment of endocrine-related malignancies such as breast and prostate cancer. Expression of HSP90 has been shown recently to be elevated in breast cancer and

its increased expression is associated with decreased survival (Pick et al., 2007). In addition, many of the proteins implicated in breast cancer progression and resistance to therapy are chaperoned by HSP90 (reviewed in Beliakoff and Whitesell, 2004). These include the oestrogen receptor, members of the ERBB receptor tyrosine kinase family, AKT, HIF1α and mutant p53 (reviewed in Beliakoff and Whitesell, 2004). This collection of potential targets suggests that HSP90 inhibitors may have therapeutic potential for the treatment of both hormone-sensitive and hormone-insensitive breast cancer. This is reinforced by the recent observation of responses to 17-AAG plus trastuzumab in patients with trastuzumab-refractory breast cancers (Modi et al., 2007). We have also obtained promising results with the isoxazole resorcinol inhibitor NVP-AUY922 in a human breast tumour xenograft (Eccles et al., 2008), a result also confirmed by others (Jensen et al., 2008). Treatment of BT474 human breast tumours with pharmacologically relevant concentrations of NVP-AUY922 induced ERα and ERBB2 depletion which was accompanied by growth arrest and/or regression of tumours (Eccles et al., 2008).

HSP90 inhibitors may also be advantageous in the treatment of prostate cancer which can be reliant on androgen receptor expression or dependent on the PI3K pathway through loss of PTEN (reviewed in Majumder and Sellers, 2005). We have recently demonstrated, for the first time, that our HSP90 inhibitor, NVP-AUY922 can reduce the growth of established orthotopic PTEN-null, hormone-independent prostate carcinoma xenografts (Eccles et al., 2008). This is consistent with earlier reports using 17-AAG which demonstrated reduced growth of androgen-dependent and androgen-independent human tumour xenografts grown subcutaneously (Solit et al., 2002).

7.6 Conclusions, Future Prospects and Challenges

The potential to simultaneously deplete malignant cells of multiple client proteins and to modulate all six hallmark traits of cancer by inhibiting a single protein target has propelled HSP90 into the spotlight as an exciting anticancer drug target. Natural product HSP90 ATPase-inhibitors have been fundamental in elucidating the mechanism of action of the molecular chaperone. The geldanamycin analogue 17-AAG has completed phase I trials and provided proof-of-concept for HSP90 inhibition in the clinical setting. 17-AAG has shown early promise as the first-in-class HSP90 inhibitor with responses being observed in melanoma, breast and prostate carcinoma and phase II trials have now been initiated. A phase III study of 17-AAG plus trastuzumab in trastuzumab-refractory, ERBB2-positive tumours has been initiated. Phase III trials have also been instigated for the combination of 17-AAG and bortezomib for patients with multiple myeloma, where promising clinical activity has been seen in earlier studies.

Tumour samples from the clinical trials are now aiding the identification of factors which may influence patient response to HSP90 inhibitors. An example is our own recent identification of NRAS/BRAF mutation status as a potential factor in

the response of melanoma patients treated with 17-AAG (Banerji et al., 2008a). The promising activity of 17-AAG in trastuzumab-resistant ERBB2 positive breast cancer may relate to the depletion of ERBB2, but effects on other clients may also play a role. Combined effects on ERBB2 and the oestrogen receptor suggests potential in tumours with these characteristics but activity in ERBB2-/ER- breast cancers is also possible. In prostate cancer, effects on the androgen receptor and on the PI3K pathway are likely to be important. Activity in multiple myeloma, most notably in combination with bortezomib, appears to involve the unfolded protein response that is important in this disease (Davenport et al., 2007). Combined administration of 17-AAG and docetaxel has demonstrated promising clinical activity in patients with NSCLC (Solit et al., 2004), potentially related to effects on mutant epidermal growth factor receptor (EGFR) which is more sensitive to HSP90 inhibitors than the wild-type form (Sawai et al., 2008; Xu et al., 2007).

Despite the promising activity of 17-AAG it is not without its limitations, which include solubility and formulation issues and significant toxicity being observed in patients. It is not known how much of the toxicity is due to non-specific off-target effects related to the particular chemical scaffold of the inhibitor. For example, the liver toxicity seen with 17-AAG may be associated with the quinone moiety. As a result there is considerable interest in searching for novel, synthetic, small-molecule inhibitors of HSP90. Several have been described including purines and the potent isoxazole resorcinols that we discovered in collaboration with Vernalis. Of the latter, NVP-AUY922 is being developed by Novartis and has just entered phase I clinical trial.

It is possible that some of the side-effects seen with HSP90 inhibitors may be a consequence of inhibiting HSP90 activity. The combinatorial effect on multiple signal transduction pathways is one of the key strengths of HSP90 inhibitors but it may also obscure factors underlying toxicities associated with their on-target effects. For the same reason it is also difficult to deconvolute the precise mechanism of action of HSP90 inhibitors in a particular cancer. Because of the potential for more specific effects on certain cancers and for reducing normal tissue toxicity, alternative methods are being investigated to inhibit particular functions of HSP90. These include targeting individual isoforms of HSP90, altering the post-translational modifications of the chaperone, preventing the association of a defined subset of client proteins by the use of substrate mimetics, or modulating the association of a particular co-chaperone. Such co-chaperones include, amongst others, AHA1, CDC37 and HSP70. Silencing the expression of these proteins results in the inhibition of HSP90 function and sensitisation to pharmacologic HSP90 inhibitors. Developing the ability to selectively target a particular subset of client proteins may facilitate the tailored use of inhibitors which target different aspects of the HSP90 chaperone for the treatment of tumours with particular molecular drivers.

Over the last few years HSP90 has progressed from a fringe target that many if not most considered too risky to one that has taken centre stage in many companies and academic drug discovery groups. Proof-of-concept for target modulation in patients and clear signs of clinical activity have reduced the perceived risk and stimulated considerable interest. A range of N-terminal ATP site inhibitors are now

emerging from screening and structure-based design programmes and are entering the clinic. This in turn has encouraged alternative technical approaches to attack HSP90 and stimulated the biomedical research community to think more widely about additional targets in chaperone biology and protein quality control. The next few years will see considerable activity in the evaluation of HSP90-targeted agents in various cancers, both endocrine and non-endocrine, as well as potentially in the treatment of other diseases. We can no doubt continue to expect to be surprised by what we do not yet know about the basic biology, as well as the pharmacology and therapeutics, of the HSP90 molecular chaperone.

7.7 Conflict of Interest

PW has been involved in a funded research collaboration with Vernalis to develop HSP90 inhibitors that were licensed to Novartis. PW has been a consultant to Novartis.

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Abbreviations

17-AAG:	17-allylamino-17-demethoxygeldanamycin
17-DMAG:	17-dimethylaminoethylamino-17-demethoxygeldanamycin
GRP:	glucose regulated protein
HSP:	Heat shock protein
siRNA:	short interfering RNA
AKT:	protein kinase B
ERBB2:	epidermal growth factor receptor 2
HSP70:	heat shock protein 70
IPI-504:	17-allylamino-17-demethoxygeldanamycin hydroquinone hydrochloride (retaspimycin)

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