Abstract  
A number of human diseases can be linked to aberrations in protein folding which cause an imbalance in protein homeostasis. Molecular chaperones, including heat shock proteins, act to assist protein folding, stability and activity in the cell. Attention has begun to focus on modulating the expression and/or activity of this group of proteins for the treatment of a wide variety of human diseases. This review will describe the progress made to date in developing pharmacological modulators of the heat shock response, including both agents which affect the entire heat shock response and those that specifically target the HSP70 and HSP90 chaperone families.
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1. Introduction: heat shock proteins in health and disease

Protein synthesis in vivo is supported by molecular chaperones which assist the folding of nascent polypeptides, avoid the formation of aggregates by preventing non-specific interactions and aid the translocation of proteins to their correct intracellular localization. When a protein is damaged, molecular chaperones may also facilitate their re-folding or, in the case of irreparably impaired proteins, their removal by the protein degradation machinery of the cell [1].

Heat shock proteins (HSPs) were originally defined according to their increased expression in response to a cellular insult such as elevated temperature, heavy metals and oxidative stress [1]. Most, but not all HSPs are molecular chaperones that are organized into families according to their molecular size or function, including HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs. The often rapid induction of HSP gene expression in response to stress is a result of a coordinated series of biochemical and genetic events which are collectively referred to as the heat shock response (HSR). Activation of the HSR results in cytoprotection to repeat exposure of the initial insult which would otherwise cause lethal molecular damage [2].

Cytoprotection is an example of increased molecular chaperone expression associated with the functioning of normal cells. However, aberrant expression of this family of proteins can also be associated with several disease states. There is increasing evidence associating molecular chaperone expression with neurodegenerative disorders including Parkinson’s, Alzheimer’s, Huntington’s and prion-related disease [3,4]. Also a large body of evidence supports the role of molecular chaperones in maintaining the cancer phenotype [5–7]. This present review will focus on the current efforts to modulate the expression and activity of molecular chaperones as a novel approach to treat protein-folding diseases, with a particular emphasis on the treatment of cancer.

2. Modulation of HSF1 and the HSR

The HSR is mediated at the transcriptional level by heat shock factors (HSFs), a group of transcription factors that are capable of specifically binding to heat shock elements within the promoter of HSP genes [8]. Among the three human HSF genes, HSF-1, -2 and -4, HSF1 is essential for the regulation of HSP expression in response to stress [8]. Although the other HSFs may contribute to the activity of HSF1, this review will focus on the regulation and modulation of HSF1 activity.

The activity of HSF1 is dependent on its ability to bind DNA, which is achieved via a series of modification steps described in Fig. 1. Through the propensity of HSF1 to control the expression of a number of different HSPs, an increasing amount of interest has focused on the development of small-molecule modulators which can alter the activity of HSF1 [2]. This may be of therapeutic benefit in the treatment of diseases which have an underlying abnormality in protein conformation or activity. So far several, structurally unrelated compounds have been identified which can induce or inhibit the HSR.

2.1. HSR inducers

There are various agents which induce the HSR and promote HSP expression without the requirement for additional forms of stress such as heat shock. These agents could have a therapeutic application in the treatment
of neurodegenerative diseases, which may benefit from increased chaperone expression to reduce the levels of misfolded or aggregated proteins that underlie the development of the pathological condition [2]. Proteasome inhibitors including MG-132, lactacystin and bortezomib, the latter clinically approved for treatment of multiple myeloma [9], activate the HSR [10,11]. This response is proposed to be a consequence of increasing the intracellular concentration of misfolded proteins which in turn cause the dissociation of HSF1 from its repressive complex with HSP90 and HSP70 [2]. Subsequently, proteasome inhibitors induce hyperphosphorylation of HSF1, DNA binding, and increased expression of HSPs [10].

The HSR can also be activated by molecules that regulate inflammation [2]. The inflammatory response involves the sequential activation of various signaling molecules, among which arachidonic acid and its metabolites such as prostaglandins play a crucial role. Phospholipase A2 stimulates the release and metabolism of arachidonic acid. Both phospholipase A2 and arachidonate induce the heat shock response by promoting increased DNA-binding by HSF1 [12]. Cyclopentenone prostaglandins of the A and J type also activate HSF1 and increase the expression of HSP70 [13].

A novel inducer of the HSR has recently been identified [14]. Tercreyclic acid A (TCA) perturbs the redox state of the cell and increases the levels of reactive oxygen species [14]. Oxidative stress, similar to thermal stress, requires the rapid and adaptive increase in HSP synthesis; therefore, the increased levels of reactive oxygen species leads to the activation of HSF1 [14].

Several inhibitors of HSP90 stimulate the activity of HSF1. The mechanism of action of these compounds, which include radicicol and the benzoquinone ansamycins, is described in more detail later in this review. They induce the HSR by stimulating the dissociation of HSF1 from its repressive complex with HSP90 [15]. This results in the increased expression of a wide range of HSPs including HSP90, HSP70 and HSP27 [16,17]. The exact mechanism underlying the dissociation of HSF1 from HSP90 in response to its inhibition by these agents has not been clearly established. However, it is proposed that inhibition of HSP90 function may, in a similar fashion to proteasome inhibitors, alter protein homeostasis by increasing the concentration of denatured proteins which compete with HSP90 for HSF1 interaction, thereby increasing the amount of active HSF1 [15].

HSF1 can also be activated by the natural triterpene, celastrol (Fig. 1). Exposure of cells to this compound induced the expression of HSP70, HSP40 and HSP27 by promoting the hyperphosphorylation, DNA binding, and transcriptional activity of HSF1 with kinetics similar to those observed following heat shock [18]. The mechanism by which celastrol induces HSF1 activity has not been clearly defined. It has been shown to have no direct activity on the chaperone function of HSP70 nor does it cause global protein denaturation [18]. However, celastrol potently inhibits the chymotrypsin-like activity of purified 20S proteasome [19]. In addition, celastrol and the structurally related triterpene, gedunin, have been recently proposed as novel HSP90 inhibitors (see later and [20]). Both of these observations may begin to explain how celastrol induces the HSR; however, further investigations are required to determine its exact mechanism of action.

All of the above compounds can act independently to affect the activity of HSF1; however, there is a class of compounds referred to as ‘co-inducers’ which are able to activate an enhanced HSR in synergy with low levels of cellular stress [2]. Non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin, indomethacin and sodium salicylate act as co-inducers by stimulating the trimerization of HSF1 and reducing the temperature required for complete activation of the transcription factor [21]. Other chaperone co-inducers are the non-toxic hydroxylamine derivatives bimoclomal and arimoclomal. Bimoclomal binds to HSF1 with low affinity to prolong the duration of HSF1 binding to DNA and has shown promise...
as a therapeutic agent in animal models of ischemia and wound healing [22]. This is postulated to be a result of the coordinated expression of all major HSPs which occur as a result of HSF1 induction [22].

2.2. HSR inhibitors

Inhibitors of the HSR may be of particular benefit for the treatment of cancer because tumor cells typically express higher levels of HSPs than normal cells and indeed may be more dependent on HSPs than normal cells. This may be a consequence of the hostile conditions created in tumor cells by the effects of deregulated oncogenes or tumor suppressor genes, along with the stressful microenvironmental features of solid tumors, which include nutrient deprivation, hypoxia and acidosi[5,7]. The increased expression of HSPs not only likely allows the cells to tolerate cumulative mutations and aberrant protein expression which may be otherwise lethal, but may also promote tumor cell survival via the well-documented anti-apoptotic function of HSPs [23]. In addition to promoting tumor cell survival, induction of the HSR may reduce the efficacy of existing anti-cancer strategies, which are complicated by the concurrent induction of this anti-apoptotic response. Studies using siRNA to target HSF1 have reinforced the potential value of developing chemical modulators of the HSR by demonstrating increased sensitization to heat shock, proteasome inhibitors, HSP90 modulators and hyperthermo-chemotherapy when HSF1 expression is reduced [24,25].

The naturally occurring flavonoid, quercetin (3,3',4',5,7-pentahydroxyflavone, Fig. 1) inhibits the heat-induced expression of HSPs in a range of different cell types by affecting several aspects of HSF1 activity, including HSF1 hyperphosphorylation, DNA binding and ultimately transcriptional activity [26]. Treatment with quercetin inhibits the growth of cancer cells both in vitro and in vivo, and also demonstrates evidence of tumor cell versus normal cell selectivity [27]. Quercetin pretreatment also renders cells more susceptible to apoptotic stimuli, including hyperthermia and chemotherapy [28]. However, the activity of quercetin is not specific because it not only modulates HSF1 activity but also inhibits the activity of several protein kinases [29,30].

Compounds which appear to inhibit HSP expression more specifically and effectively than quercetin are currently being investigated and include the benzylidene lactam, KNK437 (Fig. 1). This compound can inhibit the heat-induced expression of a variety of HSPs including HSP110/HSP105, HSP72 and HSP40 [31], but has no effect on the expression of constitutively expressed HSP family members including HSC70 and HSP90 [31]. KNK437 inhibits thermotolerance both in vitro and in vivo, resulting in increased response to hyperthermia, low dose-rate irradiation and the HSP90 inhibitor 17-AAG (see later and [32,33]). Unlike quercetin, KNK437 does not induce significant toxicity when administered alone [31] which may be because, unlike quercetin, KNK437 does not have a pleiotropic effect on protein kinase activity [31]. Although both quercetin and KNK437 have been shown to have an effect on HSF1 activity, the exact mechanism by which they prevent the acquisition of thermotolerance is not known and remains to be elucidated.

Despite their potential in vitro, quercetin and KNK437 may not be sufficiently potent for clinical use as high concentrations of compound are required to demonstrate inhibitory activity (100–500 μM). Use of such high concentrations of low potency agents increases the likelihood of off-target effects. A promising alternative to quercetin and KNK437 has recently been identified as part of a small-molecule high throughput screen for modulators of the HSR [34]. The diterpene triepoxide triptolide (Fig. 1) potently inhibits the heat shock induction of HSP70 expression by a novel mechanism which does not involve inhibition of HSF1 trimer formation, HSF1 hyperphosphorylation or the nuclear translocation and binding of HSF1 to the HSP70 gene promoter [34]. Instead, triptolide inhibits HSP expression by interfering with the activity of the C-terminal transactivation domain of HSF1 and blocking its transcriptional activity [34]. Treatment with triptolide alone was shown to significantly inhibit cytoprotection and enhance lethality when used prior to heat shock [34]. Similar to quercetin, triptolide can also modulate the activity of other cellular proteins including the transcription factors NF-κB and AP-1 [35].

Two novel inhibitors of the HSR, NZ28 and emunin, have recently been identified by a two-step high-throughput screen which utilized a cell-based luciferase refolding assay in the first step, followed by a counterscreen for toxicity [24]. Although slight inhibition of HSF1 activity was seen after treatment with both compounds it was postulated that their predominant mechanism of action was at the post-transcriptional level [24].

Because of their effects on modulating molecular chaperone expression, HSR inhibitors exhibit considerable potential as anticancer agents for use in combination with existing cancer treatments, such as hyperthermia, which are compromised by the concomitant induction of the HSR. Proof-of-concept for this strategy has been demonstrated using quercetin pretreatment to potently amplify the effects of hyperthermia in two human prostate tumor models in vivo [36]. Potency and selectivity are properties that need to be optimized to allow agents to be taken forward for clinical development.

3. Direct modulators of molecular chaperone activity

Extensive work in recent years has focused on the identification and development of small-molecule inhibitors which directly target specific HSP family members [37,38]. This intense area of research has gained momentum as a result of early indications of success with HSP90 inhibitors. This has stimulated interest in developing agents which may have efficacy in specific disease states and in particular offer the potential for increased therapeutic benefit in the treatment of cancer through selective effects on tumor versus normal tissues. The following sections will focus on the progress to date in targeting two of the major HSP families, HSP70 and HSP90.

3.1. HSP70

The HSP70 family consists of a number of highly conserved proteins which range in size from 66 kDa to 78 kDa [39]. Generally, members are structurally similar, comprising an ~45 kDa actin-like N-terminal ATPase domain and an ~15 kDa substrate-binding domain [39]. An ~10 kDa C-terminal domain is the most variable between family members and is also the position of a C-terminal EEVD motif in the majority of HSP70 isoforms, which provides the site of interaction with
co-chaperones that contain tetracopeptide repeat (TPR) motifs [39]. The orchestrated interaction of co-chaperones with HSP70 fine-tunes the balance of ATP-hydrolysis and nucleotide exchange which are critical for substrate interaction, dictating the duration over which a substrate is associated with the molecular chaperone (Fig. 2a) [39].

HSP70 chaperones interact with almost all newly synthesized, unfolded proteins [39]. This is achieved by HSP70 binding to a hydrophobic binding motif which consists of a core of five amino acids enriched with hydrophobic residues [38,39]. This binding motif is exposed during protein synthesis; however, upon protein folding it becomes buried in the hydrophobic core of the mature protein only to be re-exposed if the protein has undergone an unfolding event. Therefore, it is not surprising that HSP70 chaperones are induced in order to prevent protein aggregation and facilitate protein re-folding or degradation in response to stressful stimuli [39]. In addition, HSP70 chaperones are involved in a number of other cellular processes, including transportation of molecules across membranes, disassembly of clathrin-coated vesicles and regulation of the HSR [39].

HSP70 chaperones are also intricately involved at several stages in regulating apoptosis [23]. The anti-apoptotic function of HSP70 chaperones has been reviewed extensively elsewhere [6,23,40]. Briefly, HSP70 can prevent the loss of mitochondrial membrane potential in response to apoptotic stimuli and hence block the release of apoptotic factors including cytochrome c and AIF. This has been suggested to be mediated via HSP70 preventing the pro-apoptotic protein BAX from undergoing the conformational change and translocation to the mitochondria which are essential for its function [41]. HSP70 can also function downstream of the mitochondria by interacting directly with APAF-1 or AIF to inhibit their function. In addition, HSP70 chaperones are potent inhibitors of caspase-independent cell death via their modulation of several stages of the pro-apoptotic JNK pathway [6,23,40].

Fig. 2. The HSP70 chaperone cycle and its modulators. (a) HSP70 in the ATP-bound form adopts an open conformation which allows the rapid association and dissociation of substrates. HSP40 promotes substrate binding by stimulating HSP70 ATPase activity. This induces a conformational change which prolongs HSP70 association with substrate. The ADP bound form of HSP70 is stabilized by HIP, which interacts directly with the ATPase domain of HSP70. During nucleotide exchange ATP replaces ADP allowing HSP70 to return to its open conformation and release the mature substrate. HSP70 activity can also be regulated by co-chaperones such as CHIP and BAG-1 which inhibit HSP40-stimulated ATPase activity or stimulate nucleotide exchange, respectively (for a comprehensive review see [39]). (b) Chemical structure of 15-DSG and its structural analog NSC-630668-R/1, both of which modulate HSP70 activity.
Cancer cells commonly display impaired apoptosis pathways which may contribute to tumor initiation and progression. Many human tumor cells express elevated levels of HSP70 and to some extent the co-chaperone HSP40, suggesting that cellular transformation causes induction of these chaperone proteins or conversely that HSP70 may play a role in tumorigenesis [6,23]. In addition, a number of cancer chemotherapeutic agents, including paclitaxel, doxorubicin and gemcitabine, have been shown to induce HSP70 expression, resulting in reduced cellular sensitivity to these agents [40]. For these reasons, HSP70 has emerged in recent years as a potential new target for the development of small-molecule inhibitors which may be used alone or in combination with existing therapeutic strategies in the treatment of cancer. However, agents which modulate HSP70 expression and/or activity may also have scope for the treatment of disease states other than cancer. Because of their role in nascent protein folding, intracellular localization and prevention of aggregation, it is not surprising that the HSP70 family has been implicated in various disease states which arise due to defects in protein folding or trafficking. HSP70 chaperones have been implicated in neurological conditions including Parkinson’s, Huntington’s and Alzheimer’s disease [3,4,38]. HSP70 and HSP40 chaperones interact with and modulate the aggregation of α-synuclein and mutant huntingtin, the proteins implicated in the pathogenesis of Parkinson’s and Huntington’s disease, respectively [42,43]. HSP70 isoforms can also interact with amyloid β-peptide and tau protein, the aberrant aggregation of which is involved in the development of Alzheimer’s disease [44]. As a consequence, overexpression of HSP70 and HSP40 has been shown in several cellular models of Huntington’s, Parkinson’s and Alzheimer’s disease to have potential therapeutic benefit (reviewed in [38,39]). HSP70 has also been implicated as a possible therapeutic target for the treatment of cystic fibrosis. This is based on the role of HSP70 and HSP40 in the biogenesis of the cystic fibrosis transmembrane conductance regulator (CFTR), the protein that leads to cystic fibrosis when mutated. Decreased expression of HSP70 was shown to reduce the association of HSP70 with mutant CFTR and improve the cellular degradation of the mutant protein [45].

3.1.1. HSP70-interacting compounds. Considering the possible therapeutic potential of modulating HSP70 activity, only a small number of compounds which target HSP70 have been described so far. One compound, 15-deoxyxyspergualin (15-DSG, Fig. 2b), is currently undergoing clinical evaluation as an immunosuppressive agent and binds to HSC70 (the constitutively expressed isoform of HSP70) with a modest $K_D$ of $\sim 4 \mu M$ [46]. 15-DSG mildly stimulates the ATPase activity of HSC70 by 20–40%, but does not affect synthetic peptide binding or HSP40-stimulated ATPase activity [46]. This compound interacts in vitro with the C-terminal EEVD motif of HSC70; however, it is currently unclear how this interaction modulates HSC70 ATPase activity [47]. Importantly, 15-DSG also binds to HSP90 with a similar $K_D$ to that for HSC70 [46]. Hence, given the role of HSP90 in several cellular processes (see below), it cannot be excluded that 15-DSG may function via HSP90 rather than HSC70 [38].

With the aim of identifying novel and potentially more potent HSC70 modulators, a large-scale in silico screen of 15-DSG analogs was conducted [48]. A number of structural analogs were identified including NSC-630668-R/1 (R/1, Fig. 2b), which inhibited the endogenous and HSP40-stimulated ATPase activity of yeast HSP70 [48]. As a consequence, R/1 prevented the translocation of a pre-protein into yeast-derived ER vesicles, an in vitro assay which is dependent on HSC70 and HSP40 [48]. R/1 may act as a peptide mimic by binding to the peptide-binding domain of HSC70 to induce oligomerization which would eventually lead to its inactivation [48]. Since the initial identification of R/1, several structurally related compounds have been screened for their ability to alter the ATPase activity of HSP70 [49]. A number of those identified were similar to R/1 and stimulated HSP70 ATPase activity [49]. Interestingly, two novel cell permeable compounds were recognized which had no effect on the endogenous activity of HSP70 but instead interfered with the ability of HSP40 to enhance HSP70-mediated ATP hydrolysis [49]. The two structural analogs of 15-DSG and R/1, MAL3-39 and MAL3-101, were also shown to compromise protein translocation into ER vesicles [49].

HSP70 activity can also be modulated via direct affects on the ATPase domain. 3'-Sulfogalactolipids, containing either a ceramide or a glycerolipid, bind to the ATPase domain of HSP70 [50]. These compounds inhibit the endogenous and HSP40-stimulated ATPase cycle of HSP70 chaperones; however, the functional consequences of this type of inhibition remain unclear.

Due to limited availability of small-molecules that inhibit HSP70 function, the search for peptide aptamers, which can inhibit the function of this chaperone by acting as a substrate mimic, has begun. This is exemplified by ADD70, a construct encoding the minimum region of the HSP70 substrate AIF (see earlier) required to interact with and thereby capture endogenous HSP70 [51]. ADD70 when used alone was not cytotoxic but did display chemosensitizing properties in vitro and in vivo [51]. In addition, using syngeneic rodent models, ADD70 was demonstrated to mediate an anti-tumor effect via a cell–cell specific immune response mediated by CD8+ T-cells [51]. Interestingly, ADD70 specifically interacted with the inducible isoform of HSP70 and not HSC70 [51]. This is of particular importance because the inducible isoform is often constitutively expressed in cancer cells but relatively undetectable in the absence of stress in normal cells, suggesting that this approach may achieve tumor cell versus normal cell selectivity and hence may have promising therapeutic potential [51].

3.2. HSP90

HSP90 is an abundant molecular chaperone which constitutes 1–2% of total cellular protein. It exerts its chaperone function to ensure the correct conformation, activity, intracellular localization and proteolytic turnover of a range of proteins that are involved in cell growth, differentiation and survival [5,7,37]. Because of the large number of interesting proteins with which HSP90 has been shown to associate, a rationale exists for the therapeutic use of HSP90 inhibitors for the treatment of a wide range of human diseases [52].

However, HSP90 has probably been most widely acknowledged as a therapeutic target for the treatment of cancer [5,6]. This is because HSP90 is essential for the stability and the function of many oncogenic client proteins, which contribute to all of the hallmark traits of malignancy [6,7,37]. These so-called ‘client proteins’ include ERBB2, BCR-ABL, AKT/PKB, C-RAF, CDK4, steroid hormone receptors (estrogen and
androgen), survivin and telomerase (for an up to date list see http://www.picard.ch/). Inhibition of HSP90 function causes degradation of client proteins via the ubiquitin–proteasome pathway, which results in the combinatorial down-regulation of signals being propagated via numerous signaling pathways and modulation of all aspects of the malignant phenotype [5,7,37]. Therefore, HSP90 inhibitors have potential to treat cancers driven by multiple molecular abnormalities and their combinatorial effects could also reduce the possibility of resistance developing.

Presently, five isoforms of HSP90 have been identified which differ in their cellular localization. The two major cytoplasmic isoforms are HSP90α and HSP90β which share approximately 85% sequence identity at the protein level. Other isoforms include GRP94 in the endoplasmic reticulum, TRAP1 in the mitochondrial matrix and HSP90N which has been linked to cellular transformation via its association with RAF [53]. All isoforms, except HSP90N, have a similar overall structure consisting of three domains: an N-terminal nucleotide exchange and ATPase domain, a middle domain implicated in client protein binding, and a C-terminal dimerization domain. They are considered to exert their chaperone function via a cycle which utilizes the coordinated interaction of a number of co-chaperone proteins that are collectively involved in an orchestrated, mutually regulatory interplay with ATP/ADP exchange and ATP hydrolysis by the intrinsic and essential N-terminal ATPase domain (Fig. 3). HSP90N differs from the other HSP90 isoforms in that its N-terminal is much shorter than in other isoforms and as a result does not contain the highly conserved ATPase domain [54]. In the following sections we discuss the various classes of HSP90 inhibitors which have emerged in recent years and the novel approaches which are currently being investigated to inhibit HSP90 function.

### 3.2.1. Natural product inhibitors of HSP90

The 14-membered macrocyclic antibiotic radicicol (monorden, Fig. 4) was isolated from Monosporidium bonorden [55]. Radicicol was demonstrated to have anti-tumor activity in vitro and shown to reverse the malignant phenotype of v-SRC transformed cells [56]. Because of this radicicol was originally believed to be a tyrosine kinase inhibitor; however, it was subsequently shown using X-ray crystallography to bind to the N-terminal ATP-binding pocket of HSP90 with high affinity [57], resulting in the degradation of a number of signaling proteins [58]. Radicicol binds to all HSP90 isoforms except for HSP90N [58]. However, the strength of the interaction varies between family members with radicicol binding to HSP90α and HSP90β with 5- and 10-fold greater affinity than to GRP94 or TRAP1, respectively [58]. Although radicicol inhibits tumor cell growth in vitro, it lacks activity in vivo, most likely due to its potentially reactive epoxide moiety and other adverse chemical features that cause instability and possible toxicity [37,40]. Many oxime derivatives of radicicol have been synthesized (including KF25706 and KF58333) that retain the capacity to inhibit HSP90 function but also exhibit therapeutic activity in human tumor xenograft models [59].

The benzoquinone ansamycins are a second class of naturally occurring antibiotics which have been demonstrated to inhibit the activity of HSP90 (Fig. 4). A leading example is geldanamycin which competes with ATP for binding to the N-terminal nucleotide binding site of HSP90 [60]. Geldanamycin interacts with HSP90α, HSP90β, GRP94 and TRAP1 with similar affinities [61]. However, despite promising anti-tumor

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Fig. 3. Model of the HSP90 chaperone cycle. The client protein initially binds to a HSP70/HSP40 complex which is then transferred onto the ADP-bound HSP90 via the TPR co-chaperone HOP. When ADP is exchanged for ATP, HSP90 undergoes a conformational change which releases HSP70/HSP40 and HOP, allowing the ATP-dependent association of other co-chaperones including P23, p50CDC37 or the immunophilins (IP). The exact complement depends on the type of client protein. This is collectively referred to as the mature complex and it is here that the client protein adopts its active conformation. Other co-chaperones include AHA1 which increases the ATPase activity of HSP90. Inhibition of ATP-binding prevents the formation of the mature complex, resulting in the proteasome-dependent degradation of associated client proteins, possibly via the recruitment of the E3 ubiquitin ligase CHIP (reviewed in [93]).
activity in vitro and in vivo, the progress of geldanamycin into the clinic was stopped due to compound instability and unacceptable hepatotoxicity at therapeutic doses [62]. Further analogs were developed with the objective of finding agents with an improved therapeutic window for clinical use, including the derivative 17-allylamino-17-demethoxygeldanamycin (17-AAG or tanespimycin, Fig. 4) [63]. 17-AAG has similar cellular effects to geldanamycin, including client protein degradation, cell cycle arrest and apoptosis but with improved metabolic stability and lower toxicity [62]. Preclinical studies using 17-AAG have shown this derivative to be highly potent in vitro and to exhibit anti-tumor activity at non-toxic doses in various human tumor xenograft models [64,65]. The potency of 17-AAG in vitro has been attributed in part to its increased uptake and preferential accumulation in cancer cells, together with enhanced binding to the HSP90 super-chaperone complex that is reported to predominate in cancer but not normal cells [66,67]. Based on its biological activity, 17-AAG has recently completed several phase I clinical trials at numerous centers in the US and UK. Various dose and scheduling strategies were examined which have been discussed in detail elsewhere [52]. Doses of 17-AAG administered were well-tolerated and resulted in good pharmacokinetic exposures and pharmacodynamic responses, as demonstrated by the molecular signature of increased expression of HSP70 and decreased levels of client proteins C-RAF and CDK4 [68]. Prolonged stable disease in two patients with metastatic malignant melanoma was reported [68] along with unpublished reports of activity in prostate, breast and multiple myeloma. As a result of favorable responses during the phase I clinical trial, 17-AAG has now entered phase II clinical trial for use as a single agent therapy in various tumor types, including melanoma and breast.

Despite promising clinical activity, there are several possible factors which may reduce the clinical efficacy of 17-AAG. Preclinical studies have shown that hepatic metabolism of 17-AAG by cytochrome P450 leads to the formation of 17-amino-17-demethoxygeldanamycin (17-AG) [64]. Although 17-AG retains inhibitory activity, metabolism by CYP3A4 is likely to be a cause of variable pharmacokinetics. In addition, the activity of 17-AAG is enhanced by its conversion to the hydroquinone form, 17-AAGH2, by the reductase enzyme NQO1 or DT-diaphorase [64,69]. The polymorphic expression of both of these metabolic enzymes may pose limitations for the clinical use of 17-AAG across the population [7,37,64]. The efficacy of 17-AAG may be further reduced by its association with the multi-drug resistance protein MDR1 or P-glycoprotein [64]. Finally, 17-AAG is limited by its poor solubility, cumbersome and complex formulation and lack of oral bioavailability. Attempts to reformulate 17-AAG have resulted in clinical trials commencing with CNF1010 (http://www.biozincidec.com/) and a cremaphore-based formulation (KOS-953, http://www.kosan.com/), the latter of which has shown promising results during the phase I trial in patients with relapsed/refractory myeloma. The US National Cancer Institute and Kosan Biosciences have also developed a more water soluble and potentially orally bioavailable analog of 17-AAG, 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG or alvespimycin, Fig. 4), which is currently under preclinical and clinical evaluation [52]. 17-AAGH2, also known as IPI-504, has also entered clinical trial as a soluble derivative of 17-AAG [70].

3.2.2. Pyrazoloisoxazole resorcinols. High-throughput screening at our institution identified for the first time HSP90 inhibitors with a novel 3,4-diarylpyrazole resorcinol

Fig. 4. Chemical structures of selected HSP90 inhibitors.
scaffold. These were exemplified by the hit compound CCT018159 (Fig. 4) which inhibits the N-terminal ATPase of yeast and human HSP90 with an IC\textsubscript{50} of 7.1 μM and 3.2 μM, respectively [71]. Treatment of cancer cells with CCT018159 resulted in HSP70 induction, client protein depletion, cytostasis and apoptosis [71,72]. The pyrazoles have good solubility and, unlike 17-AAG, the cellular activity of CCT018159 was shown to be relatively independent of DT-diaphorase and P-glycoprotein [72]. Building on the X-ray crystal structure of human HSP90α in a complex with CCT018159, rational drug design was used by ourselves in collaboration with Vernalis to identify structural analogs with enhanced, nanomolar potency. This led to the identification of the 5-amide analog CCT0129397/VER-49009 (Fig. 4), which exhibited similar cellular properties to 17-AAG with the potency and potential for clinical development [73]. The resorcin isoxazole amide analog VER-50589 was recently reported to be the most potent synthetic small-molecule HSP90 inhibitor yet identified (K\textsubscript{D} 5 nM) and to show proof-of-concept for in vivo anti-tumor activity in an animal model [74].

3.2.3. Purines. Rational drug design was used by Chiosis et al. [75] to develop a novel class of HSP90 inhibitors with a purine-scaffold (Fig. 4). The first compound to be identified from this series, PU3, bound to HSP90 with moderate affinity resulting in cellular effects which are characteristic of HSP90 inhibitors [75]. An important feature of PU3 is that it is more soluble than 17-AAG; however, it is also significantly less potent against cells than the ansamycins [75]. Subsequent efforts focused on improving the potency of PU3 and led to the identification of PU24FC1 [76]. This compound exhibited biological effects on cells within a concentration range of 2–6 μM [76], and also demonstrated 10–50 times higher affinity for HSP90 from transformed cells compared to that from normal tissues [76]. Administration of PU24FC1 in human breast tumor xenograft models led to anti-tumor activity without significant toxicity [76]. A more recent study has identified 8-aryl sulfanyl, 8-arylsulfoxyl and 8-arylsulfonyl adenine derivatives of the PU class which exhibit improved water solubility and approximately 50 nM potency in cellular models, together with therapeutic activity in human tumor xenograft models [77].

3.2.4. Novobiocin. Novobiocin (Fig. 4) belongs to the family of coumarin antibiotics which bind to and inhibit bacterial DNA gyrase to block bacterial DNA synthesis. This compound is unique because unlike the HSP90 inhibitors described so far it is believed to inhibit the activity of the chaperone by interacting with a proposed cryptic ATPase domain within the C-terminal rather than N-terminal domain of HSP90 [78]. Despite the different binding site, cells exposed to novobiocin demonstrated HSP90 inhibition and client protein degradation in vitro and in vivo [78]. This was subsequently shown to be due to disruption of the interaction between HSP90 and its co-chaperones HSC70 and P23 [79]. However, in an effort to overcome the relative weak ability of novobiocin to inhibit HSP90 function and induce client protein degradation more potent analogs have been identified from a library of structural derivatives, with the most active being compound 4a (Fig. 4) [80].

3.2.5. Inhibition of HSP90 using peptide mimetics. Shepherdin is a novel peptidomimetic modeled on the region which is critical for the interaction of HSP90 with its anti-apoptotic and mitotic substrate, survivin [81]. Shepherdin mimics the survivin sequence I74-L87 and makes contact with the N-terminal ATP-binding pocket of HSP90 to cause inhibition of chaperone activity, degradation of client proteins and induction of cell death via apoptotic and non-apoptotic mechanisms [81]. Similar to the ansamycin and purine classes of HSP90 inhibitor, shepherdin showed significantly increased toxicity in tumor cells versus normal cells in vitro. Possible therapeutic selectivity was supported by in vivo observations of efficacy without toxicity following systemic administration using human tumor xenograft models [81]. A recent study has demonstrated that exposure to shepherdin resulted in dramatic cell death of acute myeloid leukemia cells in vitro and in tumor xenografts by inhibiting HSP90 function, decreasing HSP90 client proteins and disrupting mitochondrial function [82].

A novel, non-peptidic small-molecule, 5-aminimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR, Fig. 4), was designed to bind to HSP90 by mimicking the chemical and conformation properties of shepherdin bound to the N-terminal domain of HSP90 [83]. AICAR causes client protein degradation in vivo and exhibits anti-proliferative and pro-apoptotic activity in a number of tumor cell lines, but not non-tumorigenic cells [83].

3.2.6. Inhibitors of specific HSP90 isoforms. Specific isoforms of the HSP90 family have been associated with unique functions and tumor-specific expression (reviewed in [53]). Therefore, it may be therapeutically beneficial to target individual isoforms of this chaperone family. 5′-N-ethylcarboxamidoadenosine (NECA, Fig. 4) is an example of a compound which demonstrates selectivity by binding to GRP94 with a K\textsubscript{D} of 200 nM [84]. NECA does not bind to the other HSP90 isoforms and it is currently believed that a five amino acid insertion unique to within the N-terminal domain of GRP94 extends a helix to form an additional pocket adjacent to the nucleotide binding cavity which allows for this specific interaction [85]. Further studies using this compound and other methods to specifically reduce individual isoform expression/activity are required to determine if isoform-selective inhibitors could be an effective approach to increase therapeutic benefit.

3.2.7. Agents that modulate the post-translational modification of HSP90. HSP90 function can be modulated by a series of post-translational modifications including acetylation, ubiquitination and S-nitrosylation [37,86]. HSP90 acetylation has recently been refined to a specific lysine residue (K294) within the middle domain of the chaperone [87]. When acetylation of this residue was mimicked, a substantial decrease in client protein binding and the association of some but not all co-chaperones was observed [87]. Histone deacetylase (HDAC) inhibitors have been developed to target epigenetic modification of gene transcription through the hyperacetylation of cellular proteins. Following exposure to HDAC inhibitors such as LAQ824 and depsipeptide FK228, HSP90 becomes acetylated which inhibits ATP binding leading to client protein degradation both in vitro and in patients sampled as part of a Phase I clinical trial [88].

3.2.8. Other HSP90 inhibitors. Mycocgrab, developed by NeuTec Pharma Ltd. (http://www.neutecpharma.com/), is a human recombinant protein which recognizes the middle domain of fungal HSP90. Clinical data have shown that the development of HSP90 antibodies is related to patient recovery
from systemic candida [89]. Mycograb has been designed for use in combination with the anti-fungal drug amphotericin B and clinical trials are currently underway, with preliminary results indicating an 84% response rate in patients with systemic candidiasis [89]. An application for Mycograb in the treatment of cancer has been proposed based on the identification of HSP90 on the surface of certain cancer cell types [37,52].

Gene expression-based analysis was used to identify a new class of HSP90 inhibitor [20]. Using this approach celastrol and gedunin (described earlier) were shown to have a gene expression profile similar to that of existing HSP90 inhibitors [20]. Treatment of cells with celastrol and gedunin resulted in degradation of HSP90 client proteins including EGFR, BCR-ABL, androgen receptor and FLT3 [20]. Both celastrol and gedunin were found to inhibit HSP90 ATPase activity in the cell [20]. However, unlike the majority of conventional HSP90 inhibitors, neither of these compounds were found to interact with the N-terminal ATP-binding pocket of HSP90, suggesting a novel mode of action [20]. The exact mechanism of action of celastrol is currently under debate. Previous studies have shown celastrol to have potent in vitro and in vivo activity by acting as a proteasome inhibitor [19]. As described above, HSP90 activity can be modulated by ubiquitination; therefore, it is necessary to determine if celastrol could be inhibiting its chaperone function via effects on protein degradation.

3.2.9. Novel approaches to inhibit HSP90 function. So far this review has focused mainly on inhibiting HSP90 function by targeting the N-terminal ATPase domain of this chaperone directly. However, as described earlier, HSP90 functions as part of a super-chaperone complex which requires the interaction of a number of accessory co-chaperones. An increasing amount of evidence has emerged concerning the importance of these co-chaperones and the possibility of targeting them individually as a novel approach to inhibiting HSP90 function.

One example of an HSP90 co-chaperone is AHA1 (activator of HSP90 ATPase), which was identified as a stress-regulated protein that activates the essential, intrinsic ATPase of HSP90 to promote client protein maturation [90]. In particular, ε-SRC and glucocorticoid receptor have been shown to be dependent on AHA1 expression, with their activities being significantly reduced following the reduction of AHA1 expression using a variety of techniques [91]. In addition, AHA1 plays a critical role in the trafficking of the mutant form of the cystic fibrosis transmembrane conductance regulator, ΔF508, with decreased expression of AHA1 resulting in the cell surface rescue of the protein from the ER [92]. In addition, data from our own laboratory have shown that reducing the expression of AHA1 can directly affect the activity of HSP90 as demonstrated by reduced signaling via the RAS-RAF-MEK-ERK1/2 and PI3K-AKT/PKB pathways (J. Holmes, S. Sharp and P. Workman, unpublished observations). These data collectively suggest that AHA1 may be an interesting drug target with potential inhibitors being used to modulate specific activities of HSP90.

HSP70 has already been discussed as another example of a well-documented HSP90 co-chaperone. HSP70 is involved in substrate-loading onto HSP90 (Fig. 3). In our laboratory, we have reduced the expression of HSC70 and HSP72 using an siRNA approach. This resulted in HSP90 client protein deple-
tion, cytotasis and apoptosis in human colon and ovarian cell lines, the extent of which was greater than observed following treatment with 17-AAG [7].

AHA1 and HSP70 isoforms are just two examples of co-chaperones which could potentially be targeted to disrupt HSP90 function. Our laboratory, and others, are currently investigating the effects of targeting other co-chaperones including p50CDC37, P23 and CHIP.

4. Combining different modulators of the HSR

The therapeutic potential of targeting the HSR may be improved by using more than one agent to specifically target different aspects of this pathway. For example, a well known response to HSP90 inhibition is the induction of HSPs, including HSP90β, HSP72 and HSP27 [16,17]. All three chaperones have a well-documented anti-apoptotic function which may reduce the sensitivity of cells to the cell death effects of HSP90 inhibitors. In addition, increased expression of the target itself may contribute to the development of resistance to these agents [16]. Therefore, it may be of benefit to combine HSP90 inhibitors with agents which modulate the activity of these chaperones directly or those which can prevent the induction of these chaperones, e.g. HSFI inhibitors. This has been investigated using siRNA to reduce the expression of HSP72 prior to treatment with 17-AAG and by the pretreatment of cells with the HSFI inhibitor KNK437 [32]. Both approaches significantly enhanced the sensitivity of leukemia cell lines to the effects of HSP90 inhibitors [32]. Similarly, inhibition of HSP72 function by expressing the peptidomimetic of AIF, ADD70, prior to treatment with 17-AAG has been shown to increase the sensitivity of cancer cells in vitro and in vivo using syngenic animal models [51]. Finally, preventing the induction of HSP27 using siRNA techniques has been shown to increase the sensitivity to 17-AAG and to reverse resistance to this HSP90 inhibitor [17]. These data collectively suggest that if small-molecule inhibitors could be designed to modulate different aspects of the HSR, not only may they have therapeutic potential in their own right, but they might also improve patient response to existing modulators.

5. Concluding remarks

The HSR and the associated molecular players are emerging as important potential targets for drugs that may be used in the treatment of cancer and other conditions in which protein-folding and protein quality control play a key role in disease pathology. Proof-of-concept for the approach of modulating protein quality control is provided by the approval of bortezomib in multiple myeloma [9], 17-AAG was the first HSP90 molecular chaperone inhibitor to enter clinical trial and shows early promise as a result of its action on multiple oncoproteins and all of the hallmark traits of malignancy. This has generated a high level of interest in industry and academia to develop additional HSP90 ATPase inhibitors. A number of these are now entering the clinic or are in late stage preclinical development, including not only other geldanamycin analogs but also small-molecule synthetic inhibitors. Pharmacological agents that interfere with the HSR through other mechanisms
are also undergoing detailed scrutiny. The antibody Myocet, which recognizes fungal HSP90, shows promise in the treatment of systemic candidiasis and may also have potential in cancer. Given the alternative molecular approaches that could potentially be used to tackle HSP90 and other molecular chaperones, and considering the number of potential diseases in which therapeutic modulation of the HSR, molecular chaperones and protein folding is indicated, this area looks set to be a very exciting one over the next few years.

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