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Shelli R. McAlpine
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Heat Shock Protein Inhibitors

Success Stories

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19
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Editors

Heat Shock Protein Inhibitors

Success Stories

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Preface

Protein folding and degradation are key cellular processes that must be carefully regulated in the crowded and compartmentalised cellular environment. The physiological process of proteostasis must also be maintained in times of stress, where an additional burden is placed on cells due to alterations in protein structure and function. The ability of the cell to maintain appropriate protein homeostasis under both physiological conditions and during cellular upheaval is largely dependent on a phenomenon known as the stress response. The stress response is an evolutionarily conserved and often predictable response that results in the upregulation or activation of a cohort of proteins, known collectively as molecular chaperones that serve to ameliorate the consequences of protein misfolding. Selected members of the heat shock proteins (e.g. Hsp90 and Hsp70) represent the largest and best-characterised family of molecular chaperones.

Many heat shock proteins (Hsps) function as molecular chaperones, regulating a range of processes associated with protein homeostasis, including protein folding, aggregation suppression and protein degradation. The activities of these Hsps are finely tuned and usually driven by the formation of complexes with cofactors. Understanding the mechanistic details by which these Hsps function as molecular chaperones has led to their analysis in human diseases. Hsps have been implicated in either the aetiology or prevention of many human diseases, ranging from cancer to Alzheimer's and infectious diseases. Hsps have been identified as putative drug targets for therapeutic intervention. In this book, the authors provide critical insight into the identification and development of inhibitors against selected Hsps as future therapies for human disease.

Specifically, topics include discussions on Hsp90, Hsp70, Hsp47, Hsp40 and Hsp27. Describing inhibitors that modulate Hsp90 or Hsp70 or a combination of these two inhibitors provides an overview of the most recent drugs targeting these Hsps. Finally, these chapters provide insight into potential new routes for modulating these Hsps. However, despite these success stories, there are currently no Hsp inhibitors that have completed clinical trials and are in routine use for treating patients. As discussed within these chapters, there are opportunities to develop new

molecular inhibitors for these therapeutically relevant Hsps. The wide range of Hsps and their functional control of the cell make these chaperones highly relevant to all therapeutic areas.

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Targeting the C-Terminus of Hsp90 as a Cancer Therapy

Jeanette McConnell, Yao Wang, and Shelli McAlpine

Abstract Classical Hsp90 inhibitors target the N-terminal ATP binding site. While these inhibitors have had some clinical success, treatment with these molecules leads to a dramatic increase in a set of stress-related proteins, a response that is referred to as a heat shock response. The induction of a heat shock response protects the cell against the protein aggregation induced by inhibiting Hsp90 and slows down cell death. Alternatively, inhibiting Hsp90 by modulating the C-terminus does not lead to a heat shock response. Current efforts to inhibit Hsp90's C-terminus include molecules derived from natural products and mimics of native Hsp90-binding proteins. This diverse effort toward new C-terminal modulators of Hsp90 and their promising biological profile suggests that this strategy is likely the most productive future for targeting Hsp90.

Keywords ATP binding inhibitors, Cancer, C-terminus, Heat shock proteins, Hsp90, Natural products, Natural product small molecules

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1 Introduction

Recent knowledge in cancer biology has produced a new chemotherapy approach. Instead of using broadly toxic drugs, new cancer chemotherapies are often focused on a single molecular target, with the goal that only this target is inhibited [1]. Therapies focused on a single target have had numerous successes and include (a) drugs targeting human epidermal growth factor receptor 2 (HER2), (b) hormone therapies, and (c) epidermal growth factor receptor (EGFR) inhibitors [1]. Heat shock protein 90 (Hsp90) is one molecular target being clinically tested for its effectiveness as a cancer therapy. Hsp90 is a molecular chaperone broken down into three domains with distinct functions. The amino (N) domain is responsible for binding ATP, the middle (M) domain binds many of Hsp90's client proteins, and the carboxy (C) domain contains the site of homodimerization essential for protein function. The Hsp90 protein is a highly effective anticancer target because it plays a key role in many oncogenic pathways, promoting the growth and survival of cancer cells. By inhibiting Hsp90, multiple oncogenic pathways are likely to be shut down simultaneously, decreasing the likelihood of cancer cell survival [2–4].

The first Hsp90 inhibitor, geldanamycin (GA), was identified in 1994, and its derivative, 17-*N*-allylamino-17-demethoxygeldanamycin (17-AAG), entered clinical trials as an anticancer therapy in 1999 [5]. Since then, Hsp90 inhibitor drug candidates have been steadily entering clinical trials, with a total of 15 different drugs entering the clinic since 1999 [6, 7]. A wide variety of cancer types have been treated using Hsp90 inhibitors, including breast cancer, non-small cell lung cancer (NSCLC), melanoma, renal cell carcinoma (RCC), multiple myeloma (MM), gastrointestinal stromal tumor (GIST), castrate-resistant prostate cancer (CRPC), and several types of leukemia [6, 7]. However, clinical trial results showed that when used as single agents, these Hsp90 inhibitors have not been effective, and they have faced significant side effects [8].

Three exceptions to the ineffectiveness of single-agent Hsp90 inhibitors were observed in patients when treatments involved (a) 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) to treat CRPC, melanoma, or acute myeloid leukemia, (b) ganetespib to treat breast cancer and NSCLC, and (c) IPI-504 to treat NSCLC and GIST (Fig. 1) [9, 10]. However, patient responses were modest, where 17-DMAG caused a response in only 7% of HER2+ breast cancer patients, ganetespib caused a partial response in only 9% of breast cancer patients, and IPI-504 caused a partial response in just 7% NSCLC patients and 3% of GIST patients [7, 10, 11]. 17-DMAG was also reported to cause a single complete response in CRPC, three complete responses in acute myeloid leukemia, and one

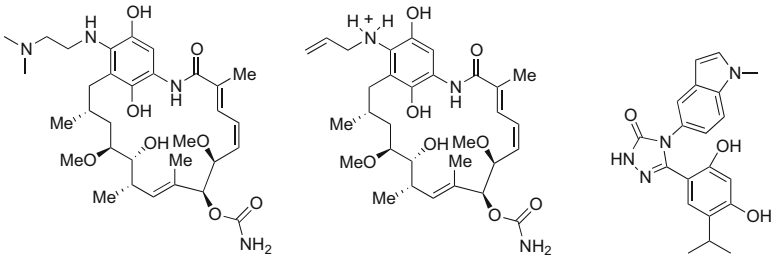

Fig. 1 Structure of Hsp90 inhibitors that have had moderate success as single-agent therapeutics in the clinic. 17-DMAG, IPI-504, and ganetespib

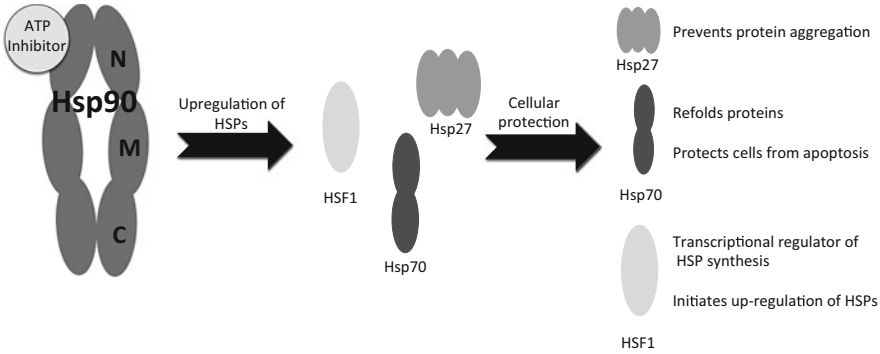

Fig. 2 Depiction of the cytoprotective consequences of ATP binding site Hsp90 inhibitors. All N-terminal ATP binding site inhibitors induce elevated levels of heat shock proteins leading to cellular protection. The key players and their role in cellular protection are depicted here

partial response in melanoma [8, 11]. While these few Hsp90 inhibitors have had some positive clinical benefit for patients, the low response rate is a significant concern.

In addition to the limited effectiveness of single-agent Hsp90 inhibitors, hepatotoxicity, ocular toxicity, and in one case mortality have caused the suspension of many clinical trials involving Hsp90 inhibitors [12, 13]. Indeed, despite their limited success, both 17-DMAG and IPI-504 have been removed from the clinic (clinicaltrials.gov). The ineffectiveness of these Hsp90 inhibitors may be due to a resistance mechanism that is activated immediately upon treatment. It is well understood that all of the clinical Hsp90 inhibitors target the ATP binding site of Hsp90, located in the N-terminal domain of the protein (Fig. 2). Inhibiting Hsp90 at this site initiates a heat shock response (HSR), which ultimately causes cellular protection (Fig. 2) [14–20].

There are currently two approaches being taken to address the cellular protection caused by induction of the heat shock response in patients after treatment with N-terminal Hsp90 inhibitors. The first, which is already being used in the clinic, is

combining Hsp90 inhibitors with other anticancer drugs, including several kinase inhibitors (clinicaltrials.gov). Indeed, clinical trial data demonstrate that Hsp90 inhibitors that target the N-terminus of Hsp90 are most effective when combined with other forms of therapy [7]. The second approach, and the focus of this chapter, is the development of Hsp90 inhibitors that target sites other than the ATP binding pocket of Hsp90. Specifically, data has demonstrated that inhibiting activity at the C-terminus of Hsp90 does not induce a heat shock response, nor does it lead to the upregulation of the heat shock proteins [15–24]. Thus, it is possible that targeting the C-terminus may succeed in a clinical setting as a single treatment.

The development of mechanistically unique Hsp90 inhibitors will also allow for combinations of multiple types of Hsp90 inhibitors, where the advantages of using different Hsp90 inhibitors together would include more effective inhibition of Hsp90 and a reduction in the cytoprotective heat shock response. However, first Hsp90 inhibitors that modulate other sites of the Hsp90 protein must be developed. This chapter discusses the status of targeting Hsp90 at sites other than the N-terminal ATP binding pocket.

2 Small Molecule Inhibitors of Hsp90

2.1 The Problem with Targeting the N-terminal ATP Binding Site as a Cancer Therapy

The disappointing clinical results of Hsp90 inhibitors as anticancer drugs are connected to increased levels of heat shock proteins 70 (Hsp70) and 27 (Hsp27) and heat shock factor 1 (HSF1) [25–27]. The induction of these heat shock proteins produces the undesirable effect of counteracting the efficiency of Hsp90-based treatment, and it has been identified as a hallmark of Hsp90 inhibitors that target the N-terminus (Figs. 2 and 3) [28–38]. On the other hand, molecules that modulate the C-terminus do not induce HSF1 nor overexpression of Hsp70 and Hsp27 (Fig. 3) [15–21, 23, 24, 39–43]. Thus, these molecules represent a promising new way to inhibit Hsp90 without simultaneously inducing the protective stress response.

There are several rescue mechanisms activated when targeting Hsp90 in addition to the heat shock response. First, induction of HSF1 supports malignant cancers by driving transcription of proteins that protect the cell, separate from the heat shock proteins [44]. HSF1 is also critical for tumor progression [45–47]. Second, Hsp70 can facilitate protein folding, prevent protein aggregation, and regulate protein complex assembly or disassembly alleviating some of the damage caused by Hsp90 inhibition [48, 49]. Third, Hsp70 actively participates in the protection of cancer cells from both extrinsic and intrinsic apoptosis [48]. Ectopic overexpression or induced endogenous levels of Hsp70 promote cancer cell survival by effectively inhibiting lysosomal membrane permeabilization [50], initiation of the death

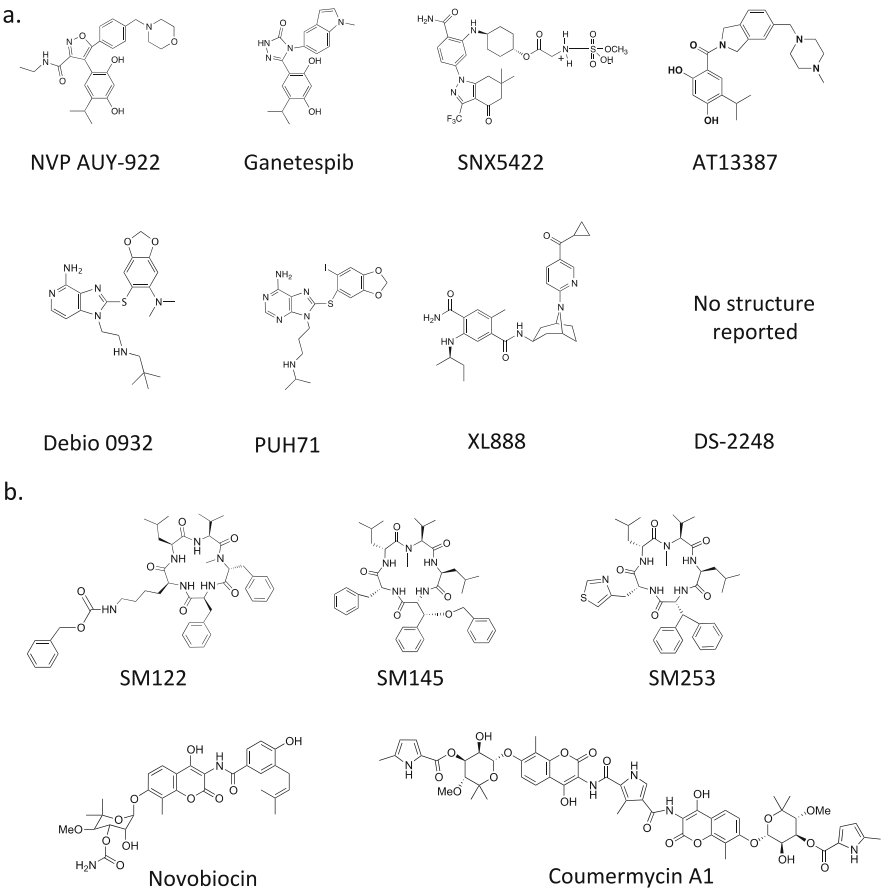

Fig. 3 (**a**) Structures of current Hsp90 inhibitors in clinical trials, all targeting the ATP binding site at the N-terminus. (**b**) Structures of molecules that modulate the C-terminus of Hsp90 inhibitors (SM122, SM145, and SM253; novobiocin and coumermycin A1)

receptor pathway [51], mitochondria-initiated signaling for caspase-dependent apoptosis [52–57], as well as apoptosis-inducing factor (AIF)-associated caspase-independent apoptosis [58, 59]. Fourth, induction of Hsp27 activates other resistance mechanisms, thereby facilitating the rescue of multiple cancer types upon the increase in Hsp27 levels [60–65].

2.2 Small Molecules that Block Access to the C-Terminus of Hsp90

The use of natural products as drug discovery leads is a well-known process and has led to many successful drugs [66]. Currently, there are two classes of molecules that

inhibit Hsp90 function without inducing high levels of Hsp70, Hsp27, or HSF1: cyclic peptide analogs (SM122, SM145, and SM253) and coumarin antibiotic derivatives (Fig. 3). The coumarin antibiotics directly bind to the C-terminus of Hsp90 and disrupt its function. There are two main coumarin derivatives that inhibit Hsp90, coumermycin A1 and novobiocin (Fig. 3). While these two molecules are related, they have different impacts on Hsp90. Coumermycin A1 disrupts the formation of the Hsp90 dimer, where a 50% reduction in dimerization was seen with 0.4 mM of coumermycin A1 [22]. Disrupting Hsp90 dimerization prevents binding between Hsp90 and several important C-terminal co-chaperones that contain a tetratricopeptide repeat (TPR) domain (CHIP, Tom70, CYP40) (where coumermycin A1 has an IC_{50} of ~5 μM [23]). When HeLa cells were treated with coumermycin A1 (5 μM), there was a decrease in the amount of glucocorticoid receptor and a slight reduction in the immunophilin FKBP52 [22, 23]. Unlike when using an N-terminal inhibitor, these inhibitory effects were not paired with induction of a heat shock response. Indeed, coumermycin A1 did not change Hsp70 levels and caused a 50% reduction in both Hsp90 and HSF1 protein levels [22, 23]. There has been some effort made toward creating new derivatives of coumermycin A1, and while one derivative caused a reduction in HER2 levels, the effect on Hsp90's interaction with TPR-containing protein or the levels of heat shock proteins has not been investigated [67].

In addition to coumermycin A1, the coumarin antibiotic novobiocin disrupts the function of Hsp90. However, novobiocin is not as potent as coumermycin A1. This is reflected in the almost 100-fold higher concentration required to disrupt the binding of TPR-containing proteins (novobiocin IC_{50} ~3 mM). Novobiocin also disrupts a different set of TPR proteins (CYP40, PP5, FKBP51/FKBP52), suggesting that the two coumarin derivatives are affecting Hsp90 in different ways, despite being from the same family of antibiotics. Unlike coumermycin A1, much work has been done investigating the SAR of novobiocin and creating new derivatives [21, 42, 68]. Two of the best derivatives created are known as F-4 and KU174 (no structures published). These molecules have a better binding affinity for Hsp90 than novobiocin (K_d novobiocin = 211 μM, F-4 = 100 μM and KU174 = 80 μM). In addition, they caused the degradation of many key Hsp90 client proteins, including Akt and HER2 without a subsequent increase in heat shock proteins [21, 68]. However, their effect on the Hsp90's C-terminal interactions has not been investigated. Using the coumarin antibiotics as a starting point for Hsp90 inhibition is a valid strategy for creating new Hsp90 inhibitors, and further investigation of their biochemical impact on Hsp90 would provide valuable insight.

Unlike the coumarin derivatives that directly block the C-terminus of Hsp90, the SM analogs block access to the C-terminus via an allosteric mechanism. Binding to the N-middle domain of Hsp90, the SM derivatives impact the conformation of Hsp90 so that the C-terminal end (MEEVD region) is no longer accessible to bind to TPR proteins. Three SM molecules (SM122, SM145, and SM253) have demonstrated the ability to block TPR-containing proteins (Fig. 4) [18, 23, 39, 69, 70]. SM122 blocks access between Hsp90 and HOP, as well as between Hsp90

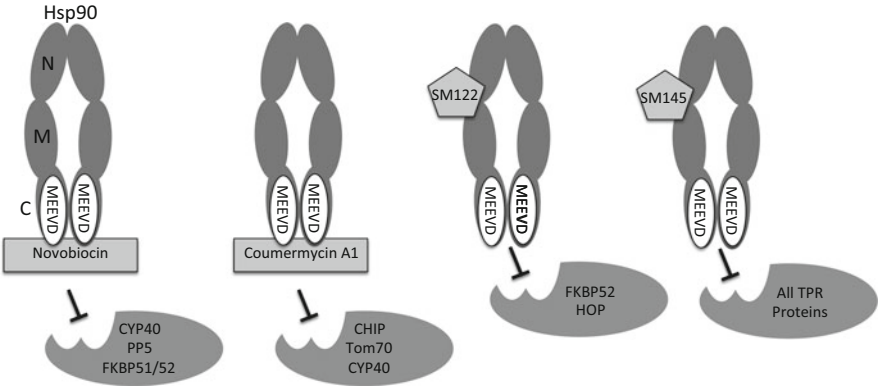

Fig. 4 Diagram depicting C-terminal-binding compounds novobiocin and coumermycin A1 as well as N-middle-binding compounds SM122 and SM145 blocking the interaction of various TPR-containing proteins with the C-terminus of Hsp90

and FKBP52, with an IC_{50} ~1 μM. SM145 blocked nine TPR-containing proteins from binding to Hsp90 with an IC_{50} = ~0.5 μM including HOP, CYP40, FKBP38, FKBP51, FKBP52, Tom70, CHIP, and UNC45 (note: PP5 did not reach 50% but was inhibited from binding to Hsp90 by 30% at 0.5 μM) [23].

Thus, the SM derivatives are significantly more potent inhibitors of the TPR-Hsp90 interaction than the coumarin antibiotics (>tenfold), and SM145 inhibits a much wider range of proteins than either SM122 or the coumarin derivatives. In addition, both SM122 and SM145 inhibit Hsp90-mediated protein refolding, as well as cause a decrease in important Hsp90 client proteins and co-chaperones, while not inducing the heat shock response [18, 23, 70]. Overall, these SM derivatives represent the under-investigated idea that targeting Hsp90 via a site other than the N-terminus is important, and it is likely that non-N-terminal inhibitors will be critical to the success of Hsp90 inhibitors in the clinic.

SM122 was investigated in an in-depth study and compared to a classic Hsp90 inhibitor, 17-AAG (Fig. 5) [16, 18, 19]. Both inhibitors 17-AAG and SM122 were evaluated at several points during the heat shock response pathway (Fig. 5). SM122 was the first C-terminal modulator used to show that inhibiting Hsp90 via modulating the C-terminus did not initiate the heat shock response via production of mRNA encoding for Hsp70 or Hsp27 or the translation and protein production of the heat shock response proteins [16, 18, 19]. The mRNA levels of Hsp70 and Hsp27 increased when cells were treated with 17-AAG, whereas mRNA levels went down for these two proteins when cells were treated with SM122. Translation of mRNA into protein was not impacted when cells were treated with 17-AAG; however, it was completely inhibited when cells were treated with SM122. Finally, Hsp70, Hsp27, and HSF1 protein levels dropped dramatically (tenfold below control) when cells were treated with SM122; however, they increased dramatically when cells were treated with 17-AAG (fivefold over control) [16, 18, 19].

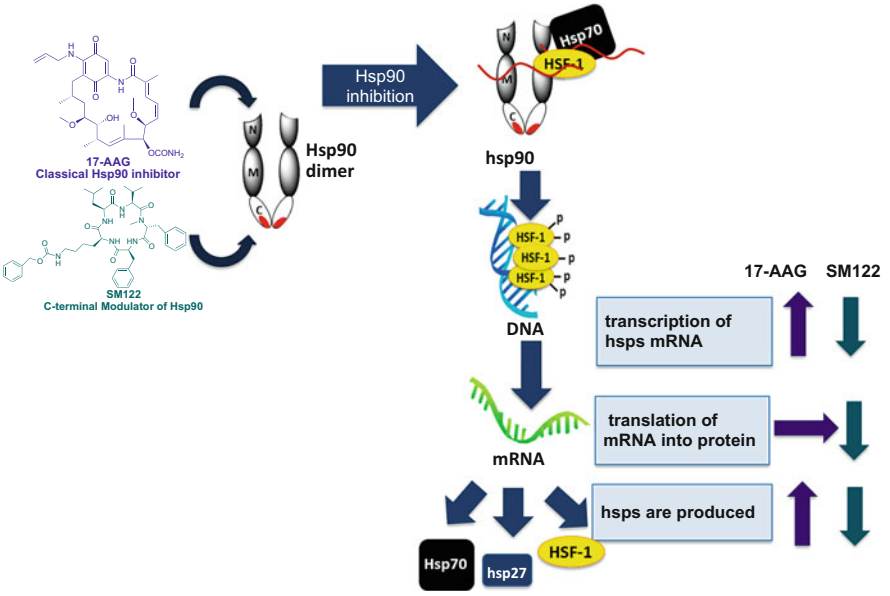

Fig. 5 N-terminal and C-terminal Hsp90 inhibition cause differing mechanistic results. Two Hsp90 inhibitors, 17-AAG and SM122, were evaluated at several points during the heat shock response pathway. The mRNA levels of Hsp70 and Hsp27 increased when cells were treated with 17-AAG, whereas mRNA production levels went down for these two proteins when cells were treated with SM122. Translation of mRNA into protein was not impacted when cells were treated with 17-AAG; however, it was completely inhibited when cells were treated with SM122. Finally, Hsp70, Hsp27, and HSF1 protein levels dropped dramatically

In addition to not initiating the HSR, treatment of HCT116 colon cancer cells with SM122 caused other effects associated with Hsp90 inhibition. For example, SM122 produced a maximum of ~ twofold decrease in protein levels of the Hsp90 co-chaperones FKBP52 and CHIP and client protein Akt. By decreasing the levels of Hsp90-interacting proteins with such varying functions, SM122 disrupts hormone receptors, protein homeostasis, and cell proliferation simultaneously. These trends were concentration dependent, with IC_{50} levels of SM122 (~10 μM) decreasing the protein levels of these molecules by ~1.25-fold, whereas treatment of HCT116 colon cancer cells with 17-AAG at its IC_{50} level (~50 nM) or even 20-fold over its IC_{50} value produced no impact on these co-chaperones and only reduced the N-terminal-binding protein Akt by fourfold [16].

SM122 also induced apoptosis and trapped cells in G0/G1 phase [19]. Specifically after 24 h, cells treated with IC_{50} values of SM122 had significant levels of cleaved PARP and induced caspase 3/caspase 7 activation threefold over control, whereas cells treated with three times the IC_{50} value of 17-AAG only induced caspase 3/caspase 7 twofold over control. These data support the hypothesis that treatment of cells with molecules that modulate the C-terminus, specifically from the SM series, immediately induces apoptosis and does not provide the cell the

opportunity to rescue itself by producing large quantities of Hsp70, Hsp27, or HSF1. In contrast, compounds that target the N-terminus of Hsp90, specifically 17-AAG, do not induce apoptosis immediately, but rather trigger a rescue mechanism, which delays the induction of apoptosis, as indicated by the delay in caspase 3/caspase 7, and low levels of PARP cleavage at 24 h.

3 Peptide Inhibitors that Mimic the TPR Domain

The small molecules discussed above that modulate the C-terminus all block the interaction between the TPR domain of the co-chaperone protein and Hsp90, initiating degradation of clients and causing cell death. All of the co-chaperones known to bind to Hsp90's C-terminus MEEVD region uses their TPR domain to facilitate this interaction (Fig. 6a). A TPR domain is made up of at least three TPR motifs, where a TPR motif is a set of 34 amino acids that are loosely conserved and fold into two 16-amino-acid helices (Fig. 6) [72, 73]. While the C-terminal MEEVD sequence of Hsp90 interacts with many TPR-containing proteins, Hsp70 also interacts with TPR-containing proteins using its C-terminal GPTIEEVD sequence (Gly-Pro-Thr-Ile-Glu-Glu-Val-Asp) (Fig. 6b) [71, 74]. Indeed, there are three TPR-containing co-chaperones (HOP, CHIP, and Tom70) that bind to both Hsp90 and Hsp70 at their C-termini [75]. Since blocking the TPR-Hsp90 interaction has numerous advantages over classic N-terminal Hsp90 inhibitors, using the conserved nature of the TPR domain to design peptide mimics to block this TPR-MEEVD/GPTIEEVD interaction is an exciting new approach. The overlap of this TPR-Hsp90/Hsp70 interaction suggests that creating molecules that mimic the TPR domain could be a good approach to inhibit both Hsp90 and Hsp70 simultaneously; however, there are differences in how Hsp90 and Hsp70 interact with the TPR domains, so this could prove challenging. Despite this difficulty, disrupting the interactions between these TPR co-chaperones and Hsp90 and/or Hsp70 will lead to inhibiting multiple oncogenic functions of one

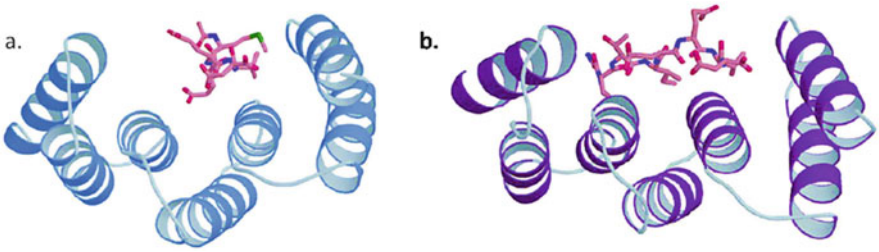

Fig. 6 Two TPR domains of HOP in complex with peptides. (**a**) TRP2A domain of HOP bound to the MEEVD sequence of Hsp90. (**b**) TRP1 domain of HOP bound to the last 12 amino acids of Hsp70. Figure taken from [71]

or both heat shock proteins and represents a viable new way to inhibit these important molecular chaperones.

3.1 TPR2A Peptide Mimics Targeting the TPR-Binding Region of Hsp90's C-Terminus

Cortajarena and co-workers reported the first example of applying an engineered Hsp90-binding TPR mimic to inhibit Hsp90 chaperone functions [76]. They created a new TPR module, CTPR390, which consists of three repeats of two alpha helices (helix A, ASAWYNLGNAYYK QG; helix B, DYQKAIEYYQKALEL). This protein is based on a computer-generated sequence of the most stable TPR domain (known as CTPR3) with the key residues for binding Hsp90 inserted creating the final CTPR390 [77, 78]. CTPR3 was selected as the basic framework because it showed remarkable stability, behaving better than TPR1 or TPR2A domains of HOP when used as a TPR mimic. Thus, this sequence could be used as a robust scaffold to investigate the interaction between Hsp90 and TPR-containing proteins or to design lead inhibitors [77]. Indeed, the design of CTPR390 was a success, and it was able to simulate the behavior of TPR-containing Hsp90 co-chaperones.

However, the interaction between CTPR390 and Hsp90 was weaker than that between HOP's TPR2A domain and Hsp90 by approximately 100-fold [78]. Therefore, it would not be effective as a competitive inhibitor. In order to improve the binding affinity of the designed TPR protein CTPR390 to Hsp90, the protein was charged by altering specific residues that appear on the outer surface of the secondary structure and would not interfere with binding (Fig. 7). These two mutant TPR domains maintained the Hsp90-binding residues from TPR2A and the same secondary structure of CTPR390, but they were negative or positively charged (CTPR390 +/-). The introduction of positive charges lowered the dissociation constant between CTPR390+ and Hsp90's MEEVD region to less than 1 μM, much lower than the dissociation constant of the original CTPR390 (K_d ~500 μM). In contrast, CTPR390- lost affinity for Hsp90, showing a $K_d > 600$ μM [77]. The improvement associated with CTPR390+ pushed the binding affinity of this engineered protein above the native binding partner HOP, which has a K_d ~5 M for Hsp90. Interestingly, CTPR390+ was very selective for the Hsp90 C-terminal

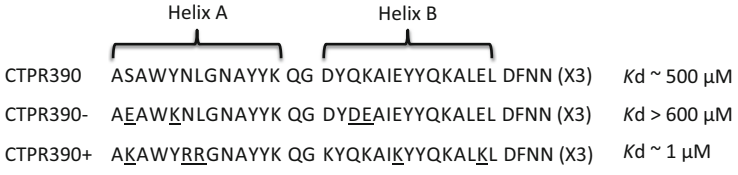

Fig. 7 Sequence of CTPR390 and the charged derivatives (CTPR390+/-) along with their *K*d values for binding to Hsp90

peptide over the Hsp70 C-terminal peptide (100-fold), whereas the native TPR2A domain of HOP only has tenfold selectivity for Hsp90 over Hsp70 [77].

Due to the high binding affinity between Hsp90 and CTPR390+, the CTPR390+ sequence was tested in cells. Successfully introducing CTPR390+ into BT474 cells (HER2-positive breast ductal carcinoma cells), using ProteoJuice (Novagen), showed that CTPR390+ could outcompete endogenous co-chaperones in an Hsp90-binding event. Specifically, after the treatment of BT474 cells with CTPR390+, the expression levels of HER2 as well as phosphorylated HER2 (the functional form of HER2) were decreased substantially, with the phosphorylated HER2 reduced to 20% of its original levels [77]. As seen with the small molecules that inhibit the interaction between Hsp90 and TPR-containing proteins, treatment of the BT474 cells with CTPR390+ did not induce Hsp70 protein.

Collectively, these results suggested that the newly engineered TPR module CTPR390+ is capable of binding to the C-terminus of Hsp90 with better stability, higher affinity, and greater specificity than the endogenous Hsp90-binding co-chaperone HOP. Inhibition of the binding event between Hsp90's C-terminus and its co-chaperones leads to Hsp90 client protein degradation and cancer cell death. Thus, CTPR390+ represents a novel class of Hsp90 inhibitors that target the interaction between Hsp90 and HOP.

3.2 Antp-TPR Hybrid Peptide-Based Treatment of Solid Tumors

Similar to the designed TPR peptide motif CTPR390+, Horibe and co-workers engineered a novel peptide (KAYARIGNSYFK, TPR peptide) that is modeled from the TPR2A domain of HOP [79]. This TPR mimic is different from CTPR390+ because it is a small peptide and consists of only 12 amino acids. The peptide sequence was obtained from the helix A3 of HOP's TPR2A domain, which is responsible for docking HOP to the C-terminus of Hsp90. When tested in binding studies, the TPR peptide successfully inhibited the interaction between Hsp90 and HOP; however, it failed to disrupt binding between Hsp90 and two other co-chaperones that contain TPR domains (FKBP5 and PP5). The TPR peptide successfully bound to both Hsp70 and Hsp90 with similar affinities; however, this binding event failed to inhibit the interaction between Hsp70 and any of the tested co-chaperones. These observations indicated that this newly designed peptide is a specific inhibitor of the interaction between Hsp90 and HOP, which is similar to the previously discussed TPR mimic CTPR390+.

Since the free TPR mimic peptide is not able to penetrate the cell membrane, the N-terminus of the peptide was fused to helix 3 of the antennapedia homeodomain protein (Antp), generating a cell membrane-penetrating variant [80]. This variant could now be used to determine the cellular consequence of treating with the TPR mimic. This new fusion peptide was designated as "hybrid Antp-TPR peptide"

(RQIKIWFQNRRMKWKK KAYARIGNSYFK) or Antp-TPR peptide for short. After structural modification, this peptidyl antagonist not only retained the capability of disrupting the interaction between Hsp90 and the TPR2A domain of HOP, but it could now penetrate cells [79]. Treating cells with this Antp-TPR peptide produced a mild anticancer effect, where it showed a concentration-dependent cytotoxicity in cancer cell lines including Caki-1 (human clear cell renal cell carcinoma), BXPC3 (human pancreatic cancer), T47D (human ductal breast epithelial cancer), and A549 (carcinomic human alveolar basal epithelial cell line) ($IC_{50} = 20–30$ μM against T47D, BXPC3, and Caki-1 cells; and ~60 μM against A549) [79].

Additionally, 24-h treatment with 68 μM Antp-TPR peptide induced extensive cell death through a caspase 3/caspase 7-mediated apoptotic mechanism in T47D breast cancer cells. Strikingly, although the IC_{50} values of Antp-TPR peptide against these tested cancer cell lines were relatively high, concentrations of Antp-TPR peptide, e.g., up to 100 μM, did not cause significant decrease in the viability of "normal" human cell line models such as HEK293T (transformed human embryonic kidney), MRC5 (normal lung fibroblast), and PE (human normal pancreatic epithelial cell) [79]. These results demonstrate that cancer cells are more sensitive to the Antp-TPR peptide than normal cells.

In T47D cells (breast cancer), 48-h treatment with 68 μM Antp-TPR peptide greatly suppressed Hsp90 client protein levels, including cyclin-dependent kinase 4 (CDK4), survivin, and Akt. These clients are usually upregulated in cancer cells, and they play essential roles in cell cycle modulation or suppression of apoptosis [80–84]. These clients also rely on the interaction between HOP and Hsp90 to facilitate their folding and maturation. Thus, suppression of these clients indicates that the Antp-TPR peptide is modulating their activity by inhibiting HOP from binding to Hsp90. This TPR peptide also had no effect on Hsp70 levels in all tested normal cell lines. Collectively, these data support the hypothesis that the Antp-TPR peptide specifically affects cancer cell survival pathways by blocking the interaction of Hsp90 with HOP and disrupting co-chaperone recruitment. Similar to the C-terminal-modulating small molecules, the Antp-TPR peptide appears to block a key interaction involving substrate loading onto Hsp90 in the molecular chaperone cycle for folding client proteins by disrupting Hsp90-TPR interactions (Fig. 8) [18, 22, 23, 39, 41].

To confirm the specificity of the TPR peptide, the authors created two Antp-TPR mutants that were designed to evaluate which amino acids were critical for cytotoxicity within the 12-amino-acid sequence. Mutant 1 involved replacing the highly conserved Arg residue and the Ile with Ala (Antp-KAYAAAGNSYFK; mutated amino acids are underlined) (Fig. 9). Mutant 2 involved replacing the Tyr-Phe-Lys of Antp-TPR peptide with a triple Gly in order to disrupt the helical structure (Antp-KAYARIGNSGGG; mutated amino acids are underlined) (Fig. 9). As expected, neither of the mutants showed any inhibitory effect on the Hsp90-TPR2A interaction, and they had no cytotoxicity against cancer cells, even at millimolar concentrations [79]. These results revealed that the mutated amino acids in both Antp-TPR mutants 1 and 2 are critical for inhibiting this protein interaction and are also indispensable for the selective antitumor activity of Antp-TPR.

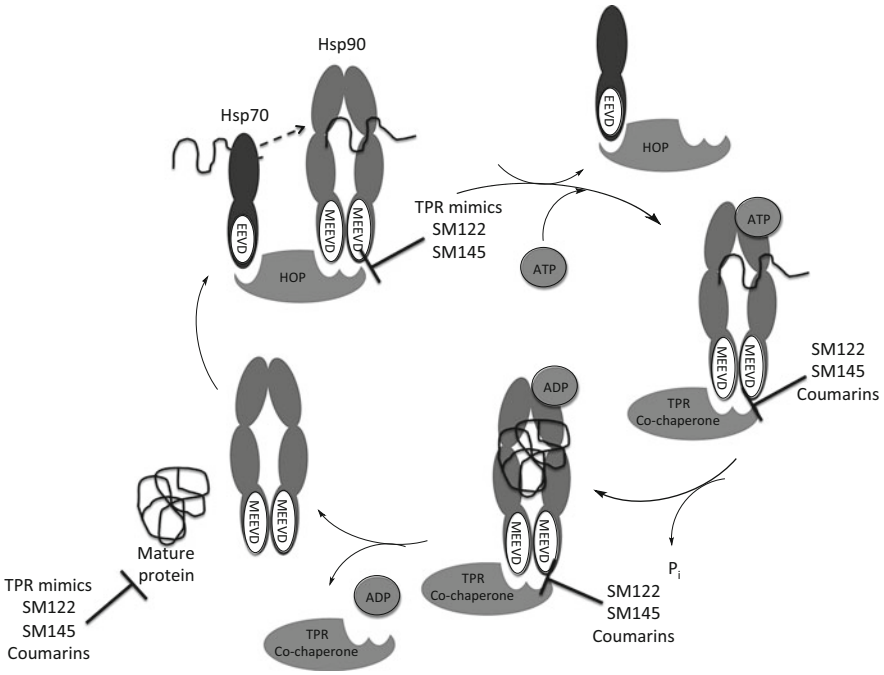

Fig. 8 Depiction of Hsp90-mediated protein folding and where the C-terminal-modulating molecules disrupt this pathway. In the normal protein-folding pathway, an unfolded protein is transferred to Hsp90 by Hsp70 via HOP. However, the TPR mimics and SM series inhibitors disrupt the interaction of Hsp90 with HOP preventing protein transfer and halting protein folding. The SM series compounds as well as the natural product C-terminal inhibitors also disrupt later in the protein-folding pathway by inhibiting the binding of essential co-chaperones to Hsp90, halting protein folding. There is no evidence yet that the TPR mimics can block the interaction of Hsp90 with TPR proteins other than HOP. All three types of C-terminal inhibitors lead to a disruption in protein folding and prevent the proper maturation of Hsp90 client proteins

The Antp-TPR peptide also displayed a significant antitumor effect in a xenograft model of BXPC3 human pancreatic cancer in mice. Administration of Antp-TPR peptide suppressed tumor growth significantly, where treatment with 1 mg/kg/day reduced tumor size by ~50% and 5 mg/kg by 73% compared to the control. Similar to the results observed in cell experiments, immunohistochemical staining of BXPC3 tumors treated with Antp-TPR peptide also demonstrated a decrease in expression levels of numerous Hsp90 client proteins, including CDK4. In addition, similar to the normal cell-based assays, this peptide showed equally unremarkable effects on the control mice, where histological examination indicated that the peptide did not impact the liver, kidney, or lungs. Thus, the Antp-TPR peptide effectively triggers tumor specific death in vivo through a mechanism of action involving degradation of Hsp90 client proteins and is an attractive new option for molecular targeted therapy of solid tumors.

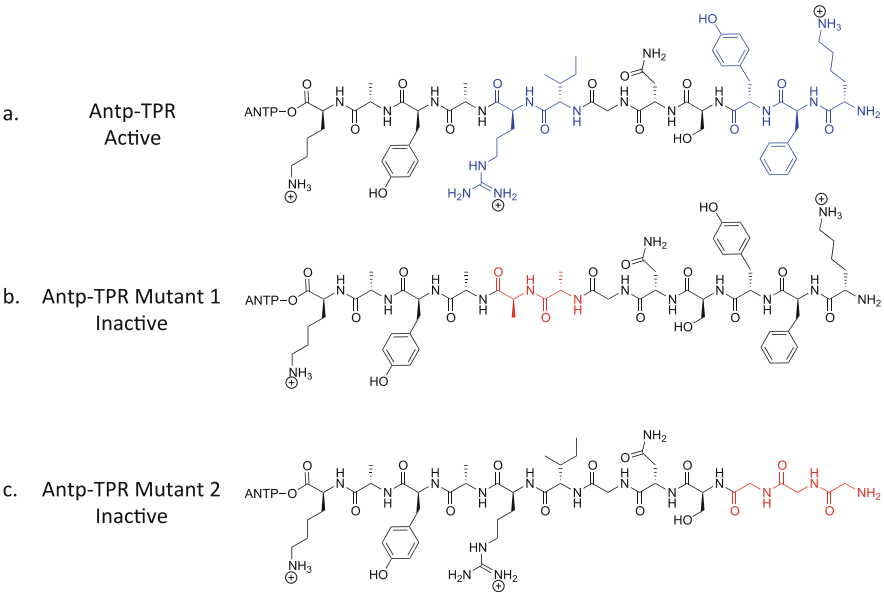

Fig. 9 (**a**) Sequence of the Antp-TPR peptide with residues essential for activity in *blue*. (**b**, **c**) Sequence of mutated versions of Antp-TPR used to determine important amino acid residues (mutated amino acids in *red*)

3.3 Antp-TPR Hybrid Peptide-Based Treatment of Leukemia

In addition to BXPC3, the Antp-TPR peptide has been used successfully against leukemia cell lines [85]. This peptide showed concentration-dependent cytotoxicity in four tested leukemia cell lines including U937 (monoblastic leukemia), K562 (chronic myelogenous leukemia), THP-1 (acute monocytic leukemia), and HL-60 (promyelocytic leukemia), with IC_{50} values from 16 to 51 μM. Importantly, the Antp-TPR peptide was much less toxic to normal cell line models including HEK293, PE, WI38, and PBMCs (peripheral blood mononuclear cells), where the IC_{50} values for normal cells were all greater than 130 μM. By comparison, 17-AAG and geldanamycin (N-terminal Hsp90 inhibitors) showed a greater cytotoxic effect on the normal cells than on cancer cells [85]. Thus, even though the Antp-TPR peptide has a somewhat high IC_{50} value, it discriminates between leukemic cells and normal cells, while the N-terminal inhibitors of Hsp90 do not.

Similar to previously tested cell lines, the Antp-TPR peptide (50 μM) affected the AML cell survival pathway by downregulating Hsp90 client proteins survivin, Akt, and CDK4 [85]. Antp-TPR peptide also selectively triggered apoptosis in AML cells but not in normal cells (WI38, HEK, PE) by activating the caspase 3/caspase 7-mediated apoptotic pathways and by disrupting the mitochondrial membrane potential. The selectivity observed for the Antp-TPR peptide is likely related to the Hsp90 client protein, survivin. Survivin is regulated by the Hsp90-HOP

binding event. It is overexpressed in cancer cells but has an extremely low expression level in tested normal cells [79]. Since cancer cells depend on survivin to control mitosis and suppress apoptosis or cell death, inducing degradation via the Hsp90 pathway will induce cell death [81, 82].

3.4 *Antp-TPR Hybrid Peptide-Based Treatment of Glioblastoma*

Among the family of solid tumors, brain cancer is more complicated than other types of solid cancer because it is located in the brain or central spinal canal. Glioblastomas (GBs) are the most commonly diagnosed malignant adult primary brain tumor [86]. GBs are usually highly malignant and the median survival is only 12–15 months [86]. That is because GB cells reproduce very quickly and are nourished by the large network of blood vessels in the nervous system. GBs also contain many different cell types and therefore have a very complex molecular pathology and heterogeneity. Thus, single targeted therapies fail to offer a long-term survival benefit [87].

GB cells rely on numerous activated oncoproteins and signaling pathways that require Hsp90 chaperone functions [88]. Therefore, Hsp90 inhibition and its related combination treatments may provide promising GB therapy. The Antp-TPR hybrid peptide has shown remarkable concentration-dependent cytotoxicity in GB cell lines U251, A172, and SN19, with IC_{50} values in range of 26–36 μM [89]. At 50 μM, the Antp-TPR hybrid peptide rapidly destroyed all GB cell types tested. Specifically, in U251 and A172 cell lines, a 6-h treatment with 50 μM of Antp-TPR peptide caused a 70–80% decrease in cell viability. Conversely, at 50 μM, the peptide did not affect the normal cell line PE, even after 24 h of treatment. In comparison, 17-AAG (0.1–0.4 μM) failed to induce significant GB cell death after 24 h of treatment, although after 48 h it reduced GB cell viability to 40–50% [89]. Finally, unlike N-terminal inhibitors, treatment of GB cells (U251, A172, and SN19) with 20–80 μM Antp-TPR peptide did not alter the expression or transcriptional levels of Hsp70 or Hsp27. These results indicate that the cytotoxic mechanism of Antp-TPR peptide differs from that of the small molecules that target the ATP binding site (e.g., N-terminus) of Hsp90.

In addition, cells treated with Antp-TPR peptide downregulated the expression of Hsp90 client proteins including p53, CDK4, Akt, and cRaf in a concentration-dependent manner [89]. Examination of cRaf, Akt, and CDK4 transcriptional levels showed that 80 μM treatments of Antp-TPR peptide induced a decrease in the mRNA levels of these three clients in A172 and SN19 cells. Thus, similar to the SM inhibitors, treatment with the Antp-TPR hybrid peptide led to a loss of Hsp90 clients in GB cells, which occurs at both protein and mRNA levels, and it does not induce the overexpression of heat shock proteins. Despite its relatively high IC_{50} value, the Antp-TPR is highly selective for malignant cells and is an exciting new Hsp90 inhibitor with several advantages over the classic N-terminal Hsp90 inhibitors.

4 Conclusion

In summary, research focused on inhibiting Hsp90's C-terminal function has proven fruitful. Both direct and allosteric inhibition of Hsp90's C-terminus have provided productive tools and potential lead structures that have advantages over the classic N-terminal inhibitors used in clinical trials. C-terminal inhibitors such as the SM series have the benefit of not inducing a cytoprotective heat shock response, as well as controlling multiple oncogenic chaperones critical for cell growth, specifically HOP, Cyp40, FKBP51, FKBP52, PP5, TOM70, CHIP, and Unc45. Regulating these TPR-containing proteins' function through Hsp90 shuts down all six hallmarks of cancer simultaneously, thus proving to be an effective oncogenic approach. TPR-containing mimics have also proven to be highly effective for controlling tumor cell growth both in cell lines and mice models. Thus, targeting the C-terminus of Hsp90 is proven to be the most successful and efficient route to control all six hallmarks of cancer simultaneously without producing the heat shock response rescue mechanism and as such represents the future of Hsp90 cancer therapy development.

References

1. Izar B, Rotow J, Gainor J, Clark J, Chabner B (2013) Pharmacokinetics, clinical indications, and resistance mechanisms in molecular targeted therapies in cancer. Pharmacol Rev 65:1351–1395
2. Bagatell R, Whitesell L (2004) Altered Hsp90 function in cancer: a unique therapeutic opportunity. Mol Cancer Ther 3:1021–1030
3. Trepel J, Mollapour M, Giaccone G et al (2010) Targeting the dynamic HSP90 complex in cancer. Nat Rev Cancer 10:537–549
4. Miyata Y, Nakamoto H, Neckers L (2013) The therapeutic target hsp90 and cancer hallmarks. Curr Pharm Des 19:347–365
5. Whitesell L, Mimnaugh EG, De Costa B et al (1994) Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. Proc Natl Acad Sci U S A 91:8324–8328
6. Jhaveri K, Modi S (2012) HSP90 inhibitors for cancer therapy and overcoming drug resistance. Adv Pharmacol 65:471–517
7. Jhaveri K, Taldone T, Modi S et al (2012) Advances in the clinical development of heat shock protein 90 (Hsp90) inhibitors in cancers. Biochim Biophys Acta 1823:742–755
8. Pacey S, Wilson RH, Walton M et al (2011) A phase I study of the heat shock protein 90 inhibitor alvespimycin (17-DMAG) given intravenously to patients with advanced solid tumors. Clin Cancer Res 17:1561–1570
9. Modi S, Stopeck A, Linden H et al (2011) HSP90 inhibition is effective in breast cancer: a phase II trial of tanespimycin (17-AAG) plus trastuzumab in patients with HER2-positive metastatic breast cancer progressing on trastuzumab. Clin Cancer Res 17:5132–5139
10. Sequist LV, Gettinger S, Senzer NN et al (2010) Activity of IPI-504, a novel heat-shock protein 90 inhibitor, in patients with molecularly defined non-small-cell lung cancer. J Clin Oncol 28:4953–4960

11. Lancet JE, Gojo I, Burton M et al (2010) Phase I study of the heat shock protein 90 inhibitor alvespimycin (KOS-1022, 17-DMAG) administered intravenously twice weekly to patients with acute myeloid leukemia. Leukemia 24:699–705
12. Rajan A, Kelly RJ, Trepel JB et al (2011) A phase I study of PF-04929113 (SNX-5422), an orally bioavailable heat shock protein 90 inhibitor, in patients with refractory solid tumor malignancies and lymphomas. Clin Cancer Res 17:6831–6839
13. Sydor JR, Normant E, Pien CS et al (2006) Development of 17-allylamino-17-demethoxygeldanamycin hydroquinone hydrochloride (IPI-504), an anti-cancer agent directed against Hsp90. Proc Natl Acad Sci U S A 103:17408–17413
14. Bagatell R, Paine-Murrieta GD, Taylor CW et al (2000) Induction of a heat shock factor 1-dependent stress response alters the cytotoxic activity of hsp90-binding agents. Clin Cancer Res 6:3312–3318
15. Wang Y, McAlpine SR (2015) C-terminal heat shock protein 90 modulators produce desirable oncogenic properties. Org Biomol Chem 13:4627–4631
16. Wang Y, McAlpine SR (2015) Combining an Hsp70 inhibitor with either an N-terminal and C-terminal hsp90 inhibitor produces mechanistically distinct phenotypes. Org Biomol Chem 13:3691–3698
17. Wang Y, McAlpine SR (2015) Heat shock protein 90 inhibitors: will they ever succeed as chemotherapeutics? Future Med Chem 7:87–90
18. Wang Y, Mcalpine SR (2015) N-terminal and C-terminal modulation of Hsp90 produce dissimilar phenotypes. Chem Comm 51:1410–1413
19. Wang Y, McAlpine SR (2015) Regulating the cytoprotective response in cancer cells using simultaneous inhibition of Hsp90 and Hsp70. Org Biomol Chem 13:2108–2116
20. Wang Y, Islam A, Davis RA et al (2015) The fungal natural product (1S, 3S)-austrocortirubin induces DNA damage via a mechanism unique from other DNA damaging agents. Bioorg Med Chem Lett 25:249–253
21. Eskew JD, Sadikot T, Morales P et al (2011) Development and characterization of a novel C-terminal inhibitor of Hsp90 in androgen dependent and independent prostate cancer cells. Bio Med Central Cancer 11:468
22. Allan RK, Mok D, Ward BK et al (2006) Modulation of chaperone function and cochaperone interaction by novobiocin in the C-terminal domain of Hsp90. J Biol Chem 281:7161–7171
23. McConnell JM, Alexander LD, McAlpine SR (2014) A heat shock protein inhibitor that modulates immunophilins and regulates hormone receptors. Bioorg Med Chem Lett 24:661–666
24. Koay YC, McConnell JR, Wang Y et al (2014) Chemically accessible Hsp90 inhibitor that does not induce a heat shock response. ACS Med Chem Lett 5:771–776
25. Powers MV, Clarke PA, Workman P (2009) Death by chaperone: HSP90, HSP70 or both? Cell Cycle 8:518–526
26. Zhang H, Chung D, Yang YC et al (2006) Identification of new biomarkers for clinical trials of Hsp90 inhibitors. Mol Cancer Ther 5:1256–1264
27. Song D, Chaerkady R, Tan AC et al (2008) Antitumor activity and molecular effects of the novel heat shock protein 90 inhibitor, IPI-504, in pancreatic cancer. Mol Cancer Ther 7:3275–3284
28. Calderwood SK, Khaleque MA, Sawyer DB et al (2006) Heat shock proteins in cancer: chaperones of tumorigenesis. Trends Biochem Sci 31:164–172
29. Mosser DD, Morimoto RI (2004) Molecular chaperones and the stress of oncogenesis. Oncogene 23:2907–2918
30. McCollum AK, TenEyck CJ, Sauer BM et al (2006) Up-regulation of heat shock protein 27 induces resistance to 17-allylamino-demethoxygeldanamycin through a glutathione-mediated mechanism. Cancer Res 66:10967–10975
31. Maloney A, Clarke PA, Naaby-Hansen S et al (2007) Gene and protein expression profiling of human ovarian cancer cells treated with the heat shock protein 90 inhibitor 17-allylamino-17-demethoxygeldanamycin. Cancer Res 67:3239–3253

32. Caldas-Lopes E, Cerchietti L, Ahn JH et al (2009) Hsp90 inhibitor PU-H71, a multimodal inhibitor of malignancy, induces complete responses in triple-negative breast cancer models. Proc Natl Acad Sci U S A 106:8368–8373
33. Gaspar N, Sharp SY, Eccles SA et al (2010) Mechanistic evaluation of the novel HSP90 inhibitor NVP-AUY922 in adult and pediatric glioblastoma. Mol Cancer Ther 9:1219–1233
34. Chatterjee M, Andrulis M, Stühmer T et al (2013) The PI3K/Akt signaling pathway regulates the expression of Hsp70, which critically contributes to Hsp90-chaperone function and tumor cell survival in multiple myeloma. Haematologica 98:1132–1141
35. Powers MV, Clarke PA, Workman P (2008) Dual targeting of Hsc70 and Hsp72 inhibits Hsp90 function and induces tumor-specific apoptosis. Cancer Cell 14:250–262
36. Stühmer T, Zöllinger A, Siegmund D et al (2008) Signalling profile and antitumour activity of the novel Hsp90 inhibitor NVP-AUY922 in multiple myeloma. Leukemia 22:1604–1612
37. Stühmer T, Chatterjee M, Grella E et al (2009) Anti-myeloma activity of the novel 2-aminothienopyrimidine Hsp90 inhibitor NVP-BEP800. Br J Haematol 47:319–327
38. Davenport EL, Zeisig A, Aronson LI et al (2010) Targeting heat shock protein 72 enhances Hsp90 inhibitor-induced apoptosis in myeloma. Leukemia 24:1804–1807
39. Ardi VC, Alexander LD, Johnson VA et al (2011) Macrocycles that inhibit the binding between heat shock protein 90 and TPR-containing proteins. ACS Chem Biol 6:1357–1367
40. Alexander LD, Partridge JR, Agard DA et al (2011) A small molecule that preferentially binds the closed Hsp90 conformation. Bioorg Med Chem Lett 21:7068–7071
41. Vasko RC, Rodriguez RA, Cunningham CN et al (2010) Mechanistic studies of Sansalvamide A-Amide: an allosteric modulator of Hsp90. ACS Med Chem Lett 1:4–8
42. Yu XM, Shen G, Cronk B et al (2005) Hsp90 inhibitors identified from a library of novobiocin analogues. J Am Chem Soc 127:12778–12779
43. Kusuma BR, Peterson LB, Zhao H et al (2011) Targeting the heat shock protein 90 dimer with dimeric inhibitors. J Med Chem 54:6234–6253
44. Mendillo ML, Santagata S, Koeva M et al (2012) HSF1 drives a transcriptional program distinct from heat shock to support highly malignant human cancers. Cell 150:549–562
45. Gabai VL, Meng L, Kim G et al (2012) Heat shock transcription factor Hsf1 is involved in tumor progression via regulation of hypoxia-inducible factor 1 and RNA-binding protein HuR. Mol Cell Biol 32:929–940
46. Santagata S, Hu R, Lin NU et al (2011) High levels of nuclear heat-shock factor 1 (HSF1) are associated with poor prognosis in breast cancer. Proc Natl Acad Sci U S A 108:18378–18383
47. Meng L, Gabai VL, Sherman MY (2010) Heat-shock transcription factor HSF1 has a critical role in human epidermal growth factor receptor-2-induced cellular transformation and tumorigenesis. Oncogene 29:5204–5213
48. Goloudina AR, Demidov ON, Garrido C (2012) Inhibition of HSP70: a challenging anti-cancer strategy. Cancer Lett 325:117–124
49. Whitesell L, Santagata S, Lin NU (2012) Inhibiting hsp90 to treat cancer: a strategy in evolution. Curr Mol Med 12:1108–1124
50. Nylandsted J, Gyrd-Hansen M, Danielewicz A et al (2004) Heat shock protein 70 promotes cell survival by inhibiting lysosomal membrane permeabilization. J Exp Med 200:425–435
51. Guo F, Sigua C, Bali P et al (2005) Mechanistic role of heat shock protein 70 in Bcr-Abl-mediated resistance to apoptosis in human acute leukemia cells. Blood 105:1246–1255
52. Creagh EM, Sheehan D, Cotter TG (2000) Heat shock proteins–modulators of apoptosis in tumour cells. Leukemia 14:1161–1173
53. Beere HM (2004) "The stress of dying": the role of heat shock proteins in the regulation of apoptosis. J Cell Sci 117:2641–2651
54. Takayama S, Reed JC, Homma S (2003) Heat-shock proteins as regulators of apoptosis. Oncogene 22:9041–9047
55. Saleh A, Srinivasula SM, Balkir L et al (2000) Negative regulation of the Apaf-1 apoptosome by Hsp70. Nat Cell Biol 2:476–483

56. Beere HM, Wolf BB, Cain K et al (2000) Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. Nat Cell Biol 2:469–475
57. Jäättelä M, Wissing D, Kokholm K et al (1998) Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases. EMBO J 17:6124–6134
58. Ravagnan L, Gurbuxani S, Susin SA et al (2001) Heat-shock protein 70 antagonizes apoptosis-inducing factor. Nat Cell Biol 3:839–843
59. Gurbuxani S, Schmitt E, Cande C et al (2003) Heat shock protein 70 binding inhibits the nuclear import of apoptosis-inducing factor. Oncogene 22:6669–6678
60. Li J, Hu W, Lan Q (2012) The apoptosis-resistance in t-AUCB-treated glioblastoma cells depends on activation of Hsp27. J Neurooncol 110:187–194
61. Bauer K, Nitsche U, Slotta-Huspenina J et al (2012) High HSP27 and HSP70 expression levels are independent adverse prognostic factors in primary resected colon cancer. Cell Oncol (Dordr) 35:197–205
62. Acunzo J, Katsogiannou M, Rocchi P (2012) Small heat shock proteins HSP27 (HspB1), αB-crystallin (HspB5) and HSP22 (HspB8) as regulators of cell death. Int J Biochem Cell Biol 44:1622–1631
63. Hsu HS, Lin JH, Huang WC et al (2011) Chemoresistance of lung cancer stemlike cells depends on activation of Hsp27. Cancer 117:1516–1528
64. Heinrich JC, Tuukkanen A, Schroeder M et al (2011) RP101 (brivudine) binds to heat shock protein HSP27 (HSPB1) and enhances survival in animals and pancreatic cancer patients. J Cancer Res Clin Oncol 137:1349–1361
65. Kang SH, Kang KW, Kim KH et al (2008) Upregulated HSP27 in human breast cancer cells reduces Herceptin susceptibility by increasing Her2 protein stability. BMC Cancer 8:286
66. Newman DJ, Cragg GM (2012) Natural products as sources of new drugs over the 30 years from 1981 to 2010. J Nat Prod 75:311–335
67. Burlison J, Blagg B (2006) Synthesis and evaluation of Coumermycin A1 analogues that inhibit the hsp90 protein machinery. Org Lett 8:4555–4558
68. Matthews SB, Vielhauer GA, Manthe CA, Chaguturu VK, Szabla K, Matts RL, Donnelly AC, Blagg BS, Holzbeierlein JM (2010) Characterization of a novel novobiocin analogue as a putative C-terminal inhibitor of heat shock protein 90 in prostate cancer cells. Prostate 70:27–36
69. Koay YC, McConnell JR, Wang Y et al (2015) Blocking the heat shock response and depleting HSF-1 levels through heat shock protein 90 (hsp90) inhibition: a significant advance on current hsp90 chemotherapies. RSC Adv. doi:10.1039/C5RA07056B
70. Wahyudi H, Wang Y, McAlpine SR (2014) Utilizing a Dimerization strategy to inhibit the dimer protein Hsp90:Synthesis and biological activity of a sansalvamide A dimer. Org Biomol Chem 12:765–773
71. Scheufler C, Brinker A, Bourenkov G et al (2000) Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. Cell 101:199–210
72. Alag R, Bharatham N, Dong A et al (2009) Crystallographic structure of the tetratricopeptide repeat domain of Plasmodium falciparum FKBP35 and its molecular interaction with Hsp90 C-terminal pentapeptide. Protein Sci 18:2115–2124
73. Zeytuni N, Zarivach R (2012) Structural and functional discussion of the tetra-trico-peptide repeat, a protein interaction module. Structure 7:397–405
74. Blatch GL, Lassle M (1999) The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. BioEssays 21:932–939
75. Caplan AJ (2003) What is a co-chaperone? Cell Stress Chaperones 8:105–107
76. Cortajarena AL, Yi F, Regan L (2008) Designed TPR modules as novel anticancer agents. ACS Chem Biol 3:161–166
77. Main ER, Xiong Y, Cocco MJ et al (2003) Design of stable alpha-helical arrays from an idealized TPR motif. Structure 11:497–508

78. Cortajarena AL, Kajander T, Pan W et al (2004) Protein design to understand peptide ligand recognition by tetratricopeptide repeat proteins. Protein Eng Des Sel 17:399–409
79. Horibe T, Kohno M, Haramoto M et al (2011) Designed hybrid TPR peptide targeting Hsp90 as a novel anticancer agent. J Transl Med 9:8
80. Kabouridis PS (2003) Biological applications of protein transduction technology. Trends Biotechnol 21:498–503
81. Salvesen GS, Duckett CS (2002) IAP proteins: blocking the road to death's door. Nat Rev Mol Cell Biol 3:401–410
82. Altieri DC (2003) Validating survivin as a cancer therapeutic target. Nat Rev Cancer 3:46–54
83. Redlak MJ, Miller TA (2011) Targeting PI3K/Akt/HSP90 signaling sensitizes gastric cancer cells to deoxycholate-induced apoptosis. Dig Dis Sci 56:323–329
84. Wu A, Wu B, Guo J et al (2011) Elevated expression of CDK4 in lung cancer. J Transl Med 9:38
85. Horibe T, Kawamoto M, Kohno M et al (2012) Cytotoxic activity to acute myeloid leukemia cells by Antp-TPR hybrid peptide targeting Hsp90. J Biosci Bioeng 114:96–103
86. Stupp R, Hegi ME, van den Bent MJ et al (2006) Changing paradigms–an update on the multidisciplinary management of malignant glioma. Oncologist 11:165–180
87. Omuro AM, Faivre S, Raymond E (2007) Lessons learned in the development of targeted therapy for malignant gliomas. Mol Cancer Ther 6:1909–1919
88. Collins V (2004) Brain tumours: classification and genes. J Neurol Neurosurg Psychiatry 75: ii2–ii11
89. Horibe T, Torisawa A, Kohno M et al (2012) Molecular mechanism of cytotoxicity induced by Hsp90-targeted Antp-TPR hybrid peptide in glioblastoma cells. Mol Cancer 11:59

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Hsp90 Co-chaperones as Drug Targets in Cancer: Current Perspectives

Adrienne L. Edkins

Abstract Hsp90 is a molecular chaperone that regulates the function of numerous oncogenic transcription factors and signalling intermediates in the cell. Inhibition of Hsp90 is sufficient to induce the proteosomal degradation of many of these proteins, and as such, the Hsp90 chaperone has been regarded as a promising drug target. The appropriate functioning of the Hsp90 chaperone is dependent on its ATPase activity and interactions with a cohort of non-substrate accessory proteins known as co-chaperones. Co-chaperones associate with Hsp90 at all stages of the chaperone cycle and regulate a range of Hsp90 functions, including ATP hydrolysis and client protein binding and release. Given the ability of co-chaperones to organise the function of the Hsp90 molecular machine, these proteins are now regarded as potential drug targets. Herein the role of selected Hsp90 co-chaperones Hop, Cdc37, p23 and Aha1 as possible drug targets is discussed with a focus on cancer.

Keywords Aha1, Cdc37, Client protein, Co-chaperone, Hop, Hsp90, p23

Contents

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1 Introduction

Heat shock protein 90 (Hsp90) is an important molecular chaperone and a promising drug target. The function of Hsp90 as a chaperone is to regulate the conformation, stability and translocation of an array of cellular proteins known as client proteins. Hsp90 clients number as many as 300 and include a range of signalling intermediates and transcription factors that are vital for cellular function [1, 2]. For a comprehensive and regularly updated list of Hsp90 clients, the reader is referred to the website maintained by the Picard group (http://www.picard.ch/downloads). Hsp90 binds to client proteins when they are in a latent state, stabilising labile intermediates while allowing immediate activation in the presence of the appropriate stimulus [3]. Hsp90 is considered a promising drug target due to the fact that many of the Hsp90 client proteins dependent on this chaperone regulate essential cellular processes and may be considered drug targets in their own right (e.g. Akt, Her2). Hsp90 inhibition is therefore regarded as a mechanism by which multiple different drug targets can be inhibited simultaneously [4]. In particular, Hsp90 inhibitors in clinical development are primarily treatments for cancer, although Hsp90 inhibitors have also shown promise for the treatment of a range of infectious human diseases caused by fungi, parasites and viruses and more recently in protein-folding diseases like Alzheimer's and Parkinson's disease [5–11].

The development of direct inhibitors of Hsp90 was initiated after the discovery that the natural ansamycin antibiotic geldanamycin (GA) was an Hsp90 inhibitor that possesses antitumour properties and could result in the degradation of oncogenic kinases [12]. GA competes with ATP for binding to the N-terminus of Hsp90 [13]. ATP binding and hydrolysis are essential for Hsp90 chaperone activity, a point highlighted by the fact that mutations that disrupt either ATP binding or hydrolysis render Hsp90 incapable of chaperoning client proteins [14–16]. Interestingly, tumour cells appeared more sensitive to GA than normal cells, leading to the analysis of this compound as a putative anticancer treatment. While the exact mechanism has not been defined, the enhanced sensitivity of tumour Hsp90 to GA is thought to result from the fact that Hsp90 in cancer cells is found almost exclusively in a complexed state with co-chaperones and client proteins [17]. The first generation of Hsp90 inhibitor series were derivatives of GA and exclusively targeted the N-terminal domain of Hsp90. However, clinical development of promising GA derivatives such as 17-AAG (tanespimycin) and 17-DMAG (alvespimycin) was prevented by poor results in human trials primarily due to problems with hepatotoxicity (which was also observed with the parent compound) [18]. Second-generation inhibitors of Hsp90 based on new scaffolds through chemical synthesis (as opposed to modification of a naturally occurring parent molecule)

are currently leading the development of Hsp90 inhibitors. These inhibitors include ganetespib (STA-9090) [19], NVP-AUY922, (2,4-dihydroxy-5-isopropylphenyl)-[5-(4-methylpiperazin-1-ylmethyl)-1,3-dihydroisoindol-2-yl]methanone (AT13387) [20, 21] and CUDC-305 [22], which incorporate a range of different chemical scaffolds and yet are exclusively targeted against the N-terminus of Hsp90. Despite the development of numerous inhibitors and clinical trials, there are currently no Hsp90 inhibitors that are routinely used in the clinic. One of the main problems with direct inhibition of Hsp90 is that with N-terminal inhibitors, there is a concomitant induction of a generalised stress response. This leads to transcriptional upregulation of other heat shock proteins (including Hsp70 and Hsp27) by HSF-1 [23]. HSF-1 is held in an inactive form in association with Hsp90, until Hsp90 inhibition leads to dissociation of HSF-1 and translocation of HSF-1 trimers to the nucleus where the induction of stress-responsive genes is the result [24]. The increased expression of an array of Hsp and molecular chaperones can have a cytoprotective effect and lead to resistance towards these N-terminal inhibitors which is undesirable in cancer therapy. Therefore, there has been an increase recently in studies aimed at identifying alternative strategies towards targeting the Hsp90 complex.

2 Co-chaperone Regulation of the Chaperone Activity of the Hsp90 Complex

The progress of Hsp90 clients through the chaperone cycle is dependent on ATP binding and ATPase activity, both of which lead to conformational changes in the protein [25]. The affinity of Hsp90 for its client proteins and co-chaperones is thus regulated by the nucleotide-bound state of the chaperone [26]. The inactive Hsp90 structure forms a “V” shape characteristic of the open conformation, in which the C-terminal regions are constitutively dimerised, while the N-terminal regions are free of nucleotide and separate from each other. In the early phases of the Hsp90 chaperone cycle, the client protein is loaded on to the chaperone by the action of a range of co-chaperones to form an intermediate complex. The binding of ATP induces a conformational change and the formation of the “closed” conformation of Hsp90 in which the N-terminal domains dimerise and hold the client protein [27]. The late stages of the cycle involve ATP hydrolysis which reverts the protein to the “open” conformational state and releases the client protein [26, 28].

Each of the stages of the Hsp90 cycle is controlled by a cohort of co-chaperones, which fine-tune the activity of Hsp90 (Fig. 1). Co-chaperones can be defined as non-client accessory proteins capable of interacting with and modulating the activity of the major molecular chaperones. Structurally, Hsp90 co-chaperones are often classified according to the presence or absence of the TPR domain [29], a degenerate 34-amino-acid repeat that forms a helix–turn–helix motif. Multiple

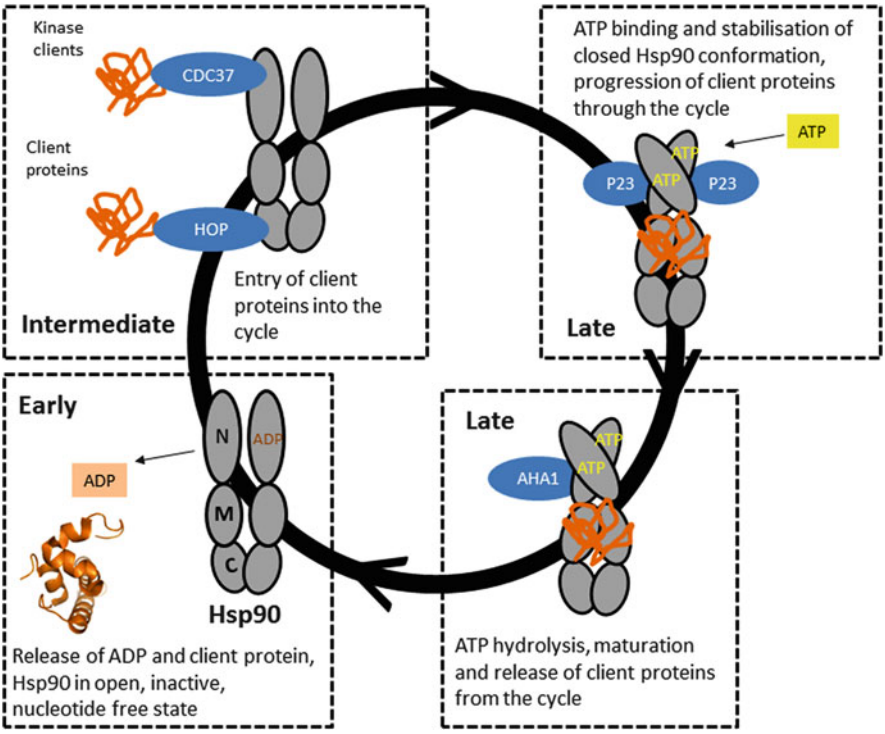

Fig. 1 Schematic diagram of the Hsp90 complex showing roles of co-chaperones Hop, Cdc37, p23 and Aha1 in terms of broad function. In the early phases of the cycle, the Hsp90 dimer is free of nucleotide, C-terminally dimerised and in the open conformation where N-terminal domains are separated. Hop and Cdc37 are intermediate-phase co-chaperones that mediate entry of client proteins into the Hsp90 cycle and bind Hsp90 in a nucleotide-free form. p23 is a late-phase co-chaperone that interacts with ATP-bound Hsp90 and stabilises the closed ATPase-active form of Hsp90. Aha1 is a late co-chaperone that accelerates ATP hydrolysis by Hsp90 and promotes release of client proteins and returns Hsp90 to its inactive conformation

TPR motifs assemble to form a TPR domain with a superhelical groove that forms the interface for interactions with other proteins, including chaperones [29, 30].

The core co-chaperones of Hsp90 (e.g. Hop, p23, Aha1) are considered general cofactors and regulate distinct phases of the Hsp90 cycle independent of the client protein (Fig. 1, Table 1). In addition, there exist a range of more specialised co-chaperones which recruit specific groups of client proteins (e.g. Cdc37), are involved in chaperone function in specific tissues (e.g. Unc45) or are involved in post-translational modifications of Hsp90 (e.g. PP5) [25, 52, 53]. The chaperone activity of Hsp90 is regulated by multiple different types of post-translational modification, including *s*-nitrosylation, phosphorylation and acetylation [28, 54–58]. Given that the different co-chaperones mediate distinct stages of the Hsp90 chaperone cycle, a number of them have been proposed as alternative targets for development of inhibitors.

Table 1 Summary of Hsp90 co-chaperones identified as putative drug targets in cancer

Name	TPR (Y/N)	Domains of Hsp90 bound[a,b]	Function	References
Aha1	N	N and M	Late cofactor mediating client protein release, stimulation of ATPase activity and conformational change, client protein maturation and release	[31–35]
Cdc37	N	N	Early cofactor mediating client entry, inhibition of Hsp90 ATPase activity, specific maturation factor for kinases	[36–39]
p23	N	N and M	Late cofactor, stabilisation of Hsp90-client complexes	[34, 40–44]
Hop	Y	C and N	Early cofactor mediating transfer of client proteins from the Hsp70 to the Hsp90 chaperone complexes, inhibits Hsp90 ATPase while stimulating Hsp70 ATPase	[45–51]

[a]*N* N-terminal ATPase domain, *M* middle domain, *C* C-terminal domain
[b]The main interaction domain is listed first

3 Hsp90 Co-chaperones as Alternate Targets in Cancer

Co-chaperones are a prerequisite for the Hsp90-mediated stabilisation, maturation and activation of client proteins. However, the focus on co-chaperones such as Hop, Cdc37 and Aha1 as potential cancer drug targets is relatively recent. Excitingly, new studies show that co-chaperone inhibition or depletion reverses cancer phenotypes such as drug resistance, metastasis and invasion [59–61]. Despite the fact that Hsp90 is highly conserved, there is evidence to suggest that the chaperone is biochemically different in cancer [17]. One of the main reasons for this difference is that in tumours Hsp90 is thought to exist almost exclusively as a higher-order heterocomplex, while Hsp90 in normal tissues largely exists in a latent, uncomplexed state [17, 62]. Co-chaperones are the major component of these multi-protein complexes, and hence Hsp90 may be more dependent on co-chaperones in malignant cells [63]. Indeed, changes in co-chaperones can include the sensitivity of yeast cells to Hsp90 inhibition [63]. In addition, co-chaperones may represent more selective drug targets, particularly with respect to targeting of cytosolic versus organelle isoforms of Hsp90. The structural similarity between the ATPase domains of the cytosolic and organelle Hsp90 means that organelle Hsp90, TRAP1 and Grp94 can be inhibited by N-terminal inhibitors like GA [64, 65]. In contrast, cytosolic Hsp90 has a wide range of co-chaperones, while TRAP1 and Grp94 do not appear to interact with these co-chaperones [66, 67]. As yet, there are no reports of bona fide co-chaperones for TRAP1 or Grp94, although Grp94 activity is regulated by some non-client proteins similar to co-chaperones (e.g. CNPY3 and ASNA1) [64, 68, 69].

The potential for certain co-chaperones to be drug targets is considered herein. The focus of this review is the Hop, Cdc37, Aha1 and p23 co-chaperones, for which

there is a defined role in cancer biology, preclinical data to suggest they may be putative therapeutic targets and/or inhibitors that have been identified.

4 Co-chaperones That Mediate Delivery of Client Proteins to Hsp90

4.1 Hsp90–Hsp70 Organising Protein (Hop/STIP1/STI1)

The early stages of the Hsp90 chaperone cycle involve the loading of client proteins onto Hsp90. This often involves the transfer of the client protein from the Hsp70 chaperone machine to the Hsp90 chaperone complex, a process mediated by the co-chaperone Hop [46, 70]. While Hop is not an essential gene in yeast, its role is key to development [71] and Hop knockout in mice is embryonic lethal [72]. Structurally, Hop contains two DP domains (DP1 and DP2, domains rich in proline and aspartic acid) and three TPR motifs (TPR1, TPR2A and TRP2B) that are able to discriminate between the C-terminal EEVD motifs of Hsp70 and Hsp90 [73, 74]. Binding of Hop to chaperones occurs via two-carboxylate clamp interactions with the C-terminal EEVD motif in either Hsp70 or Hsp90 [73, 75] (Fig. 2). TPR1 and TPR2B bind to the Hsp70 GPTIEEVD with high affinity, while the Hsp90 MEEVD peptide is bound by the TPR2A domain [51, 73, 75–77] (Fig. 2). Thus, monomeric Hop binds simultaneously to Hsp70 and Hsp90 through different TPR domains and acts as a scaffold for the transfer of client proteins [78]. While the primary binding site for Hop is the C-terminal region of the chaperones, there is evidence from more recent reports that Hop also interacts with N-terminal regions of Hsp90 [76]. While bound to Hsp90, Hop inhibits the ATPase activity of Hsp90 by preventing dimerisation of the N-terminal domains of the Hsp90 dimer, thus promoting its open conformation and facilitating client protein binding [51, 79, 80].

Hop has recently been shown to be the first co-chaperone to have independent ATPase activity, which might suggest independent chaperone capabilities [81]. Indeed, Hop has also been shown to interact directly with some cellular proteins apparently independently of Hsp90 [61, 82]. In particular, Hop plays a major role as a receptor for the prion protein during development which is independent of Hsp90 [46, 72].

4.1.1 Validating a Role for Hop in Cancer

There has been an increased interest in the role of Hop as an oncogenic co-chaperone, after observations that Hop levels are increased in SV40-transformed cells [48], as well as a range of solid tumours, including cancers of the pancreas [83], colon [62], breast [61] and liver [84]. A number of studies using RNA interference to deplete Hop levels have validated that Hop has pro-tumour effects

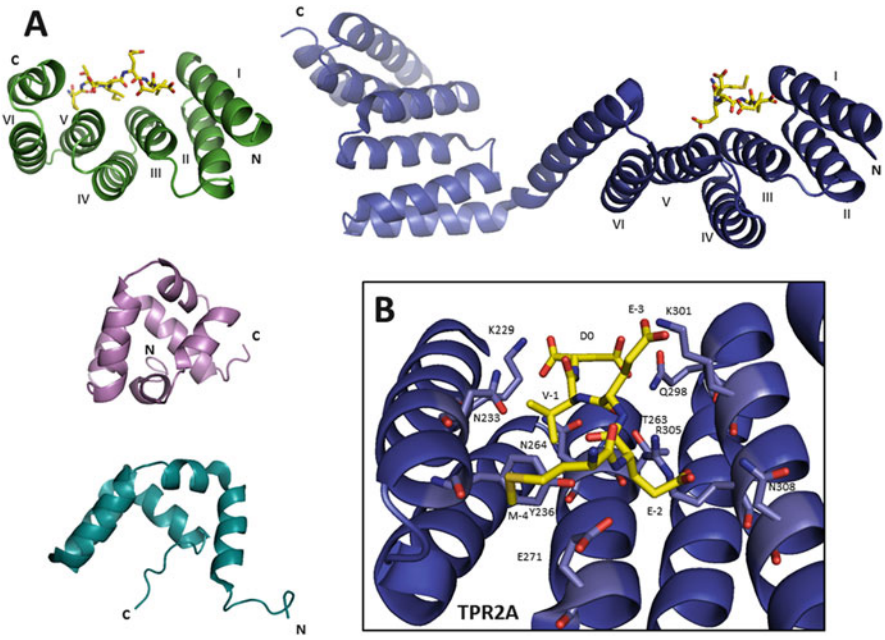

Fig. 2 Structural domains of Hop and interaction with C-terminal EEVD peptides. (**a**) Cartoon representations of TPR1 (1ELW, *green*, residues 2–116), TPR2AB (3UQ3, *dark blue*, residues 258–515), DP1 (2LLV, *pink*, residues 127–196) and DP2 (2LLW, *cyan*, residues 519–586) generated in PyMOL (DeLano Scientific). In TPR1 and TPR2AB, the GPTIEEVD (Hsp70) and MEEVD (Hsp90), respectively, are shown as *sticks* and coloured *yellow*. The three TPR motifs that comprise the TPR domains are made up of two helices and are shown for TPR1 and TPR2A (motif 1, helices I and II; motif 2, helices III and IV; motif 3, helices V and VI). The six helices of the three TPR motifs pack together to form the characteristic alpha-helical TPR domain structure. (**b**) Details of the interaction between TPR2A (1ELR, *dark blue*) and the C-terminal MEEVD peptide from Hsp90 (*yellow*, numbered D0 to M-4). Residues Y236, E271, N264, N233, K229, K301, Q298, T263, R305 and N308 from TPR2AB are involved in the interaction with the Hsp90 MEEVD motif and extend from the inner surfaces of helices I–VI. The equivalent residues in TPR1 are not shown but these are R77, K50, N43, N12, K8, S42, K73 and S76 [51, 75]

in a wide range of cancer types. Transient knockdown of Hop in murine embryonic stem cells inhibited nuclear translocation of Stat3 and prevented formation of embryoid bodies, indicating a suppression of the malignant phenotype [85]. Hop levels were higher in more aggressive pancreatic cell lines, and depletion of Hop in Panc-1 and BxPc-3 using RNA interference (RNAi) reduced invasion and migration of these cells by approximately 50% compared to the equivalent cell lines treated with non-specific short interfering (si)RNA [86]. Hop knockdown also reduced cell migration by 40% relative to control cells in both MDA-MB321 and Hs578T breast cancer cells, as well as human vascular endothelial cells (HUVEC) (by between 40 and 60% relative to controls depending on which siRNA was used) [61, 82]. The reduced invasion and migration of cancer cells upon Hop depletion is due to the fact that Hop associates with Hsp90 clients that control migratory and

invasive processes, including matrix metalloproteinase 2 (MMP2), actin and tubulin [61, 82, 86]. Interestingly, when Hop expression was reduced by RNAi, cells grew at a similar rate to cells expressing control siRNA, suggesting that Hop does not appear to play a major role in cell proliferation [82]. This is despite the fact that Hop knockout mice do not survive past E10.5 stage in embryogenesis [72]. Taken together, these data indicate a unique role for Hop in development and not just general cell growth [72]. The knockdown studies demonstrate that Hop regulates discrete cellular processes that are important for cancer biology, and therefore inhibition of Hop function may be one mechanism by which to develop anticancer agents.

4.1.2 Targeting the Interaction of Hop with Hsp90

Hop-dependent client transfer to the Hsp90 chaperone is the first step in the chaperone cycle, and therefore modulation of the activity of Hop is a possible bottleneck at which to regulate client proteins that associate with Hsp90 and prevent their proper folding and maturation [78]. The most common approach to inhibit Hop has been to target the interaction between Hop and Hsp90 or Hsp70. TPR peptide analogues or small molecules capable of blocking the binding of the Hop TPR domains from interacting with Hsp70 or Hsp90 are currently the main strategy employed to achieve this inhibition [87–91].

Horibe and colleagues designed a peptidomimetic based on the binding interface of the TPR2A domain in an attempt to block the main interaction site between Hop and Hsp90 [89]. The peptide (sequence KAYARIGNSYFK) was designed to incorporate two critical residues required for binding to Hsp90 (K301 and R305, underlined in the peptide sequence) (Fig. 3). The TPR peptide bound to Hsp90 in vitro with an affinity of 1.42×10^{-6} M, similar to the affinity of the in vitro interaction between Hsp90 and full-length Hop (4.43×10^{-6} M). Hsp70 was also able to bind the TPR peptide, although its binding ability was approximately half of that of Hsp90 [89]. Despite the fact that the TPR peptide bound to Hsp70, it did not disrupt the interaction between Hsp70 and Hop in vitro. In contrast, the TPR peptide specifically inhibited the in vitro interaction of Hsp90 and Hop but not the TPR-containing FKBP or PPP5 co-chaperones at concentrations of higher than 140 μM. In order to assess the activity of this peptide in cell lines, the TPR peptide was subsequently fused to a sequence from helix III of the cell-penetrating Antennapedia homeodomain protein to render it cell permeable (generating the hybrid Antp-TPR peptide of sequence RQIKIWFQNRRMKWKK-KAYARIGNSYFK). This Antp-TPR peptide was compared to three control peptides (RQIKIWFQNRRMKWKKKAYAAAGNSYFK, Antp-TPR mutant 1; RQIKIWFQNRRMKWKKKAYARIGNSGGG, Antp-TPR mutant 2; and RQIKIWFQNRRMKWKKRKFSAAIGYNKY, Antp-scramble) for its anticancer activity in vitro and in mouse models [88, 90].

The Antp-TPR displayed anticancer activity in a range of cell lines representative of different cancers, including renal cancer (Caki-1; IC50 47.9 μM), pancreatic

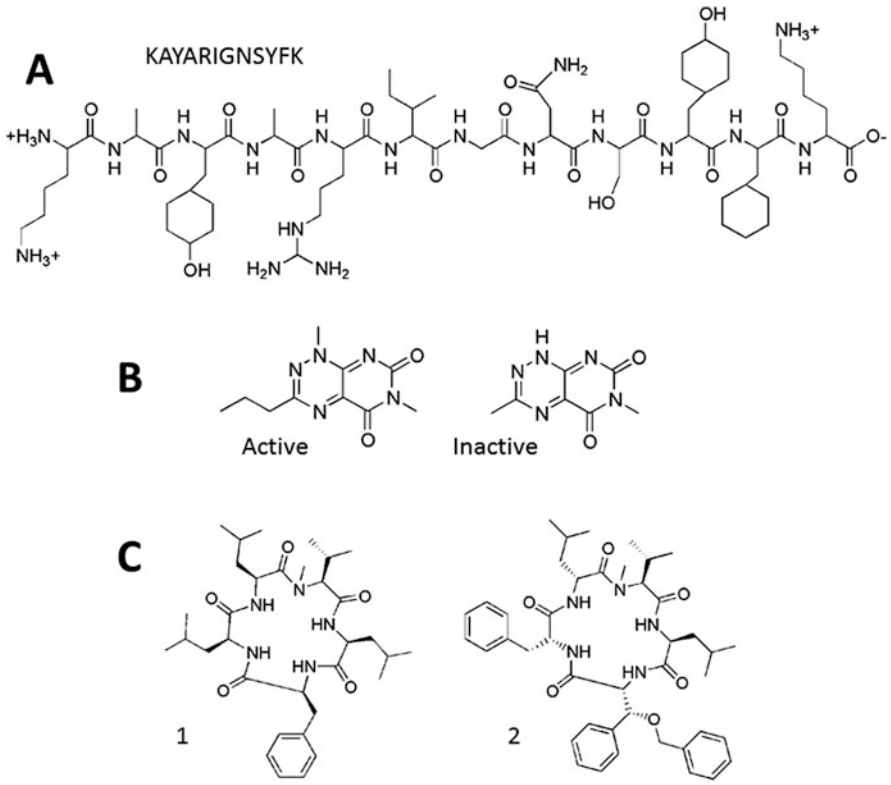

Fig. 3 Inhibitors of the interaction between Hsp90 and Hop. (**a**) Structure of the Antp-TPR peptide (KAYARIGNSYFK) that competes with Hop TPR2A for binding to Hsp90. (**b**) C9 (1,6-dimethyl-3-propylpyrimido[5,4-e][1,2,4]triazine-5,7-dione) and an inactive analogue. C9 binds to TPR2A and blocks the interaction with Hsp90 and Hop. (**c**) Sansalvamide A analogues 1 and 2 which act as allosteric inhibitors by binding to the middle domain of Hsp90 and disrupting interactions with multiple C-terminal binding co-chaperones, including Hop [88–90, 92, 93]

cancer (BXPC3; IC50 44.8 μM), breast cancer (T47D, MDA-MB-231, BT20; IC50 19.4, 56.9, 37.4 μM, respectively), lung cancer (A549; IC50 65.9 μM), prostate cancer (LNCaP; IC50 56.7 μM) and gastric cancer (OE19; IC50 33.4 μM). Equivalent concentrations of the control peptides were not toxic in the same cell lines [89]. The authors have also subsequently shown that this peptide is toxic to leukaemic and glioblastoma cell lines [88, 90]. Most interestingly, the toxic effect of the Antp-TPR peptide appeared selective to cancer cells, with IC50 values greater than 100 μM determined for cell line models representative of non-cancerous cells (HEK293T, MRC5, PE). The Antp-TPR peptide induced apoptosis in the cancer line T47D and not the non-cancerous HEK293T cell line. Treatment of cancer cell lines with the Antp-TPR peptide led to the loss of multiple cancer-associated Hsp90 clients, including protein kinases such as Akt, Bcr-Abl, v-SRC and CDK4, but importantly did not induce the upregulation of Hsp70

[89]. Antp-TPR was also shown to be effective in vivo, using the BXPC3 cell line in a xenograft mouse model of pancreatic cancer. Intravenous injections of Antp-TPR (1 or 5 mg/kg three times per week) resulted in a statistically significant reduction in tumour volume (to approximately half of the control saline-treated group) and depleted Hsp90 clients like CDK4 in the treated tumours [89]. These are promising data, despite the fact that IC50 values of the Antp-TPR peptide reported are still relatively high and that the authors did not compare the effect of the Antp-TPR peptide to that of the control peptides in the in vivo study.

Small molecules have also been shown to disrupt the interaction of Hsp90 and Hop. Pimienta and colleagues reported that the compound 1,6-dimethyl-3-propylpyrimido[5,4-e][1,2,4]triazine-5,7-dione (or C9 for short) was able to bind to the TPR2A domain of Hop and disrupt the interaction of Hop and Hsp90 [94]. C9 was also toxic to triple-negative breast cancer cell lines MDA-MB-231 (IC50 2 μM) and MDA-MB-468 (IC50 1.75 μM) within 24 h of treatment, whereas another compound containing the same 7-azapteridine ring system but that did not disrupt the Hsp90–Hop interaction was non-toxic to these cells (IC50 $>$ 100 μM). C9 induced cell cycle arrest but not caspase-3/7-mediated apoptosis. Similar concentrations of C9 were not toxic to normal fibroblast cells nor did they induce caspase-3/7-mediated apoptosis in these cells. A combination of C9 and the Hsp90 inhibitors 17-AAG and NVP-AUY922 (but not PU-H71) reduced the lethal IC50 value of C9 to 0.5 μM and 1 μM, respectively. C9 further inhibited both the linear migration (as measured by wound healing assay) and anchorage-independent growth (as measured by sphere formation assay) of MDA-MB-231 cells, whereas 17-AAG was ineffective [91]. However, it should be noted that C9 was used at 3 times the concentration of 17-AAG in these assays (i.e. 3 vs 1 μM). Importantly, C9 did not induce a stress response similar to that seen with 17-AAG. The levels of HSF-1, Hsp70 and Hsp27 were reduced in C9-treated cells compared to control and 17AAG-treated cells, as were a number of Hsp90 client proteins (CDK4, JNK1 and p38). This indicates that although C9 is effective in destabilising Hsp90 client proteins, it does not induce a compensatory stress response. The loss of these client proteins was potentiated by co-treatment of MDA-MB-231 cells with C9 and 17-AAG. Interestingly, C9 was also able to block the increases in levels of Hsp27 and HSF-1, but not Hsp70, by 17-AAG. C9 also resulted in an unexpected depletion of the levels of another Hsp90 co-chaperone, Cdc37, which may account in part for its activity. Cdc37 is a kinase-specific co-chaperone and therefore its loss may explain the reduction in the levels of JNK1 and p38 kinases seen with C9 treatment. Unfortunately, there are no data available on the in vivo anticancer activity of C9 [91].

A third class of compounds has also been shown to disrupt the interaction of multiple C-terminal binding proteins, including the co-chaperone Hop. These are macrocyclic structures that are analogues of the compound sansalvamide A (San-A) [87, 93]. Two compounds were studied in detail for their effect on the interaction of Hsp90 and C-terminal binding proteins. These compounds were compound 1, which is a single peptide analogue of the natural product sansalvamide A (henceforth referred to as San-A 1), and compound 2, which includes the

macrocyclic core with three D-amino acids and a phenyl residue at position 1 (henceforth referred to as San-A 2) (Fig. 3). San-A amide analogue 1 binds to Hsp90 with an affinity of 20 μM, while the San-A amide analogue 2 binds with a greater affinity of 3.6 μM and is ten times more cytotoxic than San-A 1 [87, 95]. The toxicity of San-A 1 (at 50 μM) and San-A 2 (5 μM) to HeLa cells was enhanced upon RNAi-mediated depletion of Hsp90 and reduced upon Hsp90 overexpression. A similar although less pronounced effect was observed with the Hsp90 inhibitor 17-AAG. These data suggested that the compounds act via an Hsp90-dependent mechanism. This was further supported by the fact that concentrations over 5 μM of San-A 1 and San-A 2 both increased Hsp70 levels in HCT-116 cells (by 25% and 85%, respectively, compared to 17-AAG which was taken as 100%). The mechanism of cell death by San-A 2 was determined to be through induction of caspase 3-dependent apoptosis in treated HCT-116 colon carcinoma cells culminating in the cleavage of PARP [87]. In contrast to the Horibe and Pimienta studies [89, 91], these compounds do not compete directly with Hop for binding to the Hsp90 EEVD motif. Instead, the San-A analogues are allosteric inhibitors that bind to the middle domain of Hsp90 [93]. This binding induces a conformational change that displaces four C-terminal Hsp90-binding proteins, three of which are TPR-containing co-chaperones (FKBP38, FKBP52 and Hop) [87, 93]. San-A 1 reduced the proportion of Hsp90 bound to FKBP52 and FKBP38 to a maximum of 20% of the control at concentrations above 5 μM and 3 μM, respectively, while San-A2 reduced the proportion of Hsp90 bound to FKBP52 and FKBP38 to a maximum of 20% of the control at concentrations above 2 μM and 1 μM, respectively. In terms of Hop, San-A 1 and San-A 2 reduced binding of Hsp90 to a maximum of 50% and 35%, respectively, at concentrations above 1 μM. Control compounds used at similar concentrations did not affect the interaction of Hsp90 and any of the three co-chaperones assessed. Interestingly, the San-A analogues were able to induce a greater maximum reduction in the binding of FKBP38 and FKBP52 to Hsp90 than Hop [87]. This most likely reflects the differences in the interaction interfaces or binding sites between Hsp90 and these different co-chaperones.

In the studies described above, some of the effects of Hop knockdown appear different from the effects of disrupting the interaction between Hop and Hsp90 with a small molecule or peptide. This is most notable for the effect on cell growth, where compounds that inhibit Hsp90–Hop interaction appear toxic [87–91], while cell growth was largely unaffected by Hop knockdown [61, 82]. This may be due to off-target effects of the compounds compared to the RNAi or possibly due to hereto undefined perturbations to Hsp90 function caused by the compounds that do not occur with Hop depletion. It is tempting to speculate that there may also be compensation by other co-chaperones in the absence of Hop; a co-chaperone whose binding to Hsp90 is also inhibited directly or indirectly by TPR directed inhibitors. Irrespective of this, these reports provide proof of concept that inhibition of the interaction between Hsp90 and Hop can effectively deplete oncogenic Hsp90 client proteins and selectively inhibit the growth of cancer cells over normal cells and validate this interface as a putative drug target.

4.2 Cell Division Cycle 37 (Cdc37)

Cdc37 is a specialised co-chaperone that is involved in the selective recruitment of kinase client proteins to the Hsp90 complex [37, 96–100]. Cdc37 is a non-TPR-containing co-chaperone and binds to the lid segment of the N-terminal domain of Hsp90 via its C-terminus, while the N-terminus of Cdc37 is bound to the client kinase [97, 101]. Hsp90 and Cdc37 interact as dimers and with a 1:1 stoichiometry. The interaction interface (~1056 A) between Cdc37 and Hsp90 is largely hydrophobic in nature but is stabilised by a network of polar interactions (Fig. 4)

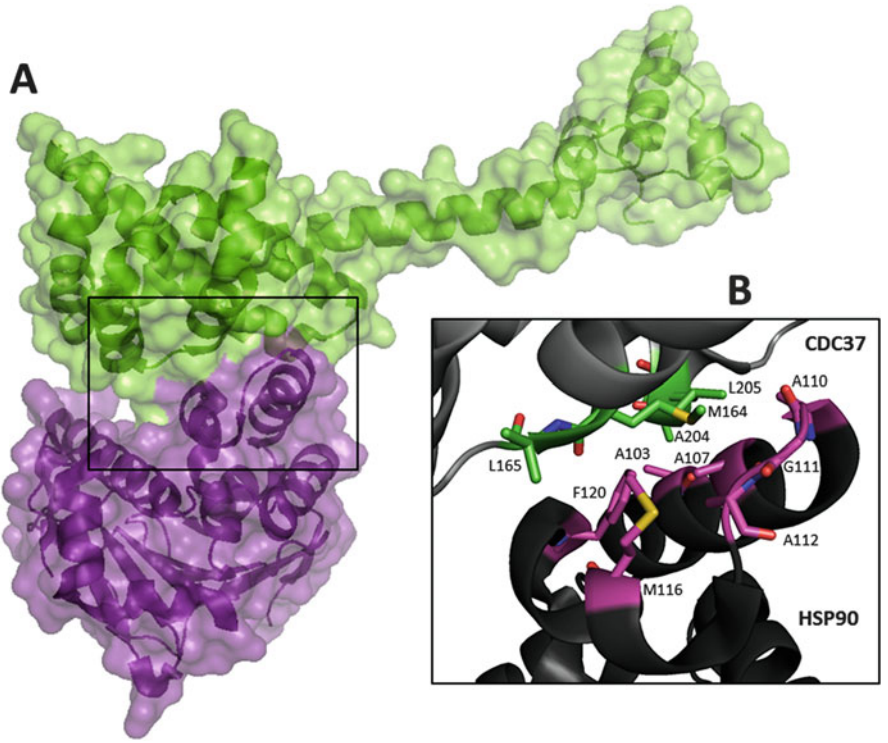

Fig. 4 Interaction of Hsp90 with the co-chaperone Cdc37. (**a**) Global view of the interaction of the N-terminal region of Hsp90 (*magenta*, residues 1–214) with the C-terminal region of Cdc37 (*green*, residues 125–378). The interaction interface is buried 1056 A of the molecular surface. (**b**) Residues involved in the interaction between Hsp90 (*dark grey*, residues shown in *magenta*) and Cdc37 (*dark grey*, residues shown in *green*) are involved in hydrophobic interactions. The interacting residues from Hsp90 (A103, A107, A110, G111, A112, M116, F120) are mainly exposed residues on the face of the Hsp90 lid structure, while the interacting residues from Cdc37 (M164, L165, A204, L205) arise from the loop between the first two helices and the beginning of the third helix. The hydrophobic interactions are stabilised by a network of polar interactions (not shown). The images were generated using PyMOL (DeLano Scientific) using the structure 1US7 [100]

[100]. Binding of Cdc37 to the N-terminal domain of Hsp90 inhibits the ATPase activity of Hsp90 by physically blocking the binding of ATP and N-terminal dimerisation of Hsp90 [100, 102]. As the kinase-binding and Hsp90-interacting domains of Cdc37 are distinct from each other, Cdc37 can bind simultaneously to the client protein and to Hsp90, thereby acting as a scaffold to recruit the kinase to Hsp90 [103]. Cdc37 and Hsp90 regulate conformational changes in the kinase client proteins, stabilising inherently labile kinases until activation [38, 60, 96, 104]. Up to 50% of the kinome of yeast has been shown to be dependent on Cdc37 for stability [105] and kinases make up the largest group of Hsp90 client proteins [106]. Inhibition of Cdc37 has therefore been regarded as an option for the selective inhibition of the family of Hsp90 kinase client proteins [107]. Recent studies have also suggested that the stability of kinases and hence the dependence on the Hsp90–Cdc37 chaperone complex vary with respect to the kinase in question and not necessarily the activation status of the kinase [108]. In addition to its role as a co-chaperone for Hsp90, Cdc37 has independent chaperone activity wherein it is able to stabilise certain client proteins independently of Hsp90 [96, 105, 109]. As such, Cdc37 can compensate for, but not replace, a reduction in Hsp90 function [96].

4.2.1 Validating a Role for Cdc37 in Cancer

Many Cdc37 clients are members of the cyclin-dependent kinase (CDK) family that are required for progression through the cell cycle and are components of the activated signalling cascades in malignancy [96, 110–114]. Some of the most important oncogenic kinases, including Src, Her2, Akt, Cdk2, Cdk4, KIT and EGFR, interact with, are stabilised by and require Cdc37 for activity [99, 106, 115–119]. Therefore, Cdc37 was expected to play an important role in malignancy. Indeed, elevated levels of Cdc37 protein have been observed in some cancers, and this overexpression has been correlated with an increase in cell proliferation through the activation of kinase pathways [107]. The highest expression of Cdc37 has been observed in tissues with high proliferation rates, consistent with the role of kinases in mediated pro-growth pathways [119]. Overexpression of Cdc37 was sufficient to induce robust and dose-dependent activation of the kinase Raf-1, an important mediator of the mitogenic MAPK signalling pathway in cells [120]. However, Cdc37 may regulate more than just cellular proliferation rates. Mouse models of breast cancer incorporating the MMTV-Cdc37 transgene show comparable rates of tumour development to mouse breast cancer models with the MMTV-cyclin D transgene, suggesting that Cdc37 functions as an oncogene [113]. Cdc37 also promoted tumour formation in response to expression of MMTV-c-myc and MMTV-cyclin D transgenes, implying that kinase stability and activation via Cdc37 are a key component of kinase-dependent tumour formation [113]. Overexpression of Cdc37 was observed in both malignant and premalignant prostate cancer, again suggesting that Cdc37 may be required for acquisition of a cancerous state [121]. Elevated levels of Cdc37 in hepatocellular carcinoma were

associated with an increase in Hsp90–Cdk4 and Cdc37–Cdk4 complexes that correlated with a poor prognosis [110], and the combined effect of Cdc37 and cyclin D1 may worsen the progression of multiple myeloma [122].

Depletion or dominant negative forms of Cdc37 inhibit cellular growth and promote apoptosis. Knockdown of Cdc37 in HCT116 colon cancer cell line by RNAi resulted in the proteasomal degradation of a number of kinases including Her2, Cdk4, Cdk6 and activated Akt, which are all important in cancer cell survival [60]. Loss of Cdc37 in these cells reduced kinase association with Hsp90 and led to a concomitant reduction in multiple kinase signalling pathways (including MAPK, PI3K and GSK3β) which inhibited cell proliferation by inducing cell cycle arrest at G1/S [60]. The effect appears specific to kinase clients, as loss of Cdc37 did not affect the levels of the non-kinase client, survivin. The combination of Cdc37 silencing with the Hsp90 inhibitor 17-AAG further enhanced the loss of kinases, reductions in kinase signalling and cell cycle arrest observed with Cdc37 silencing alone, suggesting that the effects of depletion of Cdc37 are mediated via the Hsp90 complex [60]. These data also suggest that inhibiting Cdc37 may be effective as a combination therapy with an Hsp90 inhibitor. Importantly, RNAi-mediated depletion of Cdc37 in HCT116 colon cancer cells did not induce expression of Hsp70, although depletion of Cdc37 was not able to prevent upregulation of Hsp70 in response to 17-AAG [60].

Depletion of Cdc37 by RNA interference also reduced growth in both androgen receptor (AR)-positive (LNCaP) and androgen receptor-negative (PC3, DU145) prostate cancer cell models over a 72-h period [123]. In contrast to the study in the HCT116 colon cancer cell line, Cdc37 depletion in the DU145 and PC3 prostate lines did not reduce the levels of Hsp90 kinase clients EGFR, Raf-1, Akt and Cdk4. However, while loss of Cdc37 did not deplete the levels of client proteins, it did result in the inactivation of mitogenic signalling pathways. This occurred by depletion of the active form of certain kinase client proteins, including reducing levels of phospho-Akt, phospho-ERK1/2, phospho-GSK3β and phospho-S6 ribosomal protein [123]. Similar to the study in colon cancer, Cdc37 depletion sensitised DU145 and PC3 cells to treatment with 17-AAG and led to reduced clonogenicity. However, in this context and in contrast to HCT116 colon cancer lines, Cdc37 knockdown also prevented the upregulation of Hsp70 in 17-AAG-treated prostate cancer cells [123]. AR is the only non-kinase client of Cdc37 and certain prostate cancers are known to be maintained by androgen signalling. In the same study, Cdc37 depletion also reduced androgen signalling in the LNCaP androgen receptor-positive cell line. Cdc37 depletion reduced the activation of the prostate-specific antigen (PSA) promoter, leading to reduction in the levels of PSA in shCdc37-treated cells compared to controls [123].

Cdc37 has also been shown to be an important mediator of malignancy in hepatocellular carcinoma (HCC). In a cohort of 91 HCC patients, a significant increase in the levels of both Cdc37 transcript and protein was identified in HBV-associated HCC [124]. Validating these effects in HCC cell lines (HepG2 and Huh7) in vitro and in vivo, the authors demonstrated that RNAi-mediated depletion of Cdc37 was associated with a loss of cell proliferation, increase in

apoptosis, decrease in colony formation potential and a slower rate of tumour growth in xenograft models. The anticancer effects were associated with cell cycle arrest at G1 due to a loss of cyclin D1 and Cdk4 [124]. Cdk4 is known to be stabilised by Cdc37 and Hsp90 [119, 125]; Cdc37 is involved in cyclin D1-induced transformation of mammary tissues [113], and both Cdk4 and cyclin D1 occur in a common complex with Cdc37 [125, 126].

Cdc37 has also been specifically associated with the development of gastrointestinal stromal tumours (GIST), particularly those associated with gain-of-function mutations in the oncogenic kinases KIT or PDGFRA [118]. These oncogenic mutations mean that GIST has a requirement for Hsp90 chaperoning activity and can often be treated successfully with tyrosine kinase inhibitors. The development of resistance to tyrosine kinase inhibition through heterogeneous mutations in the kinase domain of KIT renders the kinase highly sensitive to Hsp90 inhibition. However, Hsp90 inhibition has not been used successfully for GIST in a clinical setting. In a study to identify alternative drug targets for KIT-dependent GIST, Cdc37 was identified as a potential target [118]. Survival of GIST lines required Cdc37, which could be attributed to the requirement for stability and activation of mutant KIT and its downstream signalling pathways by Cdc37. This was true for both kinase inhibitor-sensitive and, importantly, kinase inhibitor-resistant KIT tumours. In addition, knockdown of Cdc37 resulted in sustained (>20 days) loss of KIT, which was in contrast to the effects of Hsp90 inhibition [118].

Taken together, these studies demonstrate a role for Cdc37 as a promoter of cellular growth and malignancy in a wide range of cancer types, particularly those dependent on oncogenic protein kinases, and suggest that Cdc37 may therefore be a putative drug target.

4.2.2 Targeting Cdc37 in Cancer

As tumour cells are often dependent on activation of kinase signalling pathways for survival, it is predicted that cancers will be more sensitive to Cdc37 inhibition than normal cells [107]. Indeed, targeting kinases directly has been a clinically successful strategy for cancer treatment [127]. Many kinases that are mutated in cancer may be more dependent on Hsp90 and, given the role of Cdc37 in mediating entry of kinases to the Hsp90 complex, could make them more susceptible to inhibition of Cdc37. As multiple kinase clients of Hsp90 have been shown to interact with Cdc37, it is possible that targeting Cdc37 may be a strategy to simultaneously inhibit multiple kinases [38, 97, 99, 104, 107, 109, 116, 119, 128–130]. Cdc37 activity has been linked to the efficacy of clinical kinase inhibitors, including vemurafenib and lapatinib, which act by competing with ATP for binding to protein kinases [131]. Studies demonstrate that the effect of these compounds may, at least in part, be due to the prevention of Cdc37 binding. The kinase inhibitors were able to prevent binding of kinases to Cdc37, thereby preventing access to the Hsp90 chaperone complex [131]. This suggests that some kinase inhibitors may act both directly, on the kinase itself, and indirectly, via inhibiting the interaction with

Cdc37. Taken together, this may suggest that an inhibitor of Cdc37 may enhance the activity of kinase inhibitors and therefore may be a useful strategy for combination therapy.

Similar to Hop, a main strategy of pharmacological inhibition of Cdc37 has been directed at the interaction with Hsp90. Celastrol is a natural quinone methide triterpene from compound isolated from the bark of *Tripterygium* species (Fig. 7). Co-immunoprecipitation studies from pancreatic cancer cell lines indicated that celastrol was able to disrupt the direct interaction between Hsp90 and Cdc37 [132]. The action of this compound is predicted to occur by blocking the interaction site between Hsp90 and Cdc37 and preventing Hsp90 ATPase activity without inhibiting ATP binding [133]. Celastrol induced degradation of kinases Akt and Cdk4, induced apoptosis in pancreatic cancer cell lines (Panc-1) and prevented metastasis in a mouse model of pancreatic cancer [132]. However, celastrol also induced expression of Hsp70 and other Hsp, due to its ability to upregulate HSF-1 [133, 134], which is undesirable in a cancer therapy.

Interestingly, depletion of Cdc37 and inhibition of the Cdc37-Hsp90 interaction with celastrol do not necessarily produce the same effects. In GIST cancers dependent on KIT kinase mutations, Cdc37 knockdown of the co-chaperone resulted in prolonged depletion of KIT and reductions in GIST viability [118]. In contrast, treatment of GIST with celastrol did not substantially reduce the levels of KIT. Additionally, celastrol treatment did result in reduced cell viability, but this was not kinase selective and occurred in both KIT-dependent and KIT-independent GIST [118]. These data could be interpreted to suggest that either the effects of celastrol are not selective to the inhibition of the Cdc37–Hsp90 interaction or that kinase clients are more dependent on Cdc37 itself rather than the interaction between Cdc37 and Hsp90. A recent report from the Workman group has suggested that targeting the interaction between Cdc37 and Hsp90 does not necessarily substantially compromise the stability of certain kinase client proteins [135]. The authors demonstrated in colon cancer cell lines (HCT116 and HT29) that overexpression or depletion of Cdc37 increased or decreased, respectively, the levels of the model Hsp90 client kinase, Cdk4. While Cdc37 overexpression increased the proportion of Cdk4 in Hsp90 complexes, overexpression of truncated or mutated Cdc37 proteins showing reduced or no Hsp90 binding unexpectedly did not affect Cdk4 expression or activity [135]. These Cdc37 variants in fact enhanced loading of Cdk4 into the Hsp90 complex similar to wild-type Cdc37 and were capable of stabilising other kinase client proteins (Cdk6, Raf and ERBB2) in addition to Cdk4. Overexpression of Cdc37 was however not able to rescue the effects of Hsp90 depletion on kinase clients, suggesting that Cdc37 and Hsp90 do not have redundant roles [135]. Taken together, these data suggest that while the role of Cdc37 in chaperoning kinases does require Hsp90, this action is not necessarily dependent on the direct interaction of Cdc37 and Hsp90. From a therapeutic perspective, direct targeting of Cdc37, rather than inhibiting the interaction of Cdc37 and Hsp90, may therefore be a more viable and selective strategy for treatment of kinase-driven malignancies.

5 Co-chaperones That Mediate Hsp90 Client Protein Maturation

5.1 Activator of Hsp90 ATPase (Aha1 and Hch1)

Aha1 is a non-TPR-containing co-chaperone that is a late cofactor and the most potent activator of Hsp90 ATPase activity described to date [33, 34]. Hch1 (also known as Aha2) is considered a homologue of Aha1 and shares approximately 40% sequence identity with the N-terminal region of Aha1. Hch1 acts as a weak stimulator of Hsp90 ATPase activity, although recent experiments in yeast suggest that the activity of Hch1 is distinct from that of Aha1. Hch1 deletion in yeast cells expressing wild-type or mutant Hsp90 led to an increase in resistance of yeast strains to the Hsp90 inhibitor, NVP-AUY922 [136], while exogenous expression of Hch1 in Hch1-null yeast reversed this resistance. Interestingly, depletion of Aha1 did not have any effect on the sensitivity of yeast cells to NVP-AUY922 [136]. The loss of Hch1 also appeared to stabilise Hsp90 mutants (G313S and A587T) normally reliant on Hop for activity. The potential of Hch1 as a drug target for fungal infections is certainly suggested by these studies in yeast, particularly since expression is restricted to lower eukaryotes.

The binding groove on Hsp90 for Aha1 is formed upon N-terminal dimerisation of the chaperone, and Aha1 binding induces a stable Hsp90 dimer (Fig. 5). A single monomer of Aha1 interacts with the N and M domains of Hsp90 dimer and induces a specific conformation that favours ATP hydrolysis [33, 35, 138, 139]. The asymmetric binding of Aha1 to these sites on Hsp90 is consistent with co-binding of other co-chaperones and client proteins to other sites on the chaperone [35]. Aha1 itself does not interact directly with any of the other co-chaperones, but it does compete in part with Hop and Cdc37 for Hsp90 binding. While Hop and Aha1 could form a ternary complex with Hsp90, the Kd of in vitro binding of Aha1 to Hsp90 was increased in the presence of Hop (from 3.8 to 32 μM). There are conflicting reports on whether or not the binding of p23 and Aha1 to Hsp90 is mutually exclusive [31, 35, 140–142], although recent in vitro binding suggests that Aha1 increases the binding affinity of p23 for Hsp90 (1.25 to 35 μM). The binding of late-cycle co-chaperones Cpr6 and Cpr7 does not interfere with Aha1 binding to Hsp90 [143].

5.1.1 Validating a Role for Aha1 in Cancer

Aha1 is not an essential gene, but knockout in yeast or RNAi-mediated depletion in HEK-293 cells has been shown to affect specific Hsp90 clients, including the oncogenic kinase Src and hormone receptors [143]. In mammalian cells, Aha1 was differentially expressed in a range of melanoma, colon, ovarian, breast and prostate cancer cell lines, and expression was induced upon treatment of tumorigenic (HCT116) colon cancer and (A2780) human ovarian cancer cells

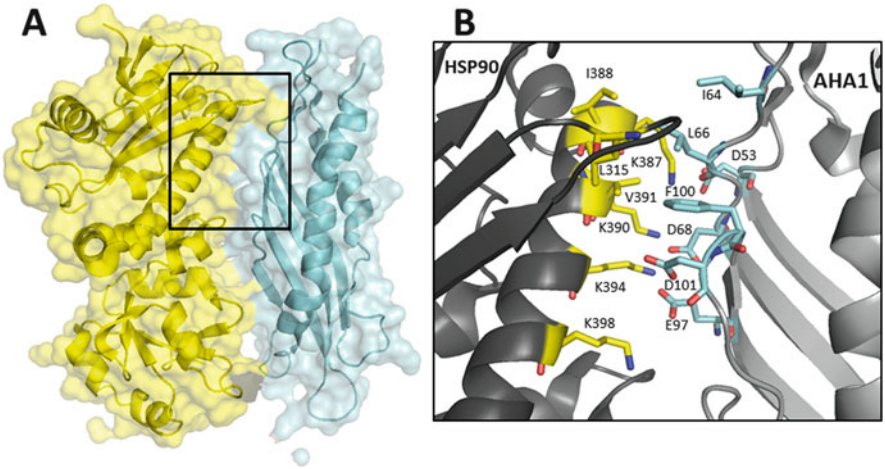

Fig. 5 Interaction of Hsp90 M domain and the N-terminal domain of the co-chaperone Aha1. (**a**) Global view of the interaction of the middle (M) domain of Hsp90 (*yellow*, residues 273–530) and the N-terminal region of Aha1 (*cyan*, residues 1–156, equivalent to Hch1) showing a large interaction interface between the two proteins. (**b**) Detailed view of the residues involved in interaction between Hsp90 (*dark grey*, with interacting residues shown in *yellow*) and Aha1 (*light grey*, with interacting residues shown in *cyan*). The core interaction involves hydrophobic interactions between I64, L66 and F100 from Aha1 and L315, I388 and V391 from Hsp90. The interaction interface also includes a ladder of ion pair interactions between D53, D101, D68 and E97 from Aha1 and K387, K390, K394 and K398 from Hsp90. Images were generated using PyMOL (DeLano Scientific) using structure 1USV [137]

with the Hsp90 inhibitor 17-AAG, in a manner dependent on the presence of HSF-1 [144]. Promisingly from a therapeutic perspective, Aha1 was not induced in the non-tumorigenic prostate epithelial cell line PNT2 upon treatment with similar concentrations of 17-AAG over a similar time frame. Consistent with its role in activation of Hsp90 ATPase activity, overexpression of Aha1 in HT29 colon cancer cells (which have a low endogenous level of the Aha1 protein) led to increased phosphorylation of Akt, MEK1/2 and ERK1/2 but did not alter the total levels of these proteins or of other Hsp90 clients like Raf and Cdk4. However, while levels of Raf were unchanged, there was an increase in the kinase activity of Raf in HT29 cells overexpressing Aha1, which explains the increase in phosphorylation of downstream signalling intermediates. In agreement with these data, selective knockdown of Aha1 in HCT116 (which constitutively express relatively high levels of Aha1) with siRNA did not influence Cdk4, Raf or ERBB2 levels but did reduce Raf kinase activity and negatively affect the phosphorylation of Akt and MEK1/2 without causing a reduction in the total level of proteins [144]. These results are consistent with studies in Aha1-null yeast, which exhibited lower levels of phosphorylated Src [33]. Overexpression of Aha1 did not change the sensitivity of HT29 or HCT116 cells to 17-AAG and could not protect Hsp90 client proteins Raf and Cdk4 from degradation in response to the inhibitor. In contrast, sensitivity to Hsp90

inhibition with 17-AAG was increased in Aha1 knockdown HCT116 colon cells. This increase in sensitivity was associated with an increase in apoptosis [144].

The association of Aha1 with Hsp90 has recently been shown to be regulated by post-translational modifications, whereby phosphorylation of Aha1 at Y223 by the kinase c-Abl promotes recruitment to the Hsp90 complex [145]. Inhibition of c-Abl or the Y223F mutation (which removes the phosphorylatable tyrosine residue in Aha1) blocks the interaction of Hsp90 with Aha1 and prevents the stimulation of the ATPase activity of Hsp90. As anticipated, the phosphomimetic Aha1 Y223E mutant displayed enhanced binding and stimulation of Hsp90 ATPase activity (approximately 8.5 times that of wild-type Aha1) [145]. Expression of the Aha1 Y223E phosphomimetic mutant compromised the chaperoning of both kinase (Src) and non-kinase (GR, HSF-1 and β-galactosidase) Hsp90 client proteins in yeast [145], which in part recapitulates the effects of Aha1 knockdown observed in mammalian colon cell lines [144]. In both yeast and mammalian cells, the effect on client proteins is predicted to occur as a result of changes in activity of the Hsp90 cycle due to influences on the Hsp90 ATPase activity.

5.1.2 Targeting Aha1 in Cancer

The links between limiting concentrations of Aha1 and increased sensitivity to Hsp90 inhibition suggest that depletion or inactivation of Aha1 levels together with Hsp90 inhibition may be a potential therapeutic strategy [144]. Targeting Aha1 may be particularly relevant in the context of treatment with Hsp90 inhibitors that activate HSF-1 that has been shown to induce Aha1 levels, which could lead to increased activation of mitogenic pathways regulated by Akt and ERK1/2 and culminate in increased cell survival or resistance [144].

While there are currently no direct small-molecule inhibitors of Aha1 described, inhibition of the c-Abl kinase was demonstrated as an effective mechanism to indirectly inhibit Aha1 phosphorylation and therefore block its interaction with Hsp90 [145]. Inhibition of c-Abl with the specific inhibitor GNF-5 resulted in hypersensitisation of prostate and renal cell carcinoma cell lines and ex vivo tumour cultures to treatment with Hsp90 inhibitors ganetespib and SNX2112, demonstrating the therapeutic benefit from targeting both Aha1 and Hsp90 [145]. The regulation of the Hsp90 cycle by c-Abl-mediated phosphorylation of Aha1 may be particularly relevant to the treatment of chronic myelogenous leukaemia (CML) since c-Abl is a proto-oncogene that undergoes genomic translocation to form the Bcr–Abl fusion protein (Philadelphia chromosome) associated with CML [146, 147]. Indeed, the recent data on the link between c-Abl and Aha1–Hsp90 interaction provide a possible mechanistic explanation for previous data that show that 17-AAG acts synergistically with imatinib (Gleevec) in both imatinib-sensitive and imatinib-resistant CML cell lines [148].

5.2 *Prostaglandin E Synthase 3 (p23)*

p23 is a late-stage, non-TPR-containing co-chaperone, which binds to Hsp90 client complexes after nucleotide binding [40, 149]. p23 has numerous Hsp90-dependent and Hsp90-independent functions in the cell [40, 41, 150]. For a comprehensive account of the role of p23 as an Hsp90 co-chaperone, the reader is directed to the recent review from the Buchner laboratory [34]. As a co-chaperone, two molecules of p23 bind to the Hsp90 dimer, making contacts with both the N and M domains of Hsp90 [140, 151]. The binding of p23 occurs in the presence of ATP when the Hsp90 dimer is in the closed conformation (Fig. 6). The effect of p23 binding is to slow the rate of Hsp90 ATPase activity and prevent client protein release [34, 140, 151–155]. The mechanism by which p23 inhibits Hsp90 ATPase remains undefined but could possibly be through either inhibition of hydrolysis or release of ADP and phosphate after hydrolysis [34]. Irrespective of which is correct, the mechanism of p23 inhibition of Hsp90 ATPase is distinct from the mechanism by which Hop prevents Hsp90 ATPase activity (by preventing N-terminal dimerisation) [34]. Binding of p23 to Hsp90 is inhibited by both Hsp90 inhibitors GA and novobiocin [156].

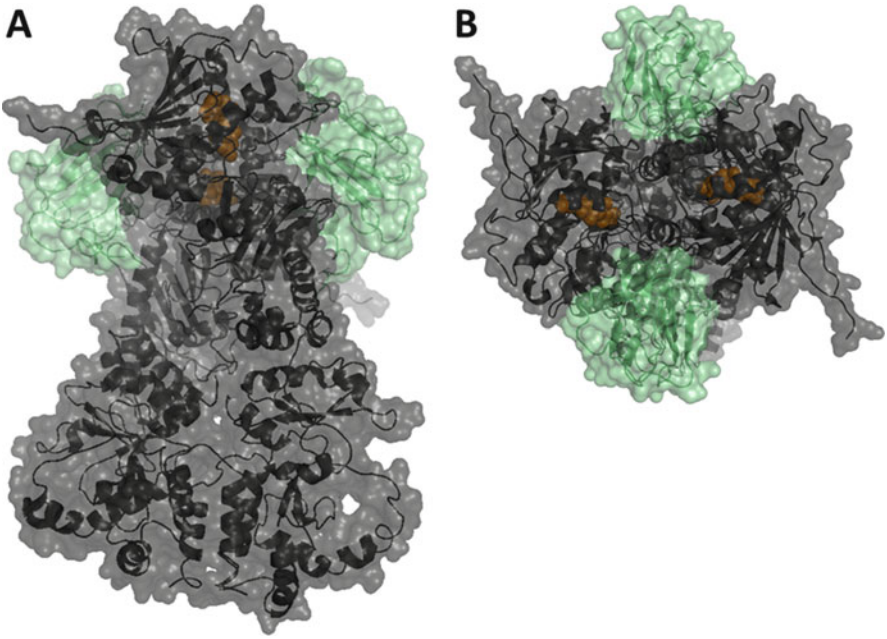

Fig. 6 Interaction of Hsp90 with the co-chaperone p23. Interaction of yeast Hsp90/Hsp82 (*grey*, residues 1–677 but with the sequence LQHMASVD replacing the charged linker at 221–255) with the yeast co-chaperone p23/Sba1 (*green*, residues 1–134). p23 interacts with the N-terminal domain of Hsp90 and Hsp90 is in the closed conformation with the N-terminal domains dimerised and ATP (*orange spheres*) bound. The images were generated using PyMOL (DeLano Scientific) using the structure 2CG9 [151]. (**a**) *Side* and (**b**) *top views* of the complex

5.2.1 Validating a Role for p23 in Cancer

Yeast with p23 knocked out are temperature sensitive, while p23 knockout in mice is lethal [157, 158]. Depletion of p23 renders yeast and mammalian cells sensitive to GA, while overexpression of p23 induces drug resistance, specifically by blocking the binding of GA to Hsp90 [159]. The well-defined role of p23 in the chaperoning of transcriptional complexes of nuclear steroid receptor client proteins (glucocorticoid receptor/GR, androgen receptor/AR, oestrogen receptor/ER) suggests that targeting p23 in hormone-driven cancers may be of therapeutic potential [43, 150, 160, 161]. Indeed, levels of p23 are elevated in prostate cancer compared to normal cells, and in prostate cancer, p23 is involved in Hsp90-dependent and Hsp90-independent chaperoning of AR which promotes tumour progression. Overexpression or depletion of p23 by RNAi enhances or reduces the transcriptional activity of AR [162]. The levels of p23 itself were increased in response to treatment with the synthetic androgen mibolerone and, to a lesser extent, the antiandrogen bicalutamide. In LNCaP prostate cancer cells, overexpression of p23 stabilised AR in the absence of hormone while RNAi-mediated depletion of p23 reduced the levels of AR [59]. Overexpression or knockdown of p23 had no significant effects on the growth of LNCaP prostate cancer cells but did affect prostate cancer motility and invasiveness. Ectopic expression of p23 increased transwell migration of LNCaP cells, while depletion of p23 by RNAi reduced cell migration in linear wound healing assays [59]. In clinical samples, high nuclear p23 was associated with development of metastases. The pro-migratory effect of p23 was associated with changes in the focal adhesion protein, vinculin, which suggests a role for p23 in modulation of the cytoskeleton. A similar reduction in wound healing was seen in PC3 cells depleted of p23. Since PC3 cells lack functional AR, these data suggest that the role of p23 in cell migration may be AR independent. The role of p23 in in vitro migration was supported by in vivo data from prostate tumours showing an inverse relationship between expression levels of nuclear p23 and patient survival. Those patients with high p23 had shorter survival times, even in the context of patients with metastatic disease. These data suggest that p23 directly related to the progression of prostate cancer and nuclear p23 in particular may be a useful biomarker for those tumours that are more likely to result in metastasis and poor outcomes [59].

Increased expression of the co-chaperone was also correlated with tumour progression and poor clinical outcomes in breast cancer caused by p23-regulated expression of a group of metastasis and drug resistance genes [163]. p23 influences ER+ breast cancer biology through its differential regulation of ER target genes [164]. In the case of genes activated by direct binding of ER to promoters containing oestrogen response elements (EREs), p23 overexpression increases gene expression. In contrast, p23 did not seem to affect genes indirectly activated by ER [164]. Simpson and colleagues showed in MCF7 cells overexpressing p23 that, of the genes regulated by p23, a number of genes commonly associated with invasive breast cancers were deregulated (including PMP22, ABCC3 and AGR2 which were upregulated and p8, TM4SF1 and Sox3 which were downregulated) [163]. The upregulation of genes in response to overexpression of p23 was associated

with increased histone H3 acetylation at the promoters of these genes indicating transcriptional activation, which is in accordance with the well-described roles of p23 in transcription [150]. MCF7 cells expressing p23 were also found to be resistant to etoposide and doxorubicin which was attributed to the increased expression of ABCC3, a multidrug resistance transporter. In breast tumours, high expression of cytoplasmic p23 in particular was associated with lymph node metastasis and decreased survival rates, which correlates with the gene expression study and indicates that p23-overexpressing breast tumours are also more aggressive in vivo [163].

5.2.2 Targeting p23 in Cancer

Celastrol, the natural compound that affects the interaction of Hsp90 with Cdc37, also inhibits p23 (Fig. 7). However, in contrast to Cdc37, the mechanism of p23 inhibition is due to a direct non-covalent interaction, which induces formation of inactive p23 fibrils [165]. The natural compound cucurbitacin D, which shows some structural similarity to celastrol (Fig. 7), has recently been shown to inhibit the interaction of Hsp90 with both p23 and Cdc37 without inducing a stress response [166]. Cucurbitacin D has also been independently shown to inhibit proliferation of endometrial, ovarian and breast carcinoma cell lines, which may in part be explained by its effect on the Hsp90 heterocomplex [167, 168].

Another structurally related natural compound is gedunin, which is isolated from the *Azadirachta indica* tree [169] and has been shown to inhibit the proliferation of both colon and ovarian cancer cell lines [170, 171]. Gedunin induced selective degradation of Hsp90 client proteins in Hs578T and MCF7 cell lines; the expression of steroid receptors PR and GR was substantially reduced, while levels of kinase clients Cdk4, Raf and Chk1 were not significantly affected [172]. These effects of gedunin in cells were similar to those observed with depletion of p23 by RNAi [59]. This differential sensitivity of steroid receptor versus kinase clients suggested that gedunin, despite structural similarity with celastrol, was selectively affecting p23 and not Cdc37 (Fig. 7). Gedunin reduced the in vitro activation of PR in RRL by reducing the proportion of p23 and Hsp90 in complex with PR. In addition to

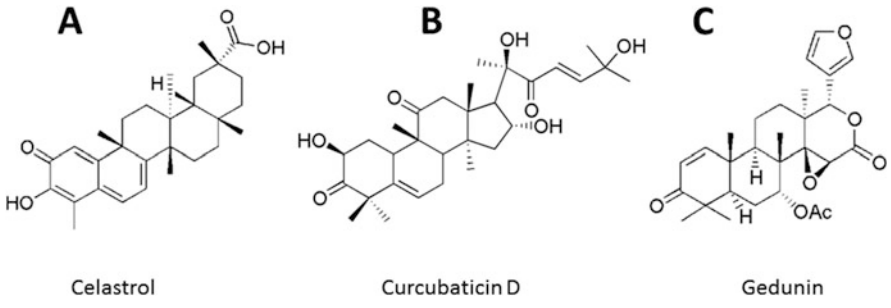

Fig. 7 Structural similarity between inhibitors celastrol, cucurbitacin D and gedunin. Celastrol and cucurbitacin D inhibit both Cdc37 and p23, while gedunin inhibits p23 and not Cdc37

directly affecting the chaperone activity of p23, gedunin treatment inhibited p23 interaction with Hsp90 and reduced p23-mediated gene regulation. Gedunin induced these effects via direct binding to p23 in vitro and in vivo; this interaction requires residues T90, K95 and A94 of p23, and unlike celastrol, gedunin did not induce fibril formation by p23. In addition to direct inhibition of p23, gedunin binding also altered the conformation of p23 making it susceptible to cleavage by caspase 7, leading to cancer cell death by apoptosis in both ER+ and ER− breast cancer cell lines (MDA-MB-231, T47D, Hs578T, MDA-MB-453 and MCF7) [172]. Importantly, from a therapeutic potential, non-transformed cell lines Hs578Bst and HME were less sensitive to gedunin treatment [172]. Gedunin, cucurbitacin D and celastrol are interesting because despite structure similarity, they appear to have differing activities. Importantly, gedunin is far less effective at inducing the stress response than celastrol, which is an undesirable consequence of Hsp90 inhibition in cancer. Whereas celastrol activates HSF-1, gedunin induced a minor increase in Hsp70 without altering the Hsp27 levels [172]. Therefore, in the context of cancer treatment, development of gedunin as a co-chaperone therapy would be preferable over celastrol.

6 Conclusion and Future Perspectives

Co-chaperones are vital for the regulation of client protein folding by the Hsp90 chaperone machine [26]. Studies on individual co-chaperones Hop, Cdc37, Aha1 and p23 have demonstrated that these proteins are involved in regulating the biology of cancer cells at numerous levels, either alone or in partnership with Hsp90, and have begun to evaluate these proteins as drug targets using small-molecule modulators or RNA interference and overexpression approaches [60, 61, 87, 144]. Targeting co-chaperones as an alternative to or in combination to targeting Hsp90 is now being considered a viable therapeutic option. However, some of the small-molecule inhibitors that are likely to be amenable to drug development are not specific for only one co-chaperone, and therefore there is an ongoing need for more selective inhibitors that can be tailored for specific contexts. There has been substantial focus in analysis of individual Hsp90 co-chaperones, and we now have some understanding of the stages at which some of these co-chaperones interact and which Hsp90-co-chaperone complexes are mutually exclusive or complementary. However, we still do not have a complete understanding of the integration of the different co-chaperones into the Hsp90 cycle, the possible ternary complexes formed under different conditions and the sequential events that occur during the Hsp90 cycle. A deep understanding of the interactions between different co-chaperones during the Hsp90 cycle will be important for drug targeting, particularly in the context of possible redundancy and compensatory functions that may predominate in the absence of one or more co-chaperones. Therefore, it may be necessary to consider dual targeting of co-chaperones to prevent the compensatory actions of co-chaperones in the cancer context. The number of Hsp90 co-chaperones continues to grow, with over twenty different co-chaperones for Hsp90 now being identified

(many of them not discussed here) [52]. As our understanding of the fundamental roles of these new co-chaperones in cancer grows, so will our opportunities to consider them as drug targets either alone or in combination with other inhibitors. In addition, the impact of post-translational modifications on co-chaperone activity is currently poorly understood. From the recent studies on Aha1, post-translational modification of co-chaperones is likely to impact greatly on Hsp90 function and may fine-tune Hsp90 function even further for specific cellular contexts [145]. In this way, targeting the post-translational modifications of Hsp90 co-chaperones may provide new avenues for therapeutic intervention.

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References

1. Hartson SD, Matts RL (2012) Approaches for defining the Hsp90-dependent proteome. Biochim Biophys Acta 1823(3):656–667. doi:10.1016/j.bbamcr.2011.08.013
2. Picard D (2012) Preface to Hsp90. Biochim Biophys Acta 1823(3):605–606. doi:10.1016/j.bbamcr.2012.02.004
3. Whitesell L, Lindquist SL (2005) HSP90 and the chaperoning of cancer. Nat Rev Cancer 5 (10):761–772
4. Whitesell L, Lin NU (2012) HSP90 as a platform for the assembly of more effective cancer chemotherapy. Biochim Biophys Acta 1823(3):756–766. doi:10.1016/j.bbamcr.2011.12.006
5. Chase G, Deng T, Fodor E, Leung BW, Mayer D, Schwemmle M, Brownlee G (2008) Hsp90 inhibitors reduce influenza virus replication in cell culture. Virology 377(2):431–439
6. Cowen LE, Singh SD, Kohler JR, Collins C, Zaas AK, Schell WA, Aziz H, Mylonakis E, Perfect JR, Whitesell L, Lindquist S (2009) Harnessing Hsp90 function as a powerful, broadly effective therapeutic strategy for fungal infectious disease. Proc Natl Acad Sci U S A 106(8):2818–2823
7. Dickey CA, Eriksen J, Kamal A, Burrows F, Kasibhatla S, Eckman CB, Hutton M, Petrucelli L (2005) Development of a high throughput drug screening assay for the detection of changes in tau levels -- proof of concept with HSP90 inhibitors. Curr Alzheimer Res 2(2):231–238
8. Geller R, Vignuzzi M, Andino R, Frydman J (2007) Evolutionary constraints on chaperone-mediated folding provide an antiviral approach refractory to development of drug resistance. Genes Dev 21(2):195–205. doi:10.1101/gad.1505307
9. Giannini G, Battistuzzi G (2015) Exploring in vitro and in vivo Hsp90 inhibitors activity against human protozoan parasites. Bioorg Med Chem Lett 25(3):462–465. doi:10.1016/j.bmcl.2014.12.048
10. Luo W, Rodina A, Chiosis G (2008) Heat shock protein 90: translation from cancer to Alzheimer's disease treatment? BMC Neurosci 9(Suppl 2):S7
11. Waza M, Adachi H, Katsuno M, Minamiyama M, Tanaka F, Doyu M, Sobue G (2006) Modulation of Hsp90 function in neurodegenerative disorders: a molecular-targeted therapy against disease-causing protein. J Mol Med 84(8):635–646

12. Zhang H, Burrows F (2004) Targeting multiple signal transduction pathways through inhibition of Hsp90. J Mol Med 82(8):488–499
13. Grenert JP, Sullivan WP, Fadden P, Haystead TA, Clark J, Mimnaugh E, Krutzsch H, Ochel HJ, Schulte TW, Sausville E, Neckers LM, Toft DO (1997) The amino-terminal domain of heat shock protein 90 (hsp90) that binds geldanamycin is an ATP/ADP switch domain that regulates hsp90 conformation. J Biol Chem 272(38):23843–23850
14. Grenert JP, Johnson BD, Toft DO (1999) The importance of ATP binding and hydrolysis by hsp90 in formation and function of protein heterocomplexes. J Biol Chem 274 (25):17525–17533
15. Obermann WM, Sondermann H, Russo AA, Pavletich NP, Hartl FU (1998) In vivo function of Hsp90 is dependent on ATP binding and ATP hydrolysis. J Cell Biol 143(4):901–910
16. Panaretou B, Prodromou C, Roe SM, O'Brien R, Ladbury JE, Piper PW, Pearl LH (1998) ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo. EMBO J 17(16):4829–4836
17. Kamal A, Thao L, Sensintaffar J, Zhang L, Boehm MF, Fritz LC, Burrows FJ (2003) A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. Nature 425 (6956):407–410
18. Drysdale MJ, Brough PA, Massey A, Jensen MR, Schoepfer J (2006) Targeting Hsp90 for the treatment of cancer. Curr Opin Drug Discov Devel 9(4):483–495
19. Wang Y, Trepel JB, Neckers LM, Giaccone G (2010) STA-9090, a small-molecule Hsp90 inhibitor for the potential treatment of cancer. Curr Opin Investig Drugs 11(12):1466–1476
20. Jensen MR, Schoepfer J, Radimerski T, Massey A, Guy CT, Brueggen J, Quadt C, Buckler A, Cozens R, Drysdale MJ, Garcia-Echeverria C, Chene P (2008) NVP-AUY922: a small molecule HSP90 inhibitor with potent antitumor activity in preclinical breast cancer models. Breast Cancer Res 10(2):R33. doi:10.1186/bcr1996
21. Voruganti S, Lacroix JC, Rogers CN, Rogers J, Matts RL, Hartson SD (2013) The anticancer drug AUY922 generates a proteomics fingerprint that is highly conserved among structurally diverse Hsp90 inhibitors. J Proteome Res. doi:10.1021/pr400321x
22. Bao R, Lai CJ, Qu H, Wang D, Yin L, Zifcak B, Atoyan R, Wang J, Samson M, Forrester J, DellaRocca S, Xu GX, Tao X, Zhai HX, Cai X, Qian C (2009) CUDC-305, a novel synthetic HSP90 inhibitor with unique pharmacologic properties for cancer therapy. Clin Cancer Res 15(12):4046–4057. doi:10.1158/1078-0432.CCR-09-0152
23. Whitesell L, Bagatell R, Falsey R (2003) The stress response: implications for the clinical development of hsp90 inhibitors. Curr Cancer Drug Targets 3(5):349–358
24. Zou J, Guo Y, Guettouche T, Smith DF, Voellmy R (1998) Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. Cell 94(4):471–480
25. Cox MB, Johnson JL (2011) The role of p23, Hop, immunophilins, and other co-chaperones in regulating Hsp90 function. Methods Mol Biol 787:45–66. doi:10.1007/978-1-61779-295-3_4
26. Li J, Soroka J, Buchner J (2012) The Hsp90 chaperone machinery: conformational dynamics and regulation by co-chaperones. Biochim Biophys Acta 1823(3):624–635. doi:10.1016/j.bbamcr.2011.09.003
27. Prodromou C, Panaretou B, Chohan S, Siligardi G, O'Brien R, Ladbury JE, Roe SM, Piper PW, Pearl LH (2000) The ATPase cycle of Hsp90 drives a molecular 'clamp' via transient dimerization of the N-terminal domains. EMBO J 19(16):4383–4392
28. Csermely P, Schnaider T, Soti C, Prohaszka Z, Nardai G (1998) The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. Pharmacol Ther 79(2):129–168
29. Blatch GL, Lassle M (1999) The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. Bioessays 21(11):932–939
30. Young JC, Obermann WM, Hartl FU (1998) Specific binding of tetratricopeptide repeat proteins to the C-terminal 12-kDa domain of hsp90. J Biol Chem 273(29):18007–18010

31. Lotz GP, Lin H, Harst A, Obermann WM (2003) Aha1 binds to the middle domain of Hsp90, contributes to client protein activation, and stimulates the ATPase activity of the molecular chaperone. J Biol Chem 278(19):17228–17235
32. Mayer MP, Nikolay R, Bukau B (2002) Aha, another regulator for hsp90 chaperones. Mol Cell 10(6):1255–1256
33. Panaretou B, Siligardi G, Meyer P, Maloney A, Sullivan JK, Singh S, Millson SH, Clarke PA, Naaby-Hansen S, Stein R, Cramer R, Mollapour M, Workman P, Piper PW, Pearl LH, Prodromou C (2002) Activation of the ATPase activity of hsp90 by the stress-regulated cochaperone aha1. Mol Cell 10(6):1307–1318
34. Rehn AB, Buchner J (2015) p23 and Aha1. Subcell Biochem 78:113–131. doi:10.1007/978-3-319-11731-7_6
35. Retzlaff M, Hagn F, Mitschke L, Hessling M, Gugel F, Kessler H, Richter K, Buchner J (2010) Asymmetric activation of the hsp90 dimer by its cochaperone aha1. Mol Cell 37 (3):344–354. doi:10.1016/j.molcel.2010.01.006
36. Abbas-Terki T, Briand PA, Donze O, Picard D (2002) The Hsp90 co-chaperones Cdc37 and Sti1 interact physically and genetically. Biol Chem 383(9):1335–1342. doi:10.1515/BC.2002.152
37. Calderwood SK (2015) Cdc37 as a co-chaperone to Hsp90. Subcell Biochem 78:103–112. doi:10.1007/978-3-319-11731-7_5
38. Farrell A, Morgan DO (2000) Cdc37 promotes the stability of protein kinases Cdc28 and Cak1. Mol Cell Biol 20(3):749–754
39. Siligardi G, Panaretou B, Meyer P, Singh S, Woolfson DN, Piper PW, Pearl LH, Prodromou C (2002) Regulation of Hsp90 ATPase activity by the co-chaperone Cdc37p/p50cdc37. J Biol Chem 277(23):20151–20159
40. Echtenkamp FJ, Zelin E, Oxelmark E, Woo JI, Andrews BJ, Garabedian M, Freeman BC (2011) Global functional map of the p23 molecular chaperone reveals an extensive cellular network. Mol Cell 43(2):229–241. doi:10.1016/j.molcel.2011.05.029
41. Freeman BC, Felts SJ, Toft DO, Yamamoto KR (2000) The p23 molecular chaperones act at a late step in intracellular receptor action to differentially affect ligand efficacies. Genes Dev 14 (4):422–434
42. Morishima Y, Kanelakis KC, Murphy PJ, Lowe ER, Jenkins GJ, Osawa Y, Sunahara RK, Pratt WB (2003) The hsp90 cochaperone p23 is the limiting component of the multiprotein hsp90/hsp70-based chaperone system in vivo where it acts to stabilize the client protein: hsp90 complex. J Biol Chem 278(49):48754–48763
43. Oxelmark E, Knoblauch R, Arnal S, Su LF, Schapira M, Garabedian MJ (2003) Genetic dissection of p23, an Hsp90 cochaperone, reveals a distinct surface involved in estrogen receptor signaling. J Biol Chem 278(38):36547–36555
44. Weaver AJ, Sullivan WP, Felts SJ, Owen BA, Toft DO (2000) Crystal structure and activity of human p23, a heat shock protein 90 co-chaperone. J Biol Chem 275(30):23045–23052
45. Alvira S, Cuellar J, Rohl A, Yamamoto S, Itoh H, Alfonso C, Rivas G, Buchner J, Valpuesta JM (2014) Structural characterization of the substrate transfer mechanism in Hsp70/Hsp90 folding machinery mediated by Hop. Nat Commun 5:5484. doi:10.1038/ncomms6484
46. Baindur-Hudson S, Edkins AL, Blatch GL (2015) Hsp70/Hsp90 organising protein (hop): beyond interactions with chaperones and prion proteins. Subcell Biochem 78:69–90. doi:10.1007/978-3-319-11731-7_3
47. Blatch GL, Lassle M, Zetter BR, Kundra V (1997) Isolation of a mouse cDNA encoding mSTI1, a stress-inducible protein containing the TPR motif. Gene 194(2):277–282
48. Honore B, Leffers H, Madsen P, Rasmussen HH, Vandekerckhove J, Celis JE (1992) Molecular cloning and expression of a transformation-sensitive human protein containing the TPR motif and sharing identity to the stress-inducible yeast protein STI1. J Biol Chem 267(12):8485–8491

49. Lassle M, Blatch GL, Kundra V, Takatori T, Zetter BR (1997) Stress-inducible, murine protein mSTI1. Characterization of binding domains for heat shock proteins and in vitro phosphorylation by different kinases. J Biol Chem 272(3):1876–1884
50. Nicolet CM, Craig EA (1989) Isolation and characterization of STI1, a stress-inducible gene from Saccharomyces cerevisiae. Mol Cell Biol 9(9):3638–3646
51. Schmid AB, Lagleder S, Grawert MA, Rohl A, Hagn F, Wandinger SK, Cox MB, Demmer O, Richter K, Groll M, Kessler H, Buchner J (2012) The architecture of functional modules in the Hsp90 co-chaperone Sti1/Hop. EMBO J 31(6):1506–1517. doi:10.1038/emboj.2011.472
52. Johnson JL (2012) Evolution and function of diverse Hsp90 homologs and cochaperone proteins. Biochim Biophys Acta 1823(3):607–613. doi:10.1016/j.bbamcr.2011.09.020
53. Sanchez ER (2012) Chaperoning steroidal physiology: lessons from mouse genetic models of Hsp90 and its cochaperones. Biochim Biophys Acta 1823(3):722–729. doi:10.1016/j.bbamcr.2011.11.006
54. Aoyagi S, Archer TK (2005) Modulating molecular chaperone Hsp90 functions through reversible acetylation. Trends Cell Biol 15(11):565–567
55. Duval M, Le Boeuf F, Huot J, Gratton JP (2007) Src-mediated phosphorylation of Hsp90 in response to vascular endothelial growth factor (VEGF) is required for VEGF receptor-2 signaling to endothelial NO synthase. Mol Biol Cell 18(11):4659–4668
56. Martinez-Ruiz A, Villanueva L, Gonzalez de Orduna C, Lopez-Ferrer D, Higueras MA, Tarin C, Rodriguez-Crespo I, Vazquez J, Lamas S (2005) S-nitrosylation of Hsp90 promotes the inhibition of its ATPase and endothelial nitric oxide synthase regulatory activities. Proc Natl Acad Sci U S A 102(24):8525–8530
57. Rao R, Fiskus W, Yang Y, Lee P, Joshi R, Fernandez P, Mandawat A, Atadja P, Bradner JE, Bhalla K (2008) HDAC6 inhibition enhances 17-AAG--mediated abrogation of hsp90 chaperone function in human leukemia cells. Blood 112(5):1886–1893. doi:10.1182/blood-2008-03-143644
58. Yang Y, Rao R, Shen J, Tang Y, Fiskus W, Nechtman J, Atadja P, Bhalla K (2008) Role of acetylation and extracellular location of heat shock protein 90alpha in tumor cell invasion. Cancer Res 68(12):4833–4842. doi:10.1158/0008-5472.CAN-08-0644
59. Cano LQ, Lavery DN, Sin S, Spanjaard E, Brooke GN, Tilman JD, Abroaf A, Gaughan L, Robson CN, Heer R, Mauri F, de Rooij J, Driouch K, Bevan CL (2015) The co-chaperone p23 promotes prostate cancer motility and metastasis. Mol Oncol 9(1):295–308. doi:10.1016/j.molonc.2014.08.014
60. Smith JR, Clarke PA, de Billy E, Workman P (2009) Silencing the cochaperone CDC37 destabilizes kinase clients and sensitizes cancer cells to HSP90 inhibitors. Oncogene 28 (2):157–169. doi:10.1038/onc.2008.380
61. Willmer T, Contu L, Blatch GL, Edkins AL (2013) Knockdown of Hop downregulates RhoC expression, and decreases pseudopodia formation and migration in cancer cell lines. Cancer Lett 328(2):252–260. doi:10.1016/j.canlet.2012.09.021
62. Kubota H, Yamamoto S, Itoh E, Abe Y, Nakamura A, Izumi Y, Okada H, Iida M, Nanjo H, Itoh H, Yamamoto Y (2010) Increased expression of co-chaperone HOP with HSP90 and HSC70 and complex formation in human colonic carcinoma. Cell Stress Chaperones 15 (6):1003–1011. doi:10.1007/s12192-010-0211-0
63. Piper PW, Millson SH, Mollapour M, Panaretou B, Siligardi G, Pearl LH, Prodromou C (2003) Sensitivity to Hsp90-targeting drugs can arise with mutation to the Hsp90 chaperone, cochaperones and plasma membrane ATP binding cassette transporters of yeast. Eur J Biochem 270(23):4689–4695
64. Felts SJ, Owen BA, Nguyen P, Trepel J, Donner DB, Toft DO (2000) The hsp90-related protein TRAP1 is a mitochondrial protein with distinct functional properties. J Biol Chem 275(5):3305–3312
65. Xu W, Mimnaugh E, Rosser MF, Nicchitta C, Marcu M, Yarden Y, Neckers L (2001) Sensitivity of mature Erbb2 to geldanamycin is conferred by its kinase domain and is

mediated by the chaperone protein Hsp90. J Biol Chem 276(5):3702–3708. doi:10.1074/jbc.M006864200
66. Altieri DC, Stein GS, Lian JB, Languino LR (2012) TRAP-1, the mitochondrial Hsp90. Biochim Biophys Acta 1823(3):767–773. doi:10.1016/j.bbamcr.2011.08.007
67. Marzec M, Eletto D, Argon Y (2012) GRP94: an HSP90-like protein specialized for protein folding and quality control in the endoplasmic reticulum. Biochim Biophys Acta 1823 (3):774–787. doi:10.1016/j.bbamcr.2011.10.013
68. Liu B, Yang Y, Qiu Z, Staron M, Hong F, Li Y, Wu S, Li Y, Hao B, Bona R, Han D, Li Z (2010) Folding of Toll-like receptors by the HSP90 paralogue gp96 requires a substrate-specific cochaperone. Nat Commun 1:79
69. Wakabayashi Y, Kobayashi M, Akashi-Takamura S, Tanimura N, Konno K, Takahashi K, Ishii T, Mizutani T, Iba H, Kouro T, Takaki S, Takatsu K, Oda Y, Ishihama Y, Saitoh S, Miyake K (2006) A protein associated with toll-like receptor 4 (PRAT4A) regulates cell surface expression of TLR4. J Immunol 177(3):1772–1779
70. Wegele H, Wandinger SK, Schmid AB, Reinstein J, Buchner J (2006) Substrate transfer from the chaperone Hsp70 to Hsp90. J Mol Biol 356(3):802–811
71. Chang HC, Nathan DF, Lindquist S (1997) In vivo analysis of the Hsp90 cochaperone Sti1 (p60). Mol Cell Biol 17(1):318–325
72. Beraldo FH, Soares IN, Goncalves DF, Fan J, Thomas AA, Santos TG, Mohammad AH, Roffe M, Calder MD, Nikolova S, Hajj GN, Guimaraes AL, Massensini AR, Welch I, Betts DH, Gros R, Drangova M, Watson AJ, Bartha R, Prado VF, Martins VR, Prado MA (2013) Stress-inducible phosphoprotein 1 has unique cochaperone activity during development and regulates cellular response to ischemia via the prion protein. FASEB J. doi:10.1096/fj.13-232280
73. Brinker A, Scheufler C, Von Der Mulbe F, Fleckenstein B, Herrmann C, Jung G, Moarefi I, Hartl FU (2002) Ligand discrimination by TPR domains. Relevance and selectivity of EEVD-recognition in Hsp70 x Hop x Hsp90 complexes. J Biol Chem 277(22):19265–19275
74. Odunuga OO, Hornby JA, Bies C, Zimmermann R, Pugh DJ, Blatch GL (2003) Tetratricopeptide repeat motif-mediated Hsc70-mSTI1 interaction. Molecular characterization of the critical contacts for successful binding and specificity. J Biol Chem 278(9):6896–6904. doi:10.1074/jbc.M206867200
75. Scheufler C, Brinker A, Bourenkov G, Pegoraro S, Moroder L, Bartunik H, Hartl FU, Moarefi I (2000) Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. Cell 101(2):199–210
76. Lee CT, Graf C, Mayer FJ, Richter SM, Mayer MP (2012) Dynamics of the regulation of Hsp90 by the co-chaperone Sti1. EMBO J 31(6):1518–1528. doi:10.1038/emboj.2012.37
77. Onuoha SC, Coulstock ET, Grossmann JG, Jackson SE (2008) Structural studies on the co-chaperone Hop and its complexes with Hsp90. J Mol Biol 379(4):732–744
78. Wegele H, Muller L, Buchner J (2004) Hsp70 and Hsp90 – a relay team for protein folding. Rev Physiol Biochem Pharmacol 151:1–44
79. Prodromou C, Siligardi G, O'Brien R, Woolfson DN, Regan L, Panaretou B, Ladbury JE, Piper PW, Pearl LH (1999) Regulation of Hsp90 ATPase activity by tetratricopeptide repeat (TPR)-domain co-chaperones. EMBO J 18(3):754–762
80. Siligardi G, Hu B, Panaretou B, Piper PW, Pearl LH, Prodromou C (2004) Co-chaperone regulation of conformational switching in the Hsp90 ATPase cycle. J Biol Chem 279 (50):51989–51998
81. Yamamoto S, Subedi GP, Hanashima S, Satoh T, Otaka M, Wakui H, Sawada K, Yokota S, Yamaguchi Y, Kubota H, Itoh H (2014) ATPase activity and ATP-dependent conformational change in the co-chaperone HSP70/HSP90-organizing protein (HOP). J Biol Chem 289 (14):9880–9886. doi:10.1074/jbc.M114.553255
82. Li J, Sun X, Wang Z, Chen L, Li D, Zhou J, Liu M (2012) Regulation of vascular endothelial cell polarization and migration by Hsp70/Hsp90-organizing protein. PLoS One 7(4), e36389. doi:10.1371/journal.pone.0036389

83. Walsh N, O'Donovan N, Kennedy S, Henry M, Meleady P, Clynes M, Dowling P (2009) Identification of pancreatic cancer invasion-related proteins by proteomic analysis. Proteome Sci 7:3
84. Sun W, Xing B, Sun Y, Du X, Lu M, Hao C, Lu Z, Mi W, Wu S, Wei H, Gao X, Zhu Y, Jiang Y, Qian X, He F (2007) Proteome analysis of hepatocellular carcinoma by two-dimensional difference gel electrophoresis: novel protein markers in hepatocellular carcinoma tissues. Mol Cell Proteomics 6(10):1798–1808. doi:10.1074/mcp.M600449-MCP200
85. Longshaw VM, Baxter M, Prewitz M, Blatch GL (2009) Knockdown of the co-chaperone Hop promotes extranuclear accumulation of Stat3 in mouse embryonic stem cells. Eur J Cell Biol 88(3):153–166. doi:10.1016/j.ejcb.2008.09.003
86. Walsh N, Larkin A, Swan N, Conlon K, Dowling P, McDermott R, Clynes M (2011) RNAi knockdown of Hop (Hsp70/Hsp90 organising protein) decreases invasion via MMP-2 down regulation. Cancer Lett 306(2):180–189. doi:10.1016/j.canlet.2011.03.004
87. Ardi VC, Alexander LD, Johnson VA, McAlpine SR (2011) Macrocycles that inhibit the binding between heat shock protein 90 and TPR-containing proteins. ACS Chem Biol 6 (12):1357–1366. doi:10.1021/cb200203m
88. Horibe T, Kawamoto M, Kohno M, Kawakami K (2012) Cytotoxic activity to acute myeloid leukemia cells by Antp-TPR hybrid peptide targeting Hsp90. J Biosci Bioeng 114(1):96–103. doi:10.1016/j.jbiosc.2012.02.016
89. Horibe T, Kohno M, Haramoto M, Ohara K, Kawakami K (2011) Designed hybrid TPR peptide targeting Hsp90 as a novel anticancer agent. J Transl Med 9:8. doi:10.1186/1479-5876-9-8
90. Horibe T, Torisawa A, Kohno M, Kawakami K (2012) Molecular mechanism of cytotoxicity induced by Hsp90-targeted Antp-TPR hybrid peptide in glioblastoma cells. Mol Cancer 11:59. doi:10.1186/1476-4598-11-59
91. Pimienta G, Herbert KM, Regan L (2011) A compound that inhibits the HOP-Hsp90 complex formation and has unique killing effects in breast cancer cell lines. Mol Pharm 8 (6):2252–2261. doi:10.1021/mp200346y
92. Kunicki JB, Petersen MN, Alexander LD, Ardi VC, McConnell JR, McAlpine SR (2011) Synthesis and evaluation of biotinylated sansalvamide A analogs and their modulation of Hsp90. Bioorg Med Chem Lett 21(16):4716–4719. doi:10.1016/j.bmcl.2011.06.083
93. Vasko RC, Rodriguez RA, Cunningham CN, Ardi VC, Agard DA, McAlpine SR (2010) Mechanistic studies of Sansalvamide A-amide: an allosteric modulator of Hsp90. ACS Med Chem Lett 1(1):4–8. doi:10.1021/ml900003t
94. Yi F, Regan L (2008) A novel class of small molecule inhibitors of Hsp90. ACS Chem Biol 3 (10):645–654
95. Sellers RP, Alexander LD, Johnson VA, Lin CC, Savage J, Corral R, Moss J, Slugocki TS, Singh EK, Davis MR, Ravula S, Spicer JE, Oelrich JL, Thornquist A, Pan CM, McAlpine SR (2010) Design and synthesis of Hsp90 inhibitors: exploring the SAR of Sansalvamide A derivatives. Bioorg Med Chem 18(18):6822–6856. doi:10.1016/j.bmc.2010.07.042
96. Kimura Y, Rutherford SL, Miyata Y, Yahara I, Freeman BC, Yue L, Morimoto RI, Lindquist S (1997) Cdc37 is a molecular chaperone with specific functions in signal transduction. Genes Dev 11(14):1775–1785
97. Lee P, Shabbir A, Cardozo C, Caplan AJ (2004) Sti1 and Cdc37 can stabilize Hsp90 in chaperone complexes with a protein kinase. Mol Biol Cell 15(4):1785–1792. doi:10.1091/mbc.E03-07-0480
98. MacLean M, Picard D (2003) Cdc37 goes beyond Hsp90 and kinases. Cell Stress Chaperones 8(2):114–119
99. Prince T, Sun L, Matts RL (2005) Cdk2: a genuine protein kinase client of Hsp90 and Cdc37. Biochemistry 44(46):15287–15295. doi:10.1021/bi051423m

100. Roe SM, Ali MM, Meyer P, Vaughan CK, Panaretou B, Piper PW, Prodromou C, Pearl LH (2004) The mechanism of Hsp90 regulation by the protein kinase-specific cochaperone p50 (cdc37). Cell 116(1):87–98
101. Zhang W, Hirshberg M, McLaughlin SH, Lazar GA, Grossmann JG, Nielsen PR, Sobott F, Robinson CV, Jackson SE, Laue ED (2004) Biochemical and structural studies of the interaction of Cdc37 with Hsp90. J Mol Biol 340(4):891–907. doi:10.1016/j.jmb.2004.05.007
102. Eckl JM, Rutz DA, Haslbeck V, Zierer BK, Reinstein J, Richter K (2013) Cdc37 (cell division cycle 37) restricts Hsp90 (heat shock protein 90) motility by interaction with N-terminal and middle domain binding sites. J Biol Chem 288(22):16032–16042. doi:10.1074/jbc.M112.439257
103. Vaughan CK, Gohlke U, Sobott F, Good VM, Ali MM, Prodromou C, Robinson CV, Saibil HR, Pearl LH (2006) Structure of an Hsp90-Cdc37-Cdk4 complex. Mol Cell 23(5):697–707. doi:10.1016/j.molcel.2006.07.016
104. Lee P, Rao J, Fliss A, Yang E, Garrett S, Caplan AJ (2002) The Cdc37 protein kinase-binding domain is sufficient for protein kinase activity and cell viability. J Cell Biol 159 (6):1051–1059. doi:10.1083/jcb.200210121
105. Mandal AK, Lee P, Chen JA, Nillegoda N, Heller A, DiStasio S, Oen H, Victor J, Nair DM, Brodsky JL, Caplan AJ (2007) Cdc37 has distinct roles in protein kinase quality control that protect nascent chains from degradation and promote posttranslational maturation. J Cell Biol 176(3):319–328. doi:10.1083/jcb.200604106
106. Taipale M, Krykbaeva I, Koeva M, Kayatekin C, Westover KD, Karras GI, Lindquist S (2012) Quantitative analysis of HSP90-client interactions reveals principles of substrate recognition. Cell 150(5):987–1001. doi:10.1016/j.cell.2012.06.047
107. Smith JR, Workman P (2009) Targeting CDC37: an alternative, kinase-directed strategy for disruption of oncogenic chaperoning. Cell Cycle 8(3):362–372
108. Kancha RK, Bartosch N, Duyster J (2013) Analysis of conformational determinants underlying HSP90-kinase interaction. PLoS One 8(7), e68394. doi:10.1371/journal.pone.0068394
109. Terasawa K, Yoshimatsu K, Iemura S, Natsume T, Tanaka K, Minami Y (2006) Cdc37 interacts with the glycine-rich loop of Hsp90 client kinases. Mol Cell Biol 26(9):3378–3389. doi:10.1128/MCB.26.9.3378-3389.2006
110. Pascale RM, Simile MM, Calvisi DF, Frau M, Muroni MR, Seddaiu MA, Daino L, Muntoni MD, De Miglio MR, Thorgeirsson SS, Feo F (2005) Role of HSP90, CDC37, and CRM1 as modulators of P16(INK4A) activity in rat liver carcinogenesis and human liver cancer. Hepatology 42(6):1310–1319. doi:10.1002/hep.20962
111. Pearl LH (2005) Hsp90 and Cdc37 – a chaperone cancer conspiracy. Curr Opin Genet Dev 15 (1):55–61. doi:10.1016/j.gde.2004.12.011
112. Schwarze SR, Fu VX, Jarrard DF (2003) Cdc37 enhances proliferation and is necessary for normal human prostate epithelial cell survival. Cancer Res 63(15):4614–4619
113. Stepanova L, Finegold M, DeMayo F, Schmidt EV, Harper JW (2000) The oncoprotein kinase chaperone CDC37 functions as an oncogene in mice and collaborates with both c-myc and cyclin D1 in transformation of multiple tissues. Mol Cell Biol 20(12):4462–4473. doi:10.1128/Mcb.20.12.4462-4473.2000
114. Zhang H, Wu W, Du Y, Santos SJ, Conrad SE, Watson JT, Grammatikakis N, Gallo KA (2004) Hsp90/p50cdc37 is required for mixed-lineage kinase (MLK) 3 signaling. J Biol Chem 279(19):19457–19463
115. Basso AD, Solit DB, Chiosis G, Giri B, Tsichlis P, Rosen N (2002) Akt forms an intracellular complex with heat shock protein 90 (Hsp90) and Cdc37 and is destabilized by inhibitors of Hsp90 function. J Biol Chem 277(42):39858–39866. doi:10.1074/jbc.M206322200
116. Dey B, Lightbody JJ, Boschelli F (1996) CDC37 is required for p60v-src activity in yeast. Mol Biol Cell 7(9):1405–1417
117. Kim H, Abd Elmageed ZY, Davis C, El-Bahrawy AH, Naura AS, Ekaidi I, Abdel-Mageed AB, Boulares AH (2014) Correlation between PDZK1, Cdc37, Akt and breast cancer

malignancy: the role of PDZK1 in cell growth through Akt stabilization by increasing and interacting with Cdc37. Mol Med 20:270–279. doi:10.2119/molmed.2013.00166
118. Marino-Enriquez A, Ou WB, Cowley G, Luo B, Jonker AH, Mayeda M, Okamoto M, Eilers G, Czaplinski JT, Sicinska E, Wang Y, Taguchi T, Demetri GD, Root DE, Fletcher JA (2014) Genome-wide functional screening identifies CDC37 as a crucial HSP90-cofactor for KIT oncogenic expression in gastrointestinal stromal tumors. Oncogene 33 (14):1872–1876. doi:10.1038/onc.2013.127
119. Stepanova L, Leng X, Parker SB, Harper JW (1996) Mammalian p50Cdc37 is a protein kinase-targeting subunit of Hsp90 that binds and stabilizes Cdk4. Genes Dev 10 (12):1491–1502
120. Grammatikakis N, Lin JH, Grammatikakis A, Tsichlis PN, Cochran BH (1999) p50(cdc37) acting in concert with Hsp90 is required for Raf-1 function. Mol Cell Biol 19(3):1661–1672
121. Stepanova L, Yang G, DeMayo F, Wheeler TM, Finegold M, Thompson TC, Harper JW (2000) Induction of human Cdc37 in prostate cancer correlates with the ability of targeted Cdc37 expression to promote prostatic hyperplasia. Oncogene 19(18):2186–2193. doi:10.1038/sj.onc.1203561
122. Katayama Y, Sakai A, Okikawa Y, Oue N, Asaoku H, Sasaki A, Imanaka F, Tsujimoto T, Takimoto Y, Masuda R, Nakaju N, Otsuki T, Yasui W, Kimura A (2004) Cyclin D1 overexpression is not a specific grouping marker, but may collaborate with CDC37 in myeloma cells. Int J Oncol 25(3):579–595
123. Gray PJ, Stevenson MA, Calderwood SK (2007) Targeting Cdc37 inhibits multiple signaling pathways and induces growth arrest in prostate cancer cells. Cancer Res 67 (24):11942–11950. doi:10.1158/0008-5472.Cani-07-3162
124. Wang Z, Wei W, Sun CK, Chua MS, So S (2015) Suppressing the CDC37 cochaperone in hepatocellular carcinoma cells inhibits cell cycle progression and cell growth. Liver Int 35 (4):1403–1415. doi:10.1111/liv.12651
125. Lamphere L, Fiore F, Xu X, Brizuela L, Keezer S, Sardet C, Draetta GF, Gyuris J (1997) Interaction between Cdc37 and Cdk4 in human cells. Oncogene 14(16):1999–2004. doi:10.1038/sj.onc.1201036
126. Zhao Q, Boschelli F, Caplan AJ, Arndt KT (2004) Identification of a conserved sequence motif that promotes Cdc37 and cyclin D1 binding to Cdk4. J Biol Chem 279 (13):12560–12564. doi:10.1074/jbc.M308242200
127. Wu P, Nielsen TE, Clausen MH (2015) FDA-approved small-molecule kinase inhibitors. Trends Pharmacol Sci 36(7):422–439. doi:10.1016/j.tips.2015.04.005
128. Cutforth T, Rubin GM (1994) Mutations in Hsp83 and cdc37 impair signaling by the sevenless receptor tyrosine kinase in Drosophila. Cell 77(7):1027–1036
129. Gould CM, Kannan N, Taylor SS, Newton AC (2009) The chaperones Hsp90 and Cdc37 mediate the maturation and stabilization of protein kinase C through a conserved PXXP motif in the C-terminal tail. J Biol Chem 284(8):4921–4935. doi:10.1074/jbc.M808436200
130. Tatebe H, Shiozaki K (2003) Identification of Cdc37 as a novel regulator of the stress-responsive mitogen-activated protein kinase. Mol Cell Biol 23(15):5132–5142
131. Polier S, Samant RS, Clarke PA, Workman P, Prodromou C, Pearl LH (2013) ATP-competitive inhibitors block protein kinase recruitment to the Hsp90-Cdc37 system. Nat Chem Biol 9(5):307–312. doi:10.1038/nchembio.1212
132. Zhang T, Hamza A, Cao X, Wang B, Yu S, Zhan CG, Sun D (2008) A novel Hsp90 inhibitor to disrupt Hsp90/Cdc37 complex against pancreatic cancer cells. Mol Cancer Ther 7 (1):162–170. doi:10.1158/1535-7163.MCT-07-0484
133. Zhang T, Li Y, Yu Y, Zou P, Jiang Y, Sun D (2009) Characterization of celastrol to inhibit hsp90 and cdc37 interaction. J Biol Chem 284(51):35381–35389. doi:10.1074/jbc.M109.051532
134. Klaic L, Morimoto RI, Silverman RB (2012) Celastrol analogues as inducers of the heat shock response. Design and synthesis of affinity probes for the identification of protein targets. ACS Chem Biol 7(5):928–937. doi:10.1021/cb200539u

135. Smith JR, de Billy E, Hobbs S, Powers M, Prodromou C, Pearl L, Clarke PA, Workman P (2015) Restricting direct interaction of CDC37 with HSP90 does not compromise chaperoning of client proteins. Oncogene 34(1):15–26. doi:10.1038/onc.2013.519
136. Armstrong H, Wolmarans A, Mercier R, Mai B, LaPointe P (2012) The co-chaperone Hch1 regulates Hsp90 function differently than its homologue Aha1 and confers sensitivity to yeast to the Hsp90 inhibitor NVP-AUY922. PLoS One 7(11), e49322. doi:10.1371/journal.pone.0049322
137. Meyer P, Prodromou C, Liao C, Hu B, Mark Roe S, Vaughan CK, Vlasic I, Panaretou B, Piper PW, Pearl LH (2004) Structural basis for recruitment of the ATPase activator Aha1 to the Hsp90 chaperone machinery. EMBO J 23(3):511–519
138. Meyer P, Prodromou C, Hu B, Vaughan C, Roe SM, Panaretou B, Piper PW, Pearl LH (2003) Structural and functional analysis of the middle segment of hsp90: implications for ATP hydrolysis and client protein and cochaperone interactions. Mol Cell 11(3):647–658
139. Soti C, Vermes A, Haystead TA, Csermely P (2003) Comparative analysis of the ATP-binding sites of Hsp90 by nucleotide affinity cleavage: a distinct nucleotide specificity of the C-terminal ATP-binding site. Eur J Biochem 270(11):2421–2428
140. Martinez-Yamout MA, Venkitakrishnan RP, Preece NE, Kroon G, Wright PE, Dyson HJ (2006) Localization of sites of interaction between p23 and Hsp90 in solution. J Biol Chem 281(20):14457–14464
141. Meyer P, Prodromou C, Liao C, Hu B, Roe SM, Vaughan CK, Vlasic I, Panaretou B, Piper PW, Pearl LH (2004) Structural basis for recruitment of the ATPase activator Aha1 to the Hsp90 chaperone machinery. EMBO J 23(6):1402–1410
142. Sun L, Prince T, Manjarrez JR, Scroggins BT, Matts RL (2012) Characterization of the interaction of Aha1 with components of the Hsp90 chaperone machine and client proteins. Biochim Biophys Acta 1823(6):1092–1101. doi:10.1016/j.bbamcr.2012.03.014
143. Harst A, Lin H, Obermann WM (2005) Aha1 competes with Hop, p50 and p23 for binding to the molecular chaperone Hsp90 and contributes to kinase and hormone receptor activation. Biochem J 387(Pt 3):789–796. doi:10.1042/BJ20041283
144. Holmes JL, Sharp SY, Hobbs S, Workman P (2008) Silencing of HSP90 cochaperone AHA1 expression decreases client protein activation and increases cellular sensitivity to the HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin. Cancer Res 68(4):1188–1197. doi:10.1158/0008-5472.can-07-3268
145. Dunn DM, Woodford MR, Truman AW, Jensen SM, Schulman J, Caza T, Remillard TC, Loiselle D, Wolfgeher D, Blagg BS, Franco L, Haystead TA, Daturpalli S, Mayer MP, Trepel JB, Morgan RM, Prodromou C, Kron SJ, Panaretou B, Stetler-Stevenson WG, Landas SK, Neckers L, Bratslavsky G, Bourboulia D, Mollapour M (2015) c-Abl mediated tyrosine phosphorylation of Aha1 activates its co-chaperone function in cancer cells. Cell Rep 12(6):1006–1018. doi:10.1016/j.celrep.2015.07.004
146. Afar DE, Goga A, Cohen L, Sawyers CL, McLaughlin J, Mohr RN, Witte ON (1994) Genetic approaches to defining signaling by the CML-associated tyrosine kinase BCR-ABL. Cold Spring Harb Symp Quant Biol 59:589–594
147. Mauro MJ, Druker BJ (2001) STI571: targeting BCR-ABL as therapy for CML. Oncologist 6(3):233–238
148. Radujkovic A, Schad M, Topaly J, Veldwijk MR, Laufs S, Schultheis BS, Jauch A, Melo JV, Fruehauf S, Zeller WJ (2005) Synergistic activity of imatinib and 17-AAG in imatinib-resistant CML cells overexpressing BCR-ABL--Inhibition of P-glycoprotein function by 17-AAG. Leukemia 19(7):1198–1206. doi:10.1038/sj.leu.2403764
149. Hildenbrand ZL, Molugu SK, Paul A, Avila GA, Herrera N, Xiao C, Cox MB, Bernal RA (2010) High-yield expression and purification of the Hsp90-associated p23, FKBP52, HOP and SGTalpha proteins. J Chromatogr B Analyt Technol Biomed Life Sci 878(28):2760–2764. doi:10.1016/j.jchromb.2010.08.016
150. Freeman BC, Yamamoto KR (2002) Disassembly of transcriptional regulatory complexes by molecular chaperones. Science 296(5576):2232–2235. doi:10.1126/science.1073051

151. Ali MM, Roe SM, Vaughan CK, Meyer P, Panaretou B, Piper PW, Prodromou C, Pearl LH (2006) Crystal structure of an Hsp90-nucleotide-p23/Sba1 closed chaperone complex. Nature 440(7087):1013–1017
152. Johnson JL, Toft DO (1995) Binding of p23 and hsp90 during assembly with the progesterone receptor. Mol Endocrinol 9(6):670–678
153. Karagoz GE, Duarte AM, Ippel H, Uetrecht C, Sinnige T, van Rosmalen M, Hausmann J, Heck AJ, Boelens R, Rudiger SG (2011) N-terminal domain of human Hsp90 triggers binding to the cochaperone p23. Proc Natl Acad Sci U S A 108(2):580–585. doi:10.1073/pnas.1011867108
154. McLaughlin SH, Sobott F, Yao ZP, Zhang W, Nielsen PR, Grossmann JG, Laue ED, Robinson CV, Jackson SE (2006) The co-chaperone p23 arrests the Hsp90 ATPase cycle to trap client proteins. J Mol Biol 356(3):746–758
155. Young JC, Hartl FU (2000) Polypeptide release by Hsp90 involves ATP hydrolysis and is enhanced by the co-chaperone p23. EMBO J 19(21):5930–5940
156. Marcu MG, Schulte TW, Neckers L (2000) Novobiocin and related coumarins and depletion of heat shock protein 90-dependent signaling proteins. J Natl Cancer Inst 92(3):242–248
157. Bohen SP (1998) Genetic and biochemical analysis of p23 and ansamycin antibiotics in the function of Hsp90-dependent signaling proteins. Mol Cell Biol 18(6):3330–3339
158. Grad I, McKee TA, Ludwig SM, Hoyle GW, Ruiz P, Wurst W, Floss T, Miller CA 3rd, Picard D (2006) The Hsp90 cochaperone p23 is essential for perinatal survival. Mol Cell Biol 26 (23):8976–8983
159. Forafonov F, Toogun OA, Grad I, Suslova E, Freeman BC, Picard D (2008) p23/Sba1p protects against Hsp90 inhibitors independently of its intrinsic chaperone activity. Mol Cell Biol 28(10):3446–3456
160. Fliss AE, Benzeno S, Rao J, Caplan AJ (2000) Control of estrogen receptor ligand binding by Hsp90. J Steroid Biochem Mol Biol 72(5):223–230
161. Knoblauch R, Garabedian MJ (1999) Role for Hsp90-associated cochaperone p23 in estrogen receptor signal transduction. Mol Cell Biol 19(5):3748–3759
162. Reebye V, Querol Cano L, Lavery DN, Brooke GN, Powell SM, Chotai D, Walker MM, Whitaker HC, Wait R, Hurst HC, Bevan CL (2012) Role of the HSP90-associated cochaperone p23 in enhancing activity of the androgen receptor and significance for prostate cancer. Mol Endocrinol 26(10):1694–1706. doi:10.1210/me.2012-1056
163. Simpson NE, Lambert WM, Watkins R, Giashuddin S, Huang SJ, Oxelmark E, Arju R, Hochman T, Goldberg JD, Schneider RJ, Reiz LF, Soares FA, Logan SK, Garabedian MJ (2010) High levels of Hsp90 cochaperone p23 promote tumor progression and poor prognosis in breast cancer by increasing lymph node metastases and drug resistance. Cancer Res 70 (21):8446–8456. doi:10.1158/0008-5472.CAN-10-1590
164. Oxelmark E, Roth JM, Brooks PC, Braunstein SE, Schneider RJ, Garabedian MJ (2006) The cochaperone p23 differentially regulates estrogen receptor target genes and promotes tumor cell adhesion and invasion. Mol Cell Biol 26(14):5205–5213. doi:10.1128/MCB.00009-06
165. Chadli A, Felts SJ, Wang Q, Sullivan WP, Botuyan MV, Fauq A, Ramirez-Alvarado M, Mer G (2010) Celastrol inhibits Hsp90 chaperoning of steroid receptors by inducing fibrillization of the Co-chaperone p23. J Biol Chem 285(6):4224–4231. doi:10.1074/jbc.M109.081018
166. Hall JA, Seedarala S, Rice N, Kopel L, Halaweish F, Blagg BS (2015) Cucurbitacin D is a disruptor of the HSP90 chaperone machinery. J Nat Prod 78(4):873–879. doi:10.1021/acs.jnatprod.5b00054
167. Ishii T, Kira N, Yoshida T, Narahara H (2013) Cucurbitacin D induces growth inhibition, cell cycle arrest, and apoptosis in human endometrial and ovarian cancer cells. Tumour Biol 34 (1):285–291. doi:10.1007/s13277-012-0549-2
168. Ku JM, Kim SR, Hong SH, Choi HS, Seo HS, Shin YC, Ko SG (2015) Cucurbitacin D induces cell cycle arrest and apoptosis by inhibiting STAT3 and NF-kappaB signaling in doxorubicin-resistant human breast carcinoma (MCF7/ADR) cells. Mol Cell Biochem 409 (1–2):33–43. doi:10.1007/s11010-015-2509-9

169. Brandt GE, Schmidt MD, Prisinzano TE, Blagg BS (2008) Gedunin, a novel hsp90 inhibitor: semisynthesis of derivatives and preliminary structure-activity relationships. J Med Chem 51 (20):6495–6502. doi:10.1021/jm8007486
170. Kamath SG, Chen N, Xiong Y, Wenham R, Apte S, Humphrey M, Cragun J, Lancaster JM (2009) Gedunin, a novel natural substance, inhibits ovarian cancer cell proliferation. Int J Gynecol Cancer 19(9):1564–1569. doi:10.1111/IGC.0b013e3181a83135
171. Uddin SJ, Nahar L, Shilpi JA, Shoeb M, Borkowski T, Gibbons S, Middleton M, Byres M, Sarker SD (2007) Gedunin, a limonoid from Xylocarpus granatum, inhibits the growth of CaCo-2 colon cancer cell line in vitro. Phytother Res 21(8):757–761. doi:10.1002/ptr.2159
172. Patwardhan CA, Fauq A, Peterson LB, Miller C, Blagg BS, Chadli A (2013) Gedunin inactivates the co-chaperone p23 protein causing cancer cell death by apoptosis. J Biol Chem 288(10):7313–7325. doi:10.1074/jbc.M112.427328

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Evaluating Dual Hsp90 and Hsp70 Inhibition as a Cancer Therapy

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Abstract The heat shock proteins (Hsps) are a family of highly conserved proteins involved in the regulation of numerous cellular processes including those associated with cancer. Inhibiting the function of these Hsps, specifically Hsp70 and Hsp90, is a major strategy used in the development of new cancer therapies. Numerous Hsp90 inhibitors have been evaluated in the clinic, and while some have experienced success, many have produced disappointing results. One reason explaining their failure is that they induce a cytoprotective response that protects cancer cells from the negative effects of Hsp90 inhibition. In order to maximise the therapeutic outcomes, dual inhibition of Hsp70 and Hsp90 can be employed to overcome cell rescue mechanisms induced by monotherapies. In this chapter, we discuss dual inhibition of Hsp70 and Hsp90 using small molecules and evaluate the potential of this strategy for the development of cancer therapeutics.

Keywords Dual inhibitors, Heat shock proteins, Heat shock response, Hsp70, Hsp90

Contents

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1 Introduction

Heat shock protein 90 (Hsp90) is a molecular chaperone involved in the maintenance of protein homeostasis in the cell [1–3]. Hsp90 assists in the folding, stabilisation, activation and degradation of numerous cellular proteins. Hsp90 interacts with over 400 client proteins [4], many of which are associated with cancer [1]. Upon activation, these client proteins assist in disease progression, which makes Hsp90 a regulator of many disease-causing pathways. Consequently, Hsp90 inhibition has emerged as a promising strategy for the treatment of diseases involving aberrant protein structure and function, including cancer.

Hsp90 exists as a homodimer, where each monomer contains an amino (N-) terminus, a middle domain and a carboxy (C-) terminus (Fig. 1). The N-terminus contains an ATP-binding site, the middle domain contains binding sites for client proteins and co-chaperones, and the C-terminus serves as the dimerisation domain and also contains binding sites for co-chaperones. It is well understood that clinical Hsp90 inhibitors or "classical inhibitors" target the N-terminal ATP-binding site of Hsp90, impacting Hsp90's protein folding cycle (Fig. 1).

The first Hsp90 inhibitor, geldanamycin, was identified in 1994, and its derivative tanespimycin (17-AAG) entered the clinic as a cancer therapeutic in 1999 [5] (Fig. 2). Both of these analogs are from the ansamycin class of compounds. Since 1995, Hsp90 inhibitor drug candidates have been steadily entering the clinic, with a total of 15 different drugs being tested as monotherapies in clinical trials since 1999

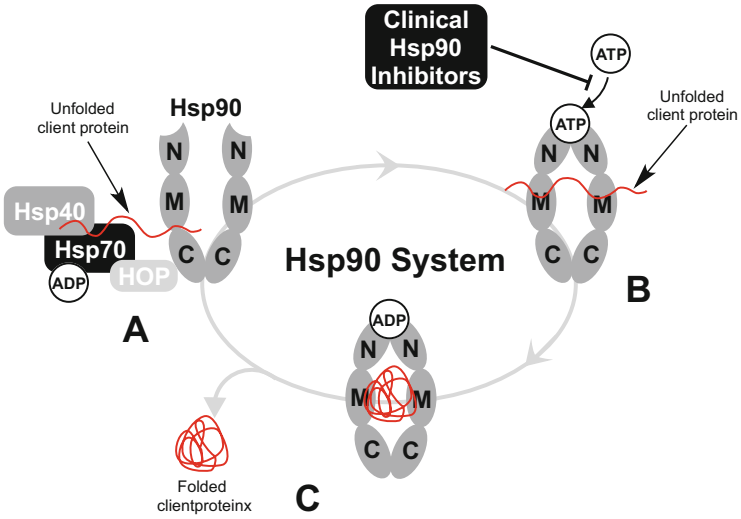

Fig. 1 *Hsp90's protein folding cycle and the mechanism of classical Hsp90 inhibition.* (**a**) Unfolded client proteins are delivered to Hsp90 by Hsp40 and Hsp70 via interactions with the co-chaperone Hsp70/Hsp90 organising protein (HOP). (**b**) Hsp90 utilises ATP hydrolysis to change conformation. (**c**) Fully folded client protein is released

[6, 7]. The ansamycin molecules are reported to inhibit ATP from binding to Hsp90 (Fig. 1b). However, recent evidence shows that they are highly promiscuous and, as such, are likely targeting many proteins, not just Hsp90, which would explain their failure in clinical trials.

More recent Hsp90 inhibitor drug candidates mimic the ATP molecule (Fig. 2). Several of these molecules are still in clinical trials and are being used to treat many cancer types including breast cancer, non-small cell lung cancer (NSCLC), melanoma, renal cell carcinoma (RCC), multiple myeloma (MM), gastrointestinal stromal tumour (GIST), castrate-resistant prostate cancer (CRPC) and several types of leukaemia [6, 7]. Clinical inhibitors block ATP from binding, leading to the inhibition of protein folding. However, clinical trial results showed that when used as single agents, these Hsp90 inhibitors were not highly efficacious and have generated disappointing patient outcomes.

As a single agent, 17-AAG had significant side effects [8] and was subsequently dropped from clinical trials. Three other recent monotherapy regiments in clinical trials involved (1) 17-DMAG to treat CRPC, melanoma or acute myeloid leukaemia; (2) ganetespib to treat breast cancer and NSCLC; and (3) IPI-504 to treat NSCLC and GIST [9, 10] (Fig. 2). However, patient's responses were modest,

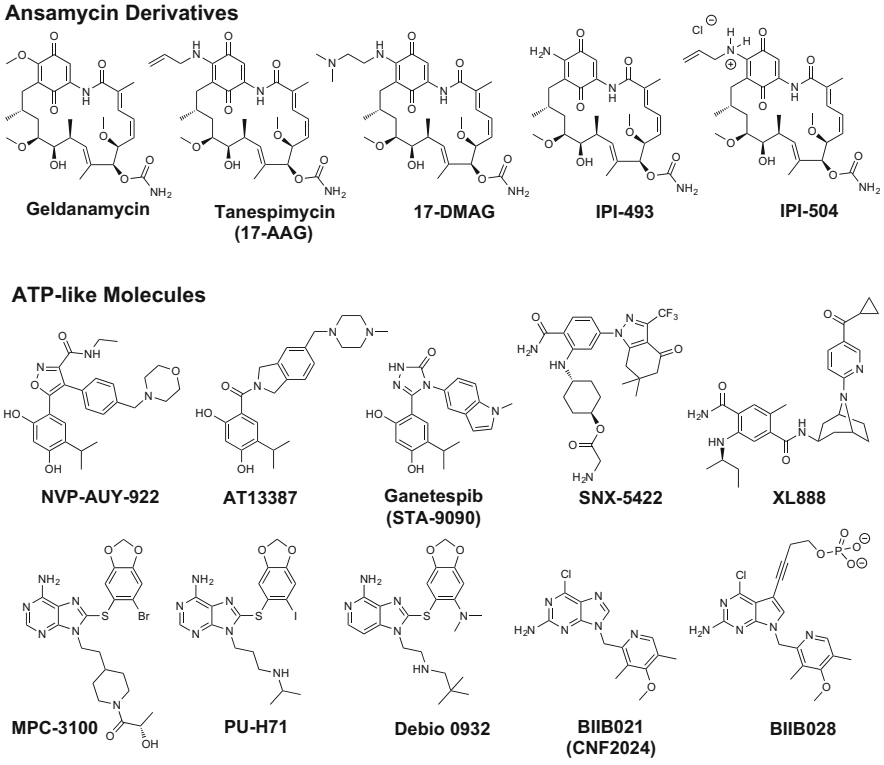

Fig. 2 The two classes of classical Hsp90 inhibitors: ansamycin analogs and ATP-like molecules

where 17-DMAG caused a response in 2 of 28 patients (7%) HER2+ breast cancer patients, ganetespib caused a partial response (PR) in 2 of 22 (9%) breast cancer patients, and IPI-504 caused a PR in 5 of 76 (7%) NSCLC patients and 1 in 36 (3%) GIST patients [7, 10, 11]. 17-DMAG was also reported to cause a complete response (CR) in a single patient that had CRPC, three CR in acute myeloid leukaemia and one PR in melanoma [8, 11]. Thus, Hsp90 inhibitors have shown positive clinical benefit for patients, although the low response rate is a significant concern. Classical Hsp90 inhibitors continue to enter into clinical trials as both single and combination therapies. Currently, there are 32 active studies evaluating the effects of these drugs on numerous cancer types (Table 1).

The low response rate coupled with hepatotoxicity, ocular toxicity and in one case mortality has caused the suspension of most clinical trials using Hsp90 inhibitors as single-agent chemotherapeutics [12, 13]. The limited effectiveness of these Hsp90 inhibitors appears to be due to several key factors. The first is that the ATP-binding site, where these clinical molecules interact with Hsp90, has a binding pocket that is similar to several classes of proteins, specifically DNA polymerases and tyrosine kinases [14]. Thus, classical inhibitors appear to bind to other proteins in addition to Hsp90, thereby producing off-target effects [15–25] and potentially contributing to the observed toxicity associated with these drugs.

Second, resistance and anti-apoptotic pathways are activated immediately upon patient treatment with these clinical Hsp90 inhibitors. This resistance is a result of the specific types of Hsp90 inhibitors activating the cellular heat shock response (HSR) [26]. The HSR is an evolutionary response that is triggered when the cell is under stress and was first discovered by subjecting cells to high temperatures. Triggering a HSR induces high levels of heat shock proteins (Hsps), which are responsible for refolding the aggregated and misfolded proteins that accumulate in the stressed or rapidly growing cell, and they aid in protein degradation [27]. The HSR facilitates cell survival by activating resistance mechanisms and anti-apoptotic pathways [28, 29].

Specifically, cellular stress leads to releasing heat shock factor 1 (HSF-1) from Hsp90 (Fig. 3b) [30–34]. Transport of HSF-1 into the cytoplasm is inhibited leading to a build-up of HSF-1 in the nucleus [35]. HSF-1 then forms a trimer in the nucleus, which is extensively phosphorylated [36]. The HSF-1 trimer binds to specific sequences known as heat shock elements (HSE) in DNA promoters and induces transcription of genes encoding for itself and multiple cellular chaperones, including heat shock protein 27 (Hsp27), heat shock protein 40 (Hsp40) and heat shock protein 70 (Hsp70), in order to rescue the cell from the accumulating unfolded proteins [37, 38] (Fig. 3c). In the absence of stress, promoters for these genes are occupied and unavailable [39, 40]. The mRNAs encoding for inducible and constitutive Hsp70 (*HSPA1A* and *HSPA8*, respectively) are produced during the HSR, as well as mRNA that encodes for HSF-1, Hsp40 and Hsp27 (Fig. 3d). These Hsps attempt to rescue the cell from the unfolded protein that is accumulating. The high levels of Hsps refold the aggregated and misfolded proteins that accumulate in the stressed cell, and selected Hsps can also aid in protein degradation [27].

Table 1 Active clinical trials involving Hsp90 inhibitors registered on clinicaltrials.gov

Molecule	Condition	Phase	Started	Treatment	Other drugs
NVP-AUY-922	Non-small cell lung cancer	II	2013	Single	–
NVP-AUY-922	Lung cancer	I	2014	Dual	Pemetrexed disodium
NVP-AUY-922	Non-small cell lung cancer	I	2013	Dual	LDK378
NVP-AUY-922	Lung cancer	II	2015	Single	–
NVP-AUY-922	GI stromal tumour	II	2011	Single	–
NVP-AUY-922	Non-small cell lung cancer	II	2013	Single	–
NVP-AUY-922	Non-small cell lung cancer	II	2014	Single	–
SNX-5422	HER2+ cancers	I/II	2013	Single	–
SNX-5422	Neuroendocrine tumours	I	2014	Single	–
SNX-5422	Haematological malignancies	I	2014	Single	–
SNX-5422	Solid tumours	I	2013	Comb.	Carboplatin, paclitaxel
STA-9090	Rectal cancer	I	2012	Dual	Capecitabine
STA-9090	Ocular melanoma	II	2010	Single	–
STA-9090	Multiple myeloma	I	2012	Comb.	Bortezomib, dexamethasone
STA-9090	Neurofibromatosis type 1	I/II	2013	Dual	Sirolimus
STA-9090	Ovarian, fallopian, peritoneal cancer	I/II	2013	Dual	Paclitaxel
STA-9090	HER2+ breast cancer	I	2014	Comb.	Paclitaxel, trastuzumab, pertuzumab
STA-9090	Head and neck cancers	I	2014	Single	–
STA-9090	Breast cancer	II	2012	Dual	Fulvestrant
STA-9090	Non-small cell lung cancer	III	2013	Dual	Docetaxel
STA-9090	Ovarian, fallopian, peritoneal cancer	I/II	2014	Dual	Paclitaxel
STA-9090	Small cell lung cancer	I/II	2014	Dual	Doxorubicin
STA-9090	Lung cancer	I/II	2013	Single	–
STA-9090	Neoplasms	I	2014	Dual	Ziv-Aflibercept
STA-9090	Breast cancer	I	2010	Single	–
STA-9090	Acute myeloid lymphoma, myelodysplastic syndrome	III	2014	Single	–
PU-H71	Lymphoma, solid tumour	I	2011	Single	–
PU-H71	Lymphoma, myeloma, solid tumour	Pre	2010	Single	–
AT13387	Non-small cell lung cancer	I/II	2012	Dual	Crizotinib
AT13387	Head and neck cancers	I	2015	Dual	Cisplatin
AT13387	Melanoma	I	2014	Comb.	Dabrafenib, trametinib
XL888	Melanoma	I	2012	Dual	Vemurafenib

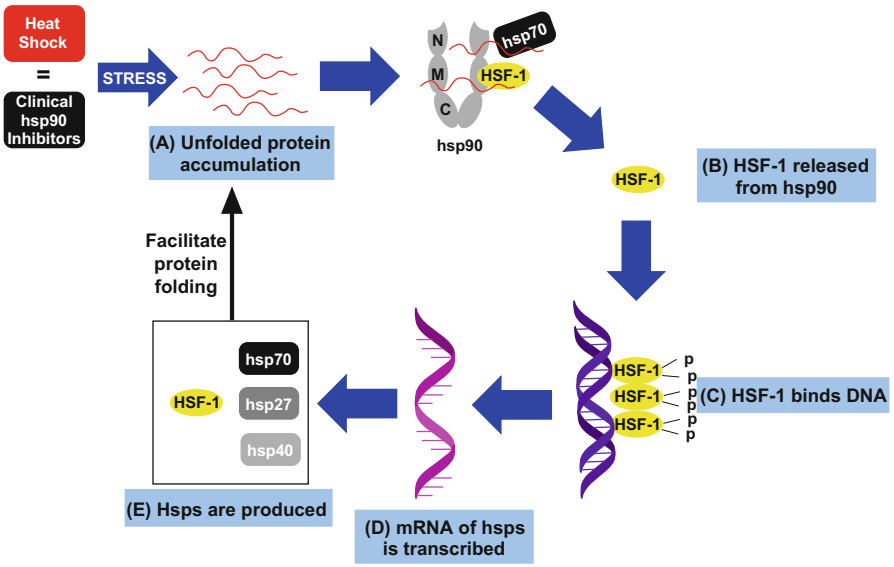

Fig. 3 Depiction of the widely accepted model of heat shock and the induction of the heat shock response (HSR). (**a**) Stress from heat shock or clinical Hsp90 inhibitors triggers an accumulation of unfolded proteins in the cell. (**b**) The Hsp90 protein complex collects these unfolded proteins, which causes the release of HSF-1 from the protein complex. (**c**) HSF-1 then forms an active trimer, which translocates to the nucleus and binds to DNA. (**d**) The mRNA of the heat shock proteins is transcribed from the DNA. (**e**) The mRNA is then translated into the heat shock proteins, which can then facilitate folding of the previously accumulated unfolded proteins

Similar to the stress caused by high temperatures, the excessive growth of cancer creates stress in cells, and thus cancer cells produce high levels of Hsps. These Hsps maintain protein folding and protein degradation and repair the large quantity of proteins required for rapid cell division, as well as stabilising mutated oncoproteins [27, 41]. This high level of Hsps, including Hsp90, is why Hsp90 inhibitors are a promising treatment for cancer. However, inhibiting Hsp90 function using the clinical inhibitors is well established to produce high levels of Hsp70; indeed, Hsp70 is often used as a pharmacodynamic marker to determine if Hsp90 is being inhibited by classical inhibitors [42–44].

Hsp70 assists in the delivery of specific clients to Hsp90 [45], as well as functioning as an independent chaperone that facilitates protein translocation; stabilises anti-apoptotic proteins; plays a key role in cellular resistance; and prevents apoptosis [46–49]. Thus, inducing high levels of Hsp70 (>6-fold over background) such as those observed when treating cells and patients with the classical Hsp90 inhibitors is problematic for killing cancer cells. Indeed, the high production of Hsp70 likely plays a key role in the disappointing clinical results [20–24]. In response to these poor results for classical inhibitors, two strategies have been employed.

The first approach, which is the development of Hsp90 inhibitors that target sites other than the ATP-binding site of Hsp90, offers alternative mechanisms for blocking Hsp90's activity. Specifically, inhibiting activity at the C-terminus of Hsp90 does not induce a HSR, nor lead to the upregulation of the Hsps [20–24, 50–55]. Thus, this approach may succeed as a single treatment as it does not produce the anti-apoptotic or resistance observed with the classical inhibitors. The second approach, which is already being used in the clinic, is a combination of classical Hsp90 inhibitors with other forms of therapy. This second approach is discussed in this chapter.

Dual inhibition is a rapidly developing area, and there are a large number of clinical trials and patents being reported in this field. Of the 32 active clinical trials, 17 are studying the effects of Hsp90 inhibitors with one or more other drugs (www.clinicaltrials.gov database). Recent patents include the use of combination treatments utilising Hsp90 inhibitors with Hsp27 or Hsp70 inhibitors (patent number WO-2007041294). Yukimasa patented results using a classic Hsp90 inhibitor (KW-2478) in combination with an Hsp70, Hsp27 or BCL2 cancer treatment drug (patent number WO-2007028387). Kyowa Hakko Kogyo patented the treatment of a classical inhibitor 17-AAG being used in combination with a kinase inhibitor such as gefitinib or a proteasome inhibitor such as bortezomib (patent number WO-2008108386). Astex Therapeutics has patented the drug combination of the classical inhibitor AT9283 with cyclin-dependent kinase inhibitors or aurora kinase inhibitors (WO2008044045). Patent activity on dual inhibitors shows that this line of investigation is being vigorously pursued.

2 Hsp90 Inhibition-Based Combination Treatment

Hsp90 is vital for most cancer cells because of its pivotal role in modulating protein conformation and maturation [56–60]. To date, more than 400 proteins are regulated by or associated with Hsp90, and as such they are called Hsp90 client proteins [4]. About half of these clients are critical for cancer cell growth [61], including transmembrane tyrosine kinases (HER2 and EGFR), metastable signalling proteins (Akt, K-ras and Raf-1), mutated signalling proteins (p53 and v-Src), chimeric signalling proteins (Bcr-Abl), cell cycle regulators (Cdk4 and Cdk6) and steroid receptors (androgen, oestrogen and progesterone receptors) [62–67]. When mutated or deregulated, these clients promote cancer growth. Cancer cell proliferation and survival [68, 69] are facilitated by Hsp90 by maintaining tumours and homeostasis and helping cells to adapt to unfavourable or stressful microenvironments that include heat, hypoxia, free radical production, radiation and chemotherapy [68–71].

Because of its key roles in tumour development, Hsp90 has emerged as a promising target for cancer therapy [63, 72–74]. Inhibiting Hsp90 has involved targeting all three domains: the N-, middle and C-domains as a paradigm of network-oriented drug discovery [63, 71, 75, 76]. Indeed, success at suppressing cancer cell growth has been reported in both preclinical and clinical studies [5, 50,

51, 54, 61, 72–74, 77–80]. Although there are currently 32 clinical trials involving Hsp90 inhibitors, only three unique structures are involved in these studies and are being tested on patients. All three target the N-terminal ATP-binding site of Hsp90 [54] and more than half of these clinical trials are using the compounds in conjunction with other therapies [81–84].

Given Hsp90's central regulating role in cancer development and its close relationship with numerous key oncogenic proteins, studies are now exploring if Hsp90 inhibitors can sensitise tumours to other chemotherapeutic agents. Developing combination therapies using Hsp90 inhibitors and other types of anticancer agents with a distinct mechanism of action is one avenue that is currently being investigated. Encouragingly, Hsp90 inhibition-based combination treatments of cancer have proven to be more effective and more successful than monotherapies in clinical trials, indicating a promising future for anticancer treatment. In this section, we focus on the investigation and achievement of combination treatments based on direct Hsp90 and Hsp70 dual inhibition.

2.1 Dual Hsp90 and Hsp70 Inhibition

The disappointing clinical results of Hsp90 inhibitors are likely connected to induction of the HSR, which upregulates Hsp70 and Hsp27 as well as HSF-1 [74, 85, 86]. Induction of Hsp70 produces the undesirable effect of counteracting the efficiency of Hsp90-based treatment, and it has been identified as a hallmark of N-terminal Hsp90 inhibitors [87–97] (Fig. 4a). The C-terminal modulators, which do not target the ATP site on Hsp90, do not induce HSF-1 nor the HSR [50, 51, 54, 77–80, 98] (Fig. 4b and c). Herein we discuss two approaches to dual inhibition of Hsp70 and Hsp90 including combining small-molecule inhibitors of both Hsp70 and Hsp90 and combining Hsp70 silencing with Hsp90 inhibitors.

There are several rescue mechanisms that are induced with the HSR. First, Hsp90 is induced and can still perform its protein folding and regulatory role. Second, Hsp70 is also induced and may compensate for some of Hsp90's inhibited functions by assisting in protein folding, preventing protein aggregation and regulating protein complex assembly or disassembly [99, 100]. Third, Hsp70 actively participates in the protection of cancer cells from both extrinsic and intrinsic apoptosis [99]. Ectopic overexpression or induced endogenous levels of Hsp70 promote cancer cell survival by effectively inhibiting lysosomal membrane permeabilization [49], death receptor pathway [48], mitochondria-initiated signalling for caspase-dependent apoptosis [46, 47, 101–104] as well as AIF-associated caspase-independent apoptosis [105, 106].

Evidence of Hsp70's critical role in apoptosis was seen when silencing Hsp70 expression using antisense oligonucleotides or ectopic transfection produced extensive apoptotic cancer cell death [48, 107, 108]. Furthermore, Hsp70 inhibition triggers an antitumour immune response by blocking the Hsp70-induced activation of myeloid suppressive cells (MDSC), which have the capacity to suppress both the

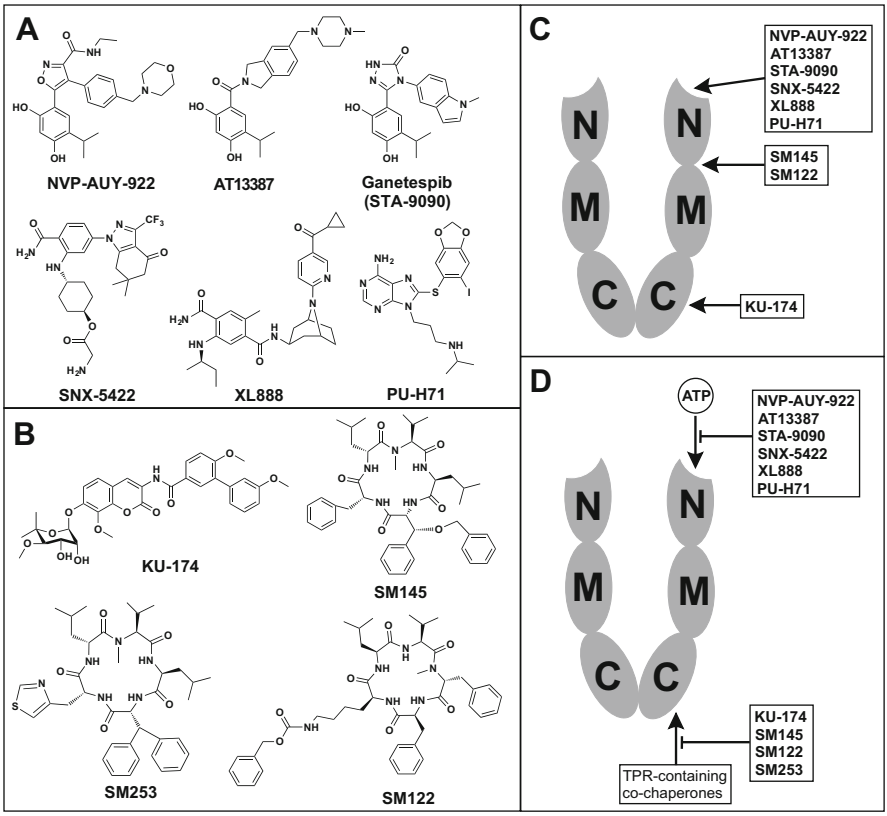

Fig. 4 *Hsp90 inhibitors in clinical trials*. (**a**) Structures of current Hsp90 inhibitors in clinical trials, all targeting the ATP-binding site at the N-terminus. (**b**) Structures of Hsp90 inhibitors targeting alternative sites on Hsp90. (**c**) Diagram of Hsp90 showing the binding locations of each inhibitor. (**d**) The interactions shown (ATP binding and TPR-containing co-chaperones) are modulated by the inhibitors

cytotoxic activities of natural killer (NK) and NKT cells and the adaptive immune response mediated by CD4+ and CD8+ T cells [109–113]. All of these factors make dual inhibition of Hsp90 and Hsp70 an optimal cancer therapy.

Using a combination treatment of Hsp90 and Hsp70 inhibitors may not only neutralise the issues associated with N-terminal Hsp90 inhibition, but it may also amplify their anticancer efficiency based on their multiple and independent mechanisms of action. Encouragingly, Hsp70 silencing using siRNA (small interfering RNA), shRNA (small hairpin RNA) or cDNA (complementary DNA in the reversed orientation) of Hsp70 has proven to successfully and synergistically potentiate Hsp90-based anticancer treatment in both solid tumours and leukaemia [48, 89, 90, 95]. However, only a few scientific studies on dual inhibition using small-molecule inhibitors have been published, mainly because only a limited

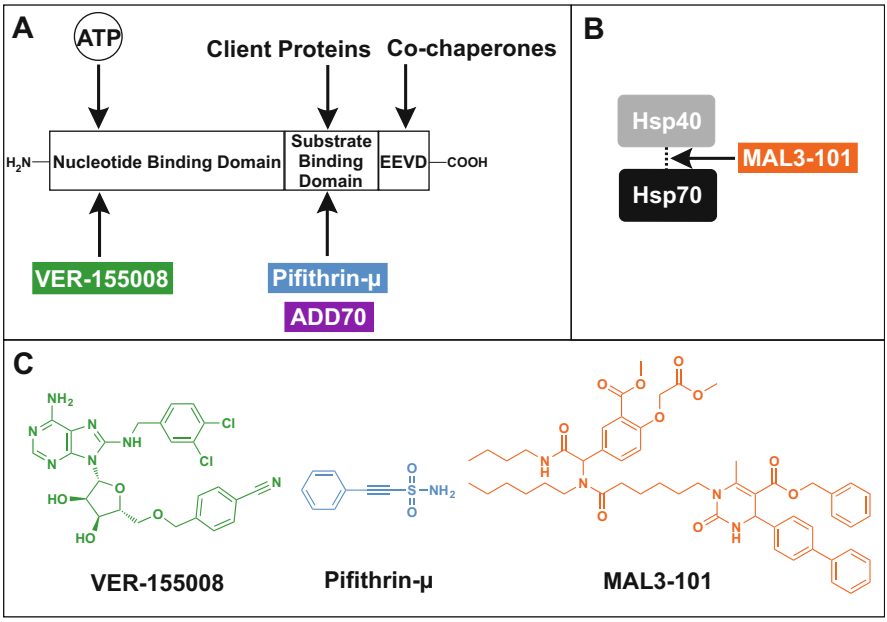

Fig. 5 *Hsp70 inhibitors.* (**a**) Diagram of the structure of Hsp70 indicating the binding sites of inhibitors: VER-155008, Pifithrin-μ and ADD70. (**b**) Hsp70 inhibitor MAL3-101 binds at the interface between the Hsp70/Hsp40 complexes. (**c**) Structures of Hsp70 inhibitors

number of compounds effectively modulate Hsp70's function [54, 90, 114]. MAL3-101, Pifithrin-μ and VER-155008 (Fig. 5c) are the only three drugs that specifically target Hsp70 and show synergism or an additive effect in combination treatment with Hsp90 inhibitors both in vitro and in vivo [89, 93, 115–117].

VER-155008 binds to the ATP-binding site in Hsp70, blocking Hsp70's access to ATP and halting Hsp70's function by denying it energy to perform (Fig. 5a). VER-155008 was developed through a structure-based X-ray crystallographic design [118]. It is also the first molecule to target the ATP-binding domain of Hsp70 protein [116]. Treatment of cancer cells with VER-155008 showed antiproliferative activity in many types of human cancer cells, including colon cancer [116], breast cancer [118], multiple myeloma [89] and acute myeloid leukaemia [117]. As expected, VER-155008 shows synergistic or additive combination effects with Hsp90 inhibitors in preclinical cancer treatments [89, 116, 117].

Pifithrin-μ binds to the substrate-binding domain of Hsp70 and blocks other substrates from effectively interacting with that site (Fig. 5). Pifithrin-μ specifically targets the inducible isoform of Hsp70, without binding to the constitutive Hsp70 or to Hsp90 [119]. It interferes with the C-terminal substrate-binding domain of Hsp70 and disrupts its association with client proteins, causing cell cycle arrest and significant apoptosis at low micromolar concentrations. This leads the loss of Hsp70 function, as it can no longer interact with substrates.

Like Pifithrin-μ, MAL3-101 (Fig. 5) binds to Hsp70 at the substrate-binding site. It blocks Hsp70's essential cellular function by inhibiting the ability of Hsp40 co-chaperones to stimulate Hsp70 ATPase activity [120, 121] (Fig. 5b). Hsp40 docks to Hsp70 during substrate transfer of unfolded client proteins (Fig. 1); thus, inhibiting this binding event halts the transfer of unfolded proteins and impacts protein homeostasis, thereby inducing cell death.

2.1.1 Combination of VER-155008 and NVP-AUY922 in Multiple Myeloma Treatment

VER-155008-based Hsp70 inhibition has been relatively successful in the treatment of multiple myeloma (MM) [89]. VER-155008 significantly decreased the cellular viability in MM cell lines, including INA-6, MM.1S, L363, KMS11 and JJN-3. The sensitivity of MM cells to VER-155008 differed between cell lines with IC_{50} values from 2.5 to 17 μM, and the drug concentrations that induce near complete cell demise in all studied cell lines are between 10 and 30 μM. VER-155008-induced Hsp70 inhibition led to apoptosis in MM with substantial accumulation of apoptosis-inducing factor (AIF) in the nucleus and with increased cleavage of pro-caspases 9/3 and the caspase substrate poly(ADP-ribose) polymerase 1 (PARP 1). Additionally, VER-155008 treatment simultaneously degraded many Hsp90 client proteins involved in a number of oncogenic signalling pathways including Ras/Raf/MAPK, JAK/STAT3, PI3K/Akt and the IKK/NFkB pathways.

When a dual inhibition approach of VER-155008 and NVP-AUY-922 was implemented into INA-6, MM.1S and primary MM cells, a synergistic mode of action was observed [89]. Specifically, the combination treatment with two inhibitors significantly enhanced apoptosis induction, where the combination effect on INA-6 cells and MM.1S cells was synergistic and additive, respectively, with combination indices (CI) less than 1 for all effect levels calculated [89, 90] (Table 2).

Table 2 Ranges of combination indices (CI) correspond to synergistic, additive or antagonistic activities

Range of CI	Description
<0.10	Very strong synergism
0.1–0.3	Strong synergism
0.3–0.7	Synergism
0.7–0.85	Moderate synergism
0.85–0.9	Slight synergism
0.9–1.1	Nearly additive
1.1–1.2	Slight antagonism
1.2–1.45	Moderate antagonism
1.45–3.3	Antagonism
3.3–10	Strong antagonism
>10	Very strong antagonism

2.1.2 Combination of VER-155008 and 17-DMAG in Leukaemia Treatment

Acute myeloid leukaemia (AML) is a biologically heterogeneous malignancy characterised by bone marrow infiltration of immature leukaemic blasts [122]. Hsp90 has emerged as a potent therapeutic target in AML, and the Hsp90 inhibitor 17-DMAG is effective in killing AML cells in vitro preclinical tests [117]. However, Hsp90 inhibition showed limited antileukaemic effects in phase I clinical trials [11, 123]. One major reason for this is the compensatory Hsp70 upregulation, which is induced by HSF-1 [74, 95, 124]. This is supported by the observation that, in a manner observed with other Hsp90 inhibitors that target the ATP pocket, 17-DMAG-based AML treatment increased Hsp70 and Hsp90 production [11].

However, combination treatment with Hsp90 and Hsp70 inhibitors to neutralise the induced Hsp70 proteins shows promise for human AML [117]. In primary human AML cells from 19 unselected patients, the Hsp70 inhibitor VER-155008 itself showed significant antileukaemic activity at 10 μM, causing a dose-dependent inhibition of cancer cell proliferation, where growth was inhibited by 72%. Additionally, Hsp90 inhibitor 17-DMAG was used to treat cells at 50 nM, which also resulted in growth inhibition of 58%. The combination treatment with both inhibitors in primary AML cells decreased cell growth to 82%, indicating an additive growth inhibition effect on AML cells. Moreover, although both VER-155008 (10 μM) and 17-DMAG (50 nM) were able to cause an inhibition in AML colony formation for most patients, the strongest and most significant decrease in colony number was observed when the two drugs were combined.

VER-155008-mediated Hsp70 inhibition in AML cells did not induce any compensatory increase in other Hsps; in fact it caused a significant reduction of both Hsp70 and Hsp90 expressions when used alone. In contrast, 17-DMAG-mediated Hsp90 inhibition resulted in a significant increase in Hsp70 and Hsp90 levels. When VER-155008 was used in combination with 17-DMAG, Hsp90 and Hsp70, expression levels increased to the same level as when cells were treated with 17-DMAG alone [117]. Thus, in contrast to silencing Hsp70 using siRNA, chemical inhibition of Hsp70 by VER-155008 fails to regulate the Hsp70 and Hsp90 protein increase that is induced by Hsp90 inhibition. These data explain why the combined effect of VER-155008 and 17-DMAG is only additive and not synergistic. Furthermore, it is possible that given that VER-155008 targets an ATP-binding pocket, it may have off-target effects.

2.1.3 Combination of VER-155008 and SM122 in Colon Cancer Treatment

SM122 (Fig. 4b) is a unique Hsp90 inhibitor that modulates the C-terminus and does not induce a HSR or produce an accumulation of Hsp70 in the human colon

cancer cell line (HCT116). Recent work by Wang and McAlpine investigated the effects of combining SM122 with Hsp70 inhibitor VER-155008 on chaperone-mediated protein folding and the induction of apoptosis, compared to a combination of 17-AAG and VER-155008 [20, 21]. Synergistic effects for both SM122/VER-155008 and 17-AAG/VER-155008 treatments were observed in multiple cell lines including HCT116, human lung adenocarcinoma epithelial cells (A549), human cervical cancer cells (HeLa) and human pancreatic cancer cells (MiaPaca-2). In addition to showing synergism, both combination treatments displayed tumour-specific effects with an acceptable therapeutic window. Analysis of chaperone-mediated protein folding was achieved using a rabbit reticulocyte lysate (RRL)-based luciferase-refolding assay. Individually, SM122 and 17-AAG have a similar impact on protein folding, where they both have an IC_{50} value of ~2 μM. However, the most effective inhibition of protein folding was observed when Hsp90 and Hsp70 were concomitantly inhibited. Combinations of 20 μM VER-155008 with increasing concentrations of either SM122 or 17-AAG showed very strong synergism. Interestingly, while 17-AAG and SM122 have very different GI_{50} values of 50 nM and 8 μM in HCT116 cells, respectively, they inhibit protein folding at a similar concentration [20, 21].

Combination treatments of SM122 or 17-AAG with VER-155008 both showed synergism in their ability to kill multiple cancer cell types and had a similar impact on protein folding. However, each combination induced apoptosis via a unique mechanism [20, 21]. HCT116 cells were treated with 50 μM VER-155008 and either SM122 or 17-AAG at two- to threefold over their GI_{50}. Apoptosis was induced in 75% of the cells treated with SM122/VER-155008, while only 50% apoptosis was induced in cells treated with 17-AAG/VER-155008. Cell death occurred via a caspase 3/7-dependent pathway with PARP-1 cleavage in both dual treatments. Interestingly, while the 17-AAG/VER-155008 treatment showed a better capacity to activate caspase 3/7, SM122/VER-155008 induced higher levels of early and late apoptosis [20, 21]. This data suggests that the primary mechanism through which 17-AAG/VER-155008 induces cell death is via caspase pathways; however, SM122/VER-155008 triggers cell death through additional pathways simultaneously, which may be beneficial in reducing the chance of tumour cells developing resistance.

The individual mechanisms by which SM122 and 17-AAG trigger cell death explains the differences in the apoptosis observed as they induce apoptosis via different cellular pathways when used in combination with VER-155008 [20–24]. Each drug combination has distinct impacts on HSR pathways. Evaluating the impact of SM122 and 17-AAG with VER-155008 on mRNA transcription, translation and protein expression levels of Hsps provided evidence of their individually unique mechanism of action [20, 21]. Activation of the HSR is characterised by an accumulation of Hsps including Hsp70, Hsp40 and Hsp27. When HCT116 cells were treated with 17-AAG and VER-155008 individually, Hsp70 mRNA levels increased by 45- and 250-fold, respectively. In contrast, SM122 produced a twofold decrease in Hsp70 mRNA [20]. These data show that

Hsp70 inhibition and N-terminal Hsp90 inhibition triggers the HSR at a transcriptional level.

When HCT116 cells were treated with 17-AAG/VER-155008 and SM122/VER-155008, Hsp70 mRNA levels increased by 3,500- and 1,500-fold, respectively [20, 21]. The SM122/VER-155008 treatment did not trigger the HSR as rapidly as 17-AAG/VER-155008, which is likely because SM122 suppresses and/or delays the transcription of Hsp70 mRNA when used in combination with VER-155008.

These phenotypic differences between SM122 and 17-AAG are also observed at the translational level, where SM122/VER-155008 synergistically inhibits protein translation, while 17-AAG/VER-155008 has no impact on translation [20]. Evaluation of heat shock protein expression levels (Hsp27, Hsp70 and Hsp40) showed that treating HCT116 cells with 17-AAG, VER-155008 or 17-AAG/VER-155008 in combination produced a large increase in Hsp70, Hsp40 and Hsp27. As discussed earlier, cells treated with SM122 decreased these protein levels [20, 21]. Dual treatment with SM122 and VER-155008 produced higher protein levels than when cells were treated with SM122 alone; however, the levels were no higher than cells treated with VER-155008 alone, showing that SM122 did not contribute to the rescue mechanism.

These results show that C-terminal modulators and N-terminal Hsp90 inhibitors have distinct mechanisms when used in combination with an Hsp70 inhibitor. Dual treatments are synergistic and induce rapid cell death in numerous cancer cell lines far more effectively than monotherapies. Thus, dual therapies have great potential as cancer treatment regimens, particularly those involving C-terminal Hsp90 modulators like SM122, which has the added benefit of reducing the HSR and limiting the ability of the cancer cell to rescue itself following treatment.

2.1.4 Combination Treatment of MAL3-101 with 17-AAG

MAL3-101 binds to an interface between Hsp40 and Hsp70, thereby impacting Hsp40-mediated stimulation of Hsp70's ATPase activity [120, 125]. Using MAL3-101 to inhibit Hsp70 alone has successfully treated preclinical MM primary tumour cells and endothelial progenitor cells (EPCs) obtained from MM patients [126]. Specifically, MAL3-101 treatment led to the inhibition of proliferation and survival in NCI-H929 cells with an IC_{50} value of 8.3 μM at 40-h exposure using an MTS assay. Cell cycle analysis showed that after 48-h treatment, MAL3-101 caused a 2.5-fold decrease at G2/M phase, with a nearly threefold increase at sub-G0/G1 phase in NCI-H929 cells, which indicated an activation of an apoptotic pathway. Confirmation by FACS analysis showed that cells treated with MAL3-101 increased apoptosis, cleavage of caspase-3 and PARP in a time-dependent manner.

In contrast to MM cells being treated with VER 155008 and 17-DMAG inhibitors, treatment of MM cells with MAL3-101 and 17-AAG led to apoptosis [126]. Specifically, in NCI-H929 MM cell line, 10 μM of MAL3-101 significantly decreased the IC_{50} of 17-AAG from 400 to 30 nM. The isobologram analysis of 10 μM MAL3-101 and 17-AAG with five different concentrations (25, 50, 100, 500

and 1,000 nM) showed tremendous synergistic effect, with combination index (CI) values from 0.008 to 0.12, where $CI < 0.1$ is "very strong synergism" and $0.1 \leq CI \geq 0.3$ is "strong synergism" (Table 2). These data support the hypothesis that VER-155008 may have off-target effects and is not only targeting Hsp70, whereas MAL3-101s may have a more selective impact on Hsp70's activity.

2.1.5 Combination Treatment of PFT-μ with 17-AAG

Pifithrin-μ (PFT-μ) has been identified as a potent Hsp70 inhibitor specifically targeting the inducible isoform of Hsp70, without binding to Hsp90 [119]. It interferes with the C-terminal substrate-binding domain of Hsp70 and disrupts its association with client proteins, causing cell cycle arrest and significant apoptosis at low micromolar concentrations in acute myeloid leukaemia (AML), acute lymphoblastic leukaemia (ALL) and primary AML blasts [119]. Importantly, normal haematopoietic cells and stromal cells exhibited a remarkably high resistance to PFT-μ compared to leukaemic blasts [115]. In bone marrow stromal cells (BMSC), the median IC_{50} value was ~4-fold higher than that in leukaemic blast cancer cells [115], which indicates that a therapeutic index can be achieved using PFT-μ.

Combination treatment with PFT-μ and the Hsp90 inhibitor 17-AAG showed synergism in reducing cell viability of all studied acute leukaemia cells including NALM-6, TOM-1 and KG-1a. Among the three cell lines, KG-1a was the least sensitive to PFT-μ and 17-AAG individual treatment, with 81% and 72% cell viability after exposure to 10 μM of PFT-μ and 5 μM of 17-AAG, respectively. However, this cell line had the most significant response to treatment by both inhibitors, showing only 29% cell viability upon treatment with these two concentrations. For the NALM-6 leukaemia cell line, their viability when treated with PFT-μ and 17-AAG monotherapies was 70% (2 μM of PFT-μ) and 70% (2 μM of 17-AAG), respectively, versus 42% when used in combination. For the TOM-1 cell line, viability was 85% (3 μM of PFT-μ) and 57% (1 μM of 17-AAG) when using monotherapies versus 36% when treating cells with both drugs.

2.1.6 Combination Treatment of AIF-Derived Peptide with 17-AAG

ADD70 is a designed peptide constructed from the amino acid residues in the AIF protein that bind to Hsp70 (amino acids 150–228) (Fig. 6). ADD70 sensitises cancer cells to apoptosis induction by capturing and neutralising the endogenous Hsp70 protein in the cytosol. ADD70 does not exert any apoptotic effects by itself [105, 127, 128]. ADD70 displayed significant anti-tumorigenic and anti-metastatic properties, as well as the ability to enhance cancer cell immunogenicity by facilitating the induction of a tumour-specific immune response, which increased the number and cytotoxic activity of $CD8^+$ tumour-infiltrating T cells [127].

The expression of ADD70 showed an additive effect when 17-AGG was used in the rat colon cancer ProB cells and mouse melanoma cancer B16F10 cells. These

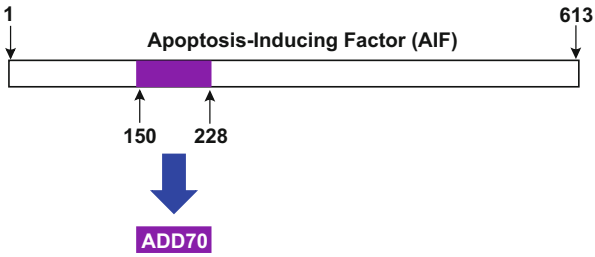

Fig. 6 *Apoptosis-inducing factor is 613 amino acids in length*. The region between amino acids 150 and 228 is essential for AIF to bind to Hsp70. ADD70 is a truncated version of AIF, where it only contains amino acids 150–228

two distinct models of tumours were developed in syngeneic rodents. The additive effect observed when using ADD70 and 17-AAG appears to be related to the reduction of inducible Hsp70 protein by ADD70, where low levels of Hsp70 protein allowed AIF-mediated caspase-independent apoptotic pathways (Fig. 6) to induce pro-apoptotic functions [105, 106, 129]. Inducing AIF-mediated apoptosis is unique to ADD70 and is not seen with any of the small molecules described above. It is thought that, since ADD70 contains the AIF sequence that binds to Hsp70, ADD70 disrupts the AIF-Hsp70-binding event inducing apoptosis via the AIF pathway. Release of AIF facilitates apoptosis and an indirect induction of the Apaf-1-mediated caspase-dependent apoptosis (Fig. 6).

Interestingly, ADD70 significantly enhanced the chemosensitizing effect of 17-AAG on cisplatin-mediated chemotherapy [127]. For example, the combination of 17-AAG and cisplatin only showed additive anticancer effects on several cancer cells. However, in the presence of ADD70, the impact of cisplatin on cell death was strongly enhanced in both cell lines, indicating that the expression of ADD70 can efficaciously potentiate the chemosensitizing effect of 17-AAG. Thus, the study of ADD70 and 17-AAG provided evidence that simultaneous targeting Hsp70 and Hsp90 can effectively provide anticancer therapy.

2.1.7 Combining Hsp70 Silencing with Hsp90 Inhibition in Human Solid Tumours

Constitutive heat shock cognate 70 (Hsc70) and inducible heat shock protein 72 (Hsp72) are two major cytoplasmic isoforms of the Hsp70 multigene family, and they have different expression patterns in mammalian cells. In non-tumour tissues, Hsc70 is abundantly and ubiquitously expressed, whereas Hsp72 is present at relatively low levels. However, under stressed conditions, Hsp72 is overexpressed, while Hsc70 is minimally impacted [89, 95, 130–132]. Selectively knocking down either Hsp72 or Hsc70 isoform using siRNA had no impact on cell proliferation in multiple cancer cells [95]. However, silencing Hsp72 significantly enhanced the antiproliferative effect of 17-AAG-mediated Hsp90 inhibition on colon cancer HCT116 cells, inducing a fivefold increase in apoptosis [95]. In contrast, when Hsc70 was silenced, there was no improved apoptosis or response to 17-AAG in any cancer cell line [95].

The differential effects of selective Hsp70 isoform silencing on the combination treatment with 17-AAG indicate that although both Hsc70 and Hsp72 can bind to Hsp90, both are induced after 17-AAG-mediated Hsp90 inhibition [92, 133–136]. Hsp72 appears to play the most important role in maintaining cell viability. These data are supported by recent evidence that 17-AAG induces 80–100-fold increases in Hsp72 mRNA levels, but only ~6-fold increase in Hsc70 [21, 23]. Thus, it appears the cell protection effects are primarily produced by an increase in Hsp72, and this isoform is heavily induced by 17-AAG. The protective effects can be silenced by knocking down Hsp72, and indeed this is the most

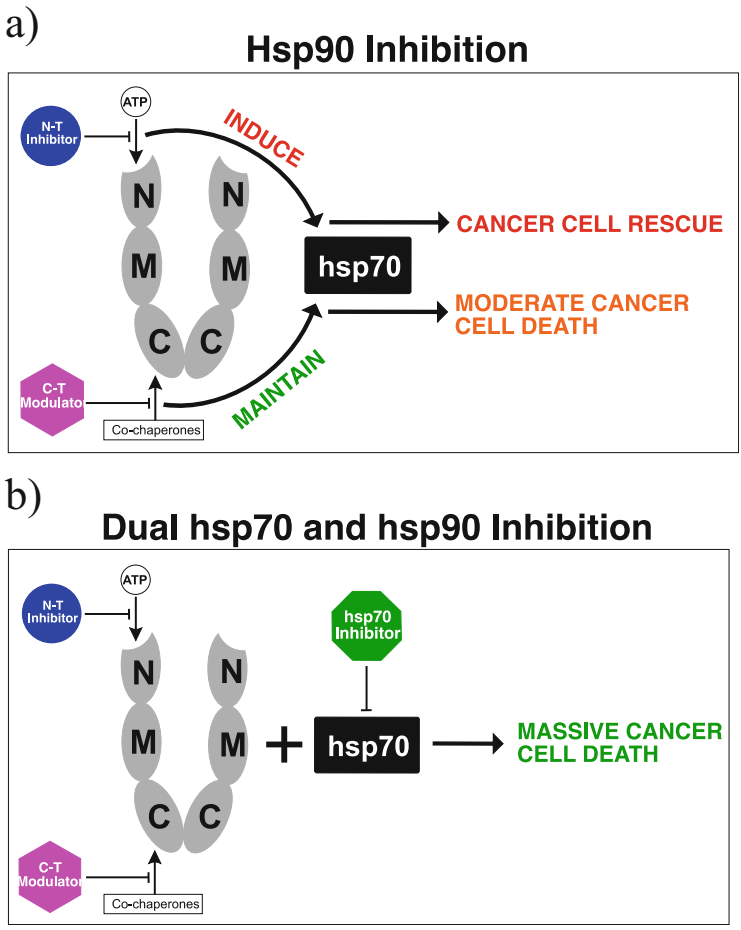

Fig. 7 (**a**) Using N-terminal inhibitors (i.e. classical inhibitors) promotes production of Hsp70 and the heat shock response, whereas using a C-terminal modulator inhibits co-chaperones from binding to Hsp90 and induces cell death. (**b**) Inhibiting both Hsp90 and Hsp70 stops the function of both proteins simultaneously, blocking the rescue response and inducing massive cancer cell death

effective route for enhancing Hsp90 inhibitors [48, 137]. Furthermore, coupling treatment of Hsp72/Hsc70 inhibition with 17-AAG also shows no effect on non-tumour cells. This observation suggests that inhibiting Hsp72 in combination with an Hsp90 inhibitor may offer a reasonable treatment with a potential therapeutic window [138].

3 Conclusions

Highlighted in this chapter are examples that indicate Hsp90 inhibition is a more effective treatment when used in combination with other chemotherapies. Successfully combining Hsp90 inhibitors with other chemotherapy drugs including molecules that target Hsps produces rapid apoptosis and cell death, which can avoid resistance and cancer metastasis. Specifically, combining Hsp90 and Hsp70 inhibitors produces large increases in apoptosis and potency of up to 92% compared to using single inhibitors. A major reason for this combination being so effective is that inhibiting Hsp90 using classical inhibitors increases the production of Hsp70 protein, which is pro-survival. We also describe how using C-terminal Hsp90 inhibitors is more effective than a classical N-terminal inhibitor when used as a dual therapy. Thus, targeting multiple points in the cell protection mechanism known as the HSR is likely to produce a highly effective new therapeutic approach (Fig. 7).

References

1. Hartl FU, Bracher A, Hayer-Hartl M (2011) Molecular chaperones in protein folding and proteostasis. Nature 475(7356):324–332
2. Schmitt E, Gehrmann M, Brunet M, Multhoff G, Garrido C (2007) Intracellular and extracellular functions of heat shock proteins: repercussions in cancer therapy. J Leukoc Biol 81 (1):15–27
3. Young JC, Agashe VR, Siegers K, Hartl FU (2004) Pathways of chaperone-mediated protein folding in the cytosol. Nat Rev Mol Cell Biol 5(10):781–791
4. Taipale M, Krykbaeva I, Koeva M, Kayatekin C, Westover KD, Karras GI, Lindquist S (2012) Quantitative analysis of HSP90-client interactions reveals principles of substrate recognition. Cell 150(5):987–1001
5. Whitesell L, Mimnaugh EG, De Costa B, Meyers CE, Neckers LM (1994) Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. Proc Natl Acad Sci U S A 91(18):8324–8328
6. Jhaveri K, Modi S (2012) HSP90 inhibitors for cancer therapy and overcoming drug resistance. Adv Pharmacol 65:471–517
7. Jhaveri K, Taldone T, Modi S, Chiosis G (2012) Advances in the clinical development of heat shock protein 90 (Hsp90) inhibitors in cancers. Biochim Biophys Acta 1823(3):742–755
8. Pacey S, Wilson RH, Walton M, Eatock MM, Hardcastle A, Zetterlund A, Arkenau HT, Moreno-Farre J, Banerji U, Roels B, Peachey H, Aherne W, de Bono JS, Raynaud F,

Workman P, Judson I (2011) A phase I study of the heat shock protein 90 inhibitor alvespimycin (17-DMAG) given intravenously to patients with advanced solid tumors. Clin Cancer Res 17(6):1561–1570
9. Modi S, Stopeck A, Linden H, Solit D, Chandarlapaty S, Rosen N, D'Andrea G, Dickler M, Moynahan ME, Sugarman S, Ma W, Patil S, Norton L, Hannah AL, Hudis C (2011) HSP90 inhibition is effective in breast cancer: a phase II trial of tanespimycin (17-AAG) plus trastuzumab in patients with HER2-positive metastatic breast cancer progressing on trastuzumab. Clin Cancer Res 17(15):5132–5139
10. Sequist LV, Gettinger S, Senzer NN, Martins RG, Janne PA, Lilenbaum R, Gray JE, Iafrate AJ, Katayama R, Hafeez N, Sweeney J, Walker JR, Fritz C, Ross RW, Grayzel D, Engelman JA, Borger DR, Paez G, Natale R (2010) Activity of IPI-504, a novel heat-shock protein 90 inhibitor, in patients with molecularly defined non-small-cell lung cancer. J Clin Oncol 28 (33):4953–4960
11. Lancet JE, Gojo I, Burton M, Quinn M, Tighe SM, Kersey K, Zhong Z, Albitar MX, Bhalla K, Hannah AL, Baer MR (2010) Phase I study of the heat shock protein 90 inhibitor alvespimycin (KOS-1022, 17-DMAG) administered intravenously twice weekly to patients with acute myeloid leukemia. Leukemia 24:699–705
12. Rajan A, Kelly RJ, Trepel JB, Kim YS, Alarcon SV, Kummar S, Gutierrez M, Crandon S, Zein WM, Jain L, Mannargudi B, Figg WD, Houk BE, Shnaidman M, Brega N, Giaccone G (2011) A phase I study of PF-04929113 (SNX-5422), an orally bioavailable heat shock protein 90 inhibitor, in patients with refractory solid tumor malignancies and lymphomas. Clin Cancer Res 17(21):6831–6839
13. Sydor JR, Normant E, Pien CS, Porter JR, Ge J, Grenier L, Pak RH, Ali JA, Dembski MS, Hudak J, Patterson J, Penders C, Pink M, Read MA, Sang J, Woodward C, Zhang Y, Grayzel DS, Wright J, Barrett JA, Palombella VJ, Adams J, Tong JK (2006) Development of 17-allylamino-17-demethoxygeldanamycin hydroquinone hydrochloride (IPI-504), an anti-cancer agent directed against Hsp90. Proc Natl Acad Sci U S A 103(46):17408–17413
14. Prodromou C, Roe SM, O'Brien R, Ladbury JE, Piper PW, Pearl LH (1997) Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. Cell 90(1):65–75
15. Chavany C, Mimnaugh E, Miller P, Bitton R, Nguyen P, Trepel J, Whitesell L, Schnur R, Moyer J, Neckers L (1996) p185erbB2 binds to GRP94 in vivo. Dissociation of the p185erbB2/GRP94 heterocomplex by benzoquinone ansamycins precedes depletion of p185erbB2. J Biol Chem 271:4974–4977
16. Johnson RD, Haber A, Rinehart KLJ (1974) Geldanamycin biosynthesis and carbon magnetic resonance. J Am Chem Soc 96:3316–3317
17. Li YH, Lu QN, Wang HQ, Tao PZ, Jiang JD (2012) Geldanamycin, a ligand of heat shock protein 90, inhibits herpes simplex virus type 2 replication both in vitro and in vivo. J Antibiot (Tokyo) 65:509–512
18. Rinehart KL, Sasaki K, Slomp G, Grostic MF, Olson EC (1970) Geldanamycin. I. Structure assignment. J Am Chem Soc 92:7591–7593
19. Schnur RC, Corman ML, Gallaschun RJ, Cooper BA, Dee MF, Doty JL, Muzzi ML, DiOrio CI, Barbacci EG, Miller PE, Pollack VA, Savage DM, Sloan DE, Pustilnik LR, Moyer JD, Moyer MP (1995) erbB-2 oncogene inhibition by geldanamycin derivatives: synthesis, mechanism of action, and structure-activity relationships. J Med Chem 38:3813–3820
20. Wang Y, McAlpine SR (2015) C-terminal heat shock protein 90 modulators produce desirable oncogenic properties. Org Biomol Chem 13:4627–4631
21. Wang Y, McAlpine SR (2015) Combining an Hsp70 inhibitor with either an N-terminal and C-terminal hsp90 inhibitor produces mechanistically distinct phenotypes. Org Biomol Chem 13:3691–3698
22. Wang Y, McAlpine SR (2015) Heat shock protein 90 inhibitors: will they ever succeed as chemotherapeutics? Future Med Chem 7(2):87–90

23. Wang Y, Mcalpine SR (2015) N-terminal and C-terminal modulation of Hsp90 produce dissimilar phenotypes. Chem Commun 51:1410–1413
24. Wang Y, McAlpine SR (2015) Regulating the cytoprotective response in cancer cells using simultaneous inhibition of Hsp90 and Hsp70. Org Biomol Chem 13:2108–2116
25. Yamaki H, Suzuki H, Choi EC, Tanaka N (1982) Inhibition of DNA synthesis in murine tumor cells by geldanamycin, an antibiotic of the benzoquinoid ansamycin group. J Antibiot (Tokyo) 35:886–892
26. Morimoto RI (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev 12:3788–3796
27. Anckar J, Sistonen L (2011) Regulation of HSF1 in the heat stress response: implications in aging and disease. Annu Rev Biochem 80:1089–1115
28. Chiosis G, JHuezo H, Rosen N, Mimgaugh E, Whitesell L, Neckers L (2003) Binding affinity and potent cell activity-finding an explanation. Mol Cancer Ther 2:123–129
29. Mahalingam D, Swords R, Carew JS, Nawrocki ST, Bhalla K, Giles FJ (2009) Targeting HSP90 for cancer therapy. Br J Cancer 100:1523–1529
30. Ali A, Bharadwaj S, O'Carroll R, Ovsenek N (1998) Hsp90 interacts with and regulates the activity of heat shock factor 1 in *Xenopus* oocytes. Mol Cell Biol 18:4949–4960
31. Bharadwaj S, Ali A, Ovsenek N (1999) Multiple components of the HSP90 chaperone complex function in regulation of heat shock factor 1 in vivo. Mol Cell Biol 19:8033–8041
32. Guo Y, Guettouche T, Fenna M, Boellmann F, Pratt WB (2001) Evidence for a mechanism of repression of heat shock factor 1 transcriptional activity by a multichaperone complex. J Biol Chem 276:45791–45799
33. Morimoto RI (2002) Dynamic remodelling of transcription complexes by molecular chaperones. Cell 110:281–284
34. Zou J, Guo Y, Guettouche T, Smith DF, Voellmy R (1998) Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP complex) that forms a stress-sensitive complex with HSF1. Cell 94:471–480
35. Vujanac M, Fenaroli A, Zimarino V (2005) Constitutive nuclear import and stress-regulated nucleocytoplasmic shuttling of mammalian heat-shock factor 1. Traffic 6:214–229
36. Kline MP, Morimoto RI (1997) Repression of the heat shock factor 1 transcriptional activation domain is modulated by constitutive phosphorylation. Mol Cell Biol 17:2107–2115
37. Sandqvist A, Bjork JK, Akerfelt M, Chitikova Z, Grichine A (2009) Heterotrimerization of heat shock factors 1 and 2 provides a transcriptional switch in response to distinct stimuli. Mol Biol Cell 20:1340–1347
38. Xiao H, Perisic O, Lis JT (1991) Cooperative binding of *Drosophila* heat shock factor to arrays of conserved 5 bp unit. Cell 64:585–593
39. Core LJ, Lis JT (2008) Transcription regulation through promoter-proximal pausing of RNA polymerase II. Science 319:1791–1792
40. Rougvie AE, Lis JT (1988) The RNA polymerase II molecule at the 5′ end of the uninduced *hsp70* gene of *D. melanogaster* is transcriptionally engaged. Cell 54:795–804
41. Morimoto RI, Tissieres A, Georgopoulos C (1990) The stress response, function of the proteins and perspectives. In: Morimoto RI, Tissieres A, Georgopoulos C (eds) Stress protein in biology and medicinal. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 1–36
42. Day JEH, Sharp SY, Rowlands MG, Aherne W, Hayes A, Raynaud FI, Lewis W, Roe SM, Prodromou C, Pearl LH, Workman P, Moody CJ (2011) Targeting the Hsp90 molecular chaperone with novel macrolactams. Synthesis, structural, binding, and cellular studies. ACS Chem Biol 6(12):1339–1347
43. Powers MV, Valenti M, Miranda S, Maloney A, Eccles SA, Thomas G, Clarke PA, Workman P (2013) Mode of cell death induced by the HSP90 inhibitor 17-AAG (tanespimycin) is dependent on the expression of pro-apoptotic bax. Oncotarget 4(11):1963–1975
44. Workman P, Al-Lazikani B (2013) Drugging cancer genomes. Nat Rev Drug Discov 12 (12):889–890

45. Powers MV, Jones K, Barillari C, Westwood I, van Montfort RL, Workman P (2010) Targeting HSP70: the second potentially druggable heat shock protein and molecular chaperone? Cell Cycle 9:1542–1550
46. Beere HM (2004) "The stress of dying": the role of heat shock proteins in the regulation of apoptosis. J Cell Sci 117:2641–2651
47. Beere HM, Wolf BB, Cain K, Mosser DD, Mahboubi A, Kuwana T, Tailor P, Morimoto RI, Cohen GM, Green DR (2000) Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. Nat Cell Biol 2:469–475
48. Guo F, Sigua C, Bali P, George P, Fiskus W, Scuto A, Annavarapu S, Mouttaki A, Sondarva G, Wei S, Wu J, Djeu J, Bhalla K (2005) Mechanistic role of heat shock protein 70 in Bcr-Abl-mediated resistance to apoptosis in human acute leukemia cells. Blood 105:1246–1255
49. Nylandsted J, Gyrd-Hansen M, Danielewicz A, Fehrenbacher N, Lademann U, Høyer-Hansen M, Weber E, Multhoff G, Rohde M, Jäättelä M (2004) Heat shock protein 70 promotes cell survival by inhibiting lysosomal membrane permeabilization. J Exp Med 200:425–435
50. Ardi VC, Alexander LD, Johnson VA, McAlpine SR (2011) Macrocycles that inhibit the binding between heat shock protein 90 and TPR-containing proteins. ACS Chem Biol 6:1357–1367
51. Eskew JD, Sadikot T, Morales P, Duren A, Dunwiddie I, Swink M, Zhang X, Hembruff S, Donnelly A, Rajewski RA, Blagg B, Manjarrez JR, Matts RL, Holzbeierlein JM, Vielhauer GA (2011) Development and characterization of a novel C-terminal inhibitor of Hsp90 in androgen dependent and independent prostate cancer cells. BMC Cancer 11:468
52. Koay YC, McConnell JR, Wang Y, Kim SJ, McAlpine SR (2014) Chemically accessible Hsp90 inhibitor that does not induce a heat shock response. ACS Med Chem Lett 5:771–776
53. Kunicki JB, Petersen MN, Alexander LD, Ardi VC, McConnell JR, McAlpine SR (2011) Synthesis and evaluation of biotinylated sansalvamide A analogs and their modulation of Hsp90. Bioorg Med Chem Lett 21:4716–4719
54. McConnell JM, Alexander LD, McAlpine SR (2014) A heat shock protein inhibitor that modulates immunophilins and regulates hormone receptors. Bioorg Med Chem Lett 24:661–666
55. Shelton SNS, Matthews ME, Lu SB, Donnelly Y, Szabla AC, Tanol K, Vielhauer M, Rajewski GA, Matts RA, Blagg RL, Robertson BS (2009) KU135, a novel novobiocin-derived C-terminal inhibitor of the 90-kDa heat shock protein, exerts potent antiproliferative effects in human leukemic cells. Mol Pharmacol 76:1314–1322
56. Hartl FU, Hayer-Hartl M (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 295:1852–1858
57. Horibe T, Kohno M, Haramoto M, Ohara K, Kawakami K (2011) Designed hybrid TPR peptide targeting Hsp90 as a novel anticancer agent. J Transl Med 9:8
58. Neckers L, Mimnaugh E, Schulte TW (1999) Hsp90 as an anti-cancer target. Drug Resist Updat 2:165–172
59. Scott MD, Frydman J (2003) Aberrant protein folding as the molecular basis of cancer. Methods Mol Biol 232:67–76
60. Workman P, Burrows F, Neckers L, Rosend N (2007) Drugging the cancer chaperone Hsp90: combinational therapeutic exploitation of oncogene addiction and tumor stress. Ann N Y Acad Sci 1113:202–216
61. Yi F, Regan L (2008) A novel class of small molecule inhibitors of Hsp90. ACS Chem Biol 3:645–654
62. Falsone SF, Gesslbauer B, Tirk F, Piccinini AM, Kungl AJ (2005) A proteomic snapshot of the human heat shock protein 90 interactome. FEBS Lett 579:6350–6354
63. Horibe T, Torisawa A, Kohno M, Kawakami K (2012) Molecular mechanism of cytotoxicity induced by Hsp90-targeted Antp-TPR hybrid peptide in glioblastoma cells. Mol Cancer 11:59

64. Kamal A, Boehm MF, Burrows FJ (2004) Therapeutic and diagnostic implications of Hsp90 activation. Trends Mol Med 10:283–290
65. McClellan AJ, Xia Y, Deutschbauer AM, Davis RW, Gerstein M, Frydman J (2007) Diverse cellular functions of the Hsp90 molecular chaperone uncovered using systems approach. Cell 131:121–135
66. Welch WJ (1991) The role of heat-shock proteins as molecular chaperones. Curr Opin Cell Biol 3:1033–1038
67. Westerheide SD, Morimoto RI (2005) Heat shock response modulators as therapeutic tools for diseases of protein conformation. J Biol Chem 280:33097–33100
68. Pearl LH, Prodromou C (2000) Structure and in vivo function of Hsp90. Curr Opin Struct Biol 10:46–51
69. Young JC, Moarefi I, Hartl FU (2001) Hsp90: a specialized but essential protein-folding tool. J Cell Biol 154:267–273
70. Horibe T, Kawamoto M, Kohno M, Kawakami K (2012) Cytotoxic activity to acute myeloid leukemia cells by Antp-TPR hybrid peptide targeting Hsp90. J Biosci Bioeng 114:96–103
71. Isaacs JS, Xu W, Neckers L (2003) Heat shock protein 90 as a molecular target for cancer therapeutics. Cancer Cell 3:213–217
72. Biamonte MA, Van de Water R, Arndt JW, Scannevin RH, Perret D, Lee WC (2010) Heat shock protein 90: inhibitors in clinical trials. J Med Chem 53:3–17
73. Janin YL (2010) ATPase inhibitors of heat-shock protein 90, second season. Drug Discov Today 15:342–353
74. Powers MV, Clarke PA, Workman P (2009) Death by chaperone: HSP90, HSP70 or both? Cell Cycle 8:518–526
75. Butcher EC (2005) Can cell systems biology rescue drug discovery? Nat Rev Drug Discov 4:461–467
76. Drysdale MJ, Brough PA, Massey A, Jensen MR, Schoepfer J (2006) Targeting Hsp90 for the treatment of cancer. Curr Opin Drug Discov Devel 9:483–495
77. Alexander LD, Partridge JR, Agard DA, McAlpine SR (2011) A small molecule that preferentially binds the closed Hsp90 conformation. Bioorg Med Chem Lett 21:7068–7071
78. Kusuma BR, Peterson LB, Zhao H, Vielhauer G, Holzberlein J, Blagg BS (2011) Targeting the heat shock protein 90 dimer with dimeric inhibitors. J Med Chem 54:6234–6253
79. Vasko RC, Rodriguez RA, Cunningham CN, Ardi VC, Agard DA, McAlpine SR (2010) Mechanistic studies of Sansalvamide A-amide: an allosteric modulator of Hsp90. ACS Med Chem Lett 1:4–8
80. Yu XM, Shen G, Cronk B, Marcu M, Holzberlein J, Neckers LM, Blagg BSJ (2005) Hsp90 inhibitors identified from a library of novobiocin analogues. J Am Chem Soc 127:12778–12779
81. Gandhi N, Wild AT, Chettiar ST, Aziz K, Kato Y, Gajula RP, Williams RD, Cades JA, Annadanam A, Song D, Zhang Y, Hales RK, Herman JM, Armour E, DeWeese TL, Schaeffer EM, Tran PT (2013) Novel Hsp90 inhibitor NVP-AUY922 radiosensitizes prostate cancer cells. Cancer Biol Ther 14:347–356
82. Goldman JW, Raju RN, Gordon GA, El-Hariry I, Teofilivici F, Vukovic VM, Bradley R, Karol MD, Chen Y, Guo W, Inoue T, Rosen LS (2013) A first in human, safety, pharmacokinetics, and clinical activity phase I study of once weekly administration of the Hsp90 inhibitor ganetespib (STA-9090) in patients with solid malignancies. BMC Cancer 13:152–161
83. Graham B, Curry J, Smyth T, Fazal L, Feltell R, Harada I, Coyle J, Williams B, Reule M, Angove H, Cross DM, Lyons J, Wallis NG, Thompson NT (2012) The heat shock protein 90 inhibitor, AT13387, displays a long duration of action in vitro and in vivo in non-small cell lung cancer. Cancer Sci 103:522–527
84. Modi S, Saura C, Henderson C, Lin NU, Mahtani R, Goddard J, Rodenas E, Hudis C, O'Shaughnessy J, Baselga J (2013) A multicenter trial evaluating retaspimycin HCL

(IPI-504) plus trastuzumab in patients with advanced or metastatic HER2-positive breast cancer. Breast Cancer Res Treat 139:107–113
85. Song D, Chaerkady R, Tan AC, García-García E, Nalli A, Suárez-Gauthier A, López-Ríos F, Zhang XF, Solomon A, Tong J, Read M, Fritz C, Jimeno A, Pandey A, Hidalgo M (2008) Antitumor activity and molecular effects of the novel heat shock protein 90 inhibitor, IPI-504, in pancreatic cancer. Mol Cancer Ther 7:3275–3284
86. Zhang H, Chung D, Yang YC, Neely L, Tsurumoto S, Fan J, Zhang L, Biamonte M, Brekken J, Lundgren K, Burrows F (2006) Identification of new biomarkers for clinical trials of Hsp90 inhibitors. Mol Cancer Ther 5:1256–1264
87. Caldas-Lopes E, Cerchietti L, Ahn JH, Clement CC, Robles AI, Rodina A, Moulick K, Taldone T, Gozman A, Guo Y, Wu N, de Stanchina E, White J, Gross SS, Ma Y, Varticovski L, Melnick A, Chiosis G (2009) Hsp90 inhibitor PU-H71, a multimodal inhibitor of malignancy, induces complete responses in triple-negative breast cancer models. Proc Natl Acad Sci U S A 106:8368–8373
88. Calderwood SK, Khaleque MA, Sawyer DB, Ciocca DR (2006) Heat shock proteins in cancer: chaperones of tumorigenesis. Trends Biochem Sci 31:164–172
89. Chatterjee M, Andrulis M, Stühmer T, Müller E, Hofmann C, Steinbrunn T, Heimberger T, Schraud H, Kressmann S, Einsele H, Bargou RC (2013) The PI3K/Akt signaling pathway regulates the expression of Hsp70, which critically contributes to Hsp90-chaperone function and tumor cell survival in multiple myeloma. Haematologica 98:1132–1141
90. Davenport EL, Zeisig A, Aronson LI, Moore HE, Hockley S, Gonzalez D, Smith EM, Powers MV, Sharp SY, Workman P, Morgan GJ, Davies FE (2010) Targeting heat shock protein 72 enhances Hsp90 inhibitor-induced apoptosis in myeloma. Leukemia 24(10):1804–1807
91. Gaspar N, Sharp SY, Eccles SA, Gowan S, Popov S, Jones C, Pearson A, Vassal G, Workman P (2010) Mechanistic evaluation of the novel HSP90 inhibitor NVP-AUY922 in adult and pediatric glioblastoma. Mol Cancer Ther 9:1219–1233
92. Maloney A, Clarke PA, Naaby-Hansen S, Stein R, Koopman J-O, Akpan A, Yang A, Zvelebil M, Cramer R, Stimson L, Aherne W, Banerji U, Judson I, Sharp S, Powers M, deBilly E, Salmons J, Walton M, Burlingame A, Waterfield M, Workman P (2007) Gene and protein expression profiling of human ovarian cancer cells treated with the heat shock protein 90 inhibitor 17-allylamino-17-demethoxygeldanamycin. Cancer Res 67:3239–3253
93. McCollum AK, TenEyck CJ, Sauer BM, Toft DO, Erlichman C (2006) Up-regulation of heat shock protein 27 induces resistance to 17-allylamino-demethoxygeldanamycin through a glutathione-mediated mechanism. Cancer Res 66:10967–10975
94. Mosser DD, Morimoto RI (2004) Molecular chaperones and the stress of oncogenesis. Oncogene 23:2907–2918
95. Powers MV, Clarke PA, Workman P (2008) Dual targeting of Hsc70 and Hsp72 inhibits Hsp90 function and induces tumor-specific apoptosis. Cancer Cell 14:250–262
96. Stühmer T, Chatterjee M, Grella E, Seggewiss R, Langer C, Müller S, Schoepfer J, Garcia-Echeverria C, Quadt C, Jensen MR, Einsele H, Bargou RC (2009) Anti-myeloma activity of the novel 2-aminothienopyrimidine Hsp90 inhibitor NVP-BEP800. Br J Haematol 47:319–327
97. Stühmer T, Zöllinger A, Siegmund D, Chatterjee M, Grella E, Knop S, Kortüm M, Unzicker C, Jensen MR, Quadt C, Chène P, Schoepfer J, García-Echeverría C, Einsele H, Wajant H, Bargou RC (2008) Signalling profile and antitumour activity of the novel Hsp90 inhibitor NVP-AUY922 in multiple myeloma. Leukemia 22:1604–1612
98. Wahyudi H, Wang Y, McAlpine SR (2014) Utilizing a Dimerization strategy to inhibit the dimer protein Hsp90: synthesis and biological activity of a sansalvamide A dimer. Org Biomol Chem 12:765–773
99. Goloudina AR, Demidov ON, Garrido C (2012) Inhibition of HSP70: a challenging anti-cancer strategy. Cancer Lett 325:117–124
100. Whitesell L, Santagata S, Lin NU (2012) Inhibiting HSP90 to treat cancer: a strategy in evolution. Curr Mol Med 12:1108–1124

101. Creagh EM, Sheehan D, Cotter TG (2000) Heat shock proteins--modulators of apoptosis in tumour cells. Leukemia 14:1161–1173
102. Jäättelä M, Wissing D, Kokholm K, Kallunki T, Egeblad M (1998) Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases. EMBO J 17:6124–6134
103. Saleh A, Srinivasula SM, Balkir L, Robbins PD, Alnemri ES (2000) Negative regulation of the Apaf-1 apoptosome by Hsp70. Nat Cell Biol 2:476–483
104. Takayama S, Reed JC, Homma S (2003) Heat-shock proteins as regulators of apoptosis. Oncogene 22:9041–9047
105. Gurbuxani S, Schmitt E, Cande C, Parcellier A, Hammann A, Daugas E, Kouranti I, Spahr C, Pance A, Kroemer G, Garrido C (2003) Heat shock protein 70 binding inhibits the nuclear import of apoptosis-inducing factor. Oncogene 22:6669–6678
106. Ravagnan L, Gurbuxani S, Susin SA, Maisse C, Daugas E, Zamzami N, Mak T, Jäättelä M, Penninger JM, Garrido C, Kroemer G (2001) Heat-shock protein 70 antagonizes apoptosis-inducing factor. Nat Cell Biol 3:839–843
107. Nylandsted J, Rohde M, Brand K, Bastholm L, Elling F, Jäättelä M (2000) Selective depletion of heat shock protein 70 (Hsp70) activates a tumor-specific death program that is independent of caspases and bypasses Bcl-2. Proc Natl Acad Sci U S A 97:7871–7876
108. Nylandsted J, Wick W, Hirt UA, Brand K, Rohde M, Leist M, Weller M, Jäättelä M (2002) Eradication of glioblastoma, and breast and colon carcinoma xenografts by Hsp70 depletion. Cancer Res 62:7139–7142
109. Ishii T, Udono H, Yamano T, Ohta H, Uenaka A, Ono T, Hizuta A, Tanaka N, Srivastava PK, Nakayama E (1999) Isolation of MHC class I-restricted tumor antigen peptide and its precursors associated with heat shock proteins hsp70, hsp90, and gp96. J Immunol 162:1303–1309
110. Multhoff G (2002) Activation of natural killer cells by heat shock protein 70. Int J Hyperthermia 18:576–585
111. Rérole AL, Gobbo J, De Thonel A, Schmitt E, Pais de Barros JP, Hammann A, Lanneau D, Fourmaux E, Deminov O, Micheau O, Lagrost L, Colas P, Kroemer G, Garrido C (2011) Peptides and aptamers targeting HSP70: a novel approach for anticancer chemotherapy. Cancer Res 71(2):484–495
112. Srivastava PK (2008) New jobs for ancient chaperones. Sci Am 299:50–55
113. Stangl S, Gehrmann M, Riegger J, Kuhs K, Riederer I, Sievert W, Hube K, Mocikat R, Dressel R, Kremmer E, Pockley AG, Friedrich L, Vigh L, Skerra A, Multhoff G (2011) Targeting membrane heat-shock protein 70 (Hsp70) on tumors by cmHsp70.1 antibody. Proc Natl Acad Sci U S A 108(2):733–738
114. Evans CG, Chang L, Gestwicki JE (2010) Heat shock protein 70 (hsp70) as an emerging drug target. J Med Chem 53:4585–4602
115. Kaiser M, Kühnl A, Reins J, Fischer S, Ortiz-Tanchez J, Schlee C, Mochmann LH, Heesch S, Benlasfer O, Hofmann WK, Thiel E, Baldus CD (2011) Antileukemic activity of the HSP70 inhibitor pifithrin-μ in acute leukemia. Blood Cancer J 1(7):e28. doi:10.1038/bcj.2011.28
116. Massey AJ, Williamson DS, Browne H, Murray JB, Dokurno P, Shaw T, Macias AT, Daniels Z, Geoffroy S, Dopson M, Lavan P, Matassova N, Francis GL, Graham CJ, Parsons R, Wang Y, Padfield A, Comer M, Drysdale MJ, Wood M (2010) A novel, small molecule inhibitor of Hsc70/Hsp70 potentiates Hsp90 inhibitor induced apoptosis in HCT116 colon carcinoma cells. Cancer Chemother Pharmacol 66(3):535–545
117. Reikvam H, Nepstad I, Sulen A, Gjertsen BT, Hatfield KJ, Bruserud Ø (2013) Increased antileukemic effects in human acute myeloid leukemia by combining HSP70 and HSP90 inhibitors. Expert Opin Investig Drugs 22:551–563
118. Williamson DS, Borgognoni J, Clay A, Daniels Z, Dokurno P, Drysdale MJ, Foloppe N, Francis GL, Graham CJ, Howes R, Macias AT, Murray JB, Parsons R, Shaw T, Surgenor AE, Terry L, Wang Y, Wood M, Massey AJ (2009) Novel adenosine-derived inhibitors of 70 kDa heat shock protein, discovered through structure-based design. J Med Chem 52:1510–1513

119. Leu JI, Pimkina J, Frank A, Murphy ME, George DL (2009) A small molecule inhibitor of inducible heat shock protein 70. Mol Cell 36(1):15–27
120. Fewell SW, Smith CM, Lyon MA, Dumitrescu TP, Wipf P, Day BW, Brodsky JL (2004) Small molecule modulators of endogenous and co-chaperone-stimulated Hsp70 ATPase activity. J Biol Chem 279(49):51131–51140
121. Rodina A, Vilenchik M, Moulick K, Aguirre J, Kim J, Chiang A, Litz J, Clement CC, Kang Y, She Y, Wu N, Felts S, Wipf P, Massague J, Jiang X, Brodsky JL, Krystal GW, Chiosis G (2007) Selective compounds define Hsp90 as a major inhibitor of apoptosis in small-cell lung cancer. Nat Chem Biol 3:498–507
122. Estey EH (2012) Acute myeloid leukemia: 2012 update on diagnosis, risk stratification, and management. Am J Hematol 87:89–99
123. Kaufmann SH, Karp JE, Litzow MR, Mesa RA, Hogan W, Steensma DP, Flatten KS, Loegering DA, Schneider PA, Peterson KL, Maurer MJ, Smith BD, Greer J, Chen Y, Reid JM, Ivy SP, Ames MM, Adjei AA, Erlichman C, Karnitz LM (2011) Phase I and pharmacological study of cytarabine and tanespimycin in relapsed and refractory acute leukemia. Haematologica 96:1619–1626
124. Reikvam H, Ersvaer E, Bruserud O (2009) Heat shock protein 90 - a potential target in the treatment of human acute myelogenous leukemia. Curr Cancer Drug Targets 9:761–776
125. Wisen S, Bertelsen EB, Thompson AD, Patury S, Ung P, Chang L, Evans CG, Walter GM, Wipf P, Carlson HA, Brodsky JL, Zuiderweg ER, Gestwicki JE (2010) Binding of a small molecule at a protein-protein interface regulates the chaperone activity of hsp70-hsp40. ACS Chem Biol 5(6):611–622
126. Braunstein MJ, Scott SS, Scott CM, Behrman S, Walter P, Wipf P, Coplan JD, Chrico W, Joseph D, Brodsky JL, Batuman O (2011) Antimyeloma effects of the heat shock protein 70 molecular chaperone inhibitor MAL3-101. J Oncol 2011:232037
127. Schmitt E, Maingret L, Puig PE, Rerole AL, Ghiringhelli F, Hammann A, Solary E, Kroemer G, Garrido C (2006) Heat shock protein 70 neutralization exerts potent antitumor effects in animal models of colon cancer and melanoma. Cancer Res 66:4191–4197
128. Schmitt E, Parcellier A, Gurbuxani S, Cande C, Hammann A, Morales MC, Hunt CR, Dix DJ, Kroemer RT, Giordanetto F, Jäättelä M, Penninger JM, Pance A, Kroemer G, Garrido C (2003) Chemosensitization by a non-apoptogenic heat shock protein 70-binding apoptosis-inducing factor mutant. Cancer Res 63(23):8233–8240
129. Matsumori Y, Hong SM, Aoyama K, Fan Y, Kayama T, Sheldon RA, Vexler ZS, Ferriero DM, Weinstein PR, Liu J (2005) Hsp70 overexpression sequesters AIF and reduces neonatal hypoxic/ischemic brain injury. J Cereb Blood Flow Metab 25:899–910
130. Daugaard M, Rohde M, Jäättelä M (2007) The heat shock protein 70 family: highly homologous proteins with overlapping and distinct functions. FEBS Lett 581:3702–3710
131. Garrido C, Brunet M, Didelot C, Zermati Y, Schmitt E, Kroemer G (2006) Heat shock proteins 27 and 70: anti-apoptotic proteins with tumorigenic properties. Cell Cycle 5:2592–2601
132. Sherman M, Multhoff G (2007) Heat shock proteins in cancer. Ann N Y Acad Sci 1113:192–201
133. Banerji U, O'Donnell A, Scurr M, Pacey S, Stapleton S, Asad Y, Simmons L, Maloney A, Raynaud F, Campbell M, Walton M, Lakhani S, Kaye S, Workman P, Judson I (2005) Phase I pharmacokinetic and pharmacodynamic study of 17-allylamino, 17-demethoxygeldanamycin in patients with advanced malignancies. J Clin Oncol 23:4152–4161
134. Banerji U, Walton M, Raynaud F, Grimshaw R, Kelland L, Valenti M, Judson I, Workman P (2005) Pharmacokinetic-pharmacodynamic relationships for the heat shock protein 90 molecular chaperone inhibitor 17-allylamino, 17-demethoxygeldanamycin in human ovarian cancer xenograft models. Clin Cancer Res 11:7023–7032
135. Goetz MP, Toft D, Reid J, Ames M, Stensgard B, Safgren S, Adjei AA, Sloan J, Atherton P, Vasile V, Salazaar S, Adjei A, Croghan G, Erlichman C (2005) Phase I trial of 17-allylamino-17-demethoxygeldanamycin in patients with advanced cancer. J Clin Oncol 23:1078–1087

136. Hostein I, Robertson D, DiStefano F, Workman P, Clarke PA (2001) Inhibition of signal transduction by the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin results in cytostasis and apoptosis. Cancer Res 61:4003–4009
137. Gabai VL, Budagova KR, Sherman MY (2005) Increased expression of the major heat shock protein Hsp72 in human prostate carcinoma cells is dispensable for their viability but confers resistance to a variety of anticancer agents. Oncogene 24:3328–3338
138. Yoon YJ, Kim JA, Shin KD, Shin DS, Han YM, Lee YJ, Lee JS, Kwon BM, Han DC (2011) KRIBB11 inhibits HSP70 synthesis through inhibition of heat shock factor 1 function by impairing the recruitment of positive transcription elongation factor b to the hsp70 promoter. J Biol Chem 286(3):1737–1747

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The Effect of Structure and Mechanism of the Hsp70 Chaperone on the Ability to Identify Chemical Modulators and Therapeutics

Alexandra Manos-Turvey, Jeffrey L. Brodsky, and Peter Wipf

Abstract The role of the Hsp70 molecular chaperone in effecting proper cellular protein folding, transport, and degradation processes, stabilizing protein complexes, and maintaining membrane integrity has long been recognized. More recently, Hsp70 has been linked to severe neurological diseases, such as Alzheimer's, Parkinson's and Huntington's disease, as well as to cystic fibrosis and cancer. As a result, there is a growing interest in the development of small-molecule modulators of Hsp70 function. While several distinct classes of Hsp70 agonists and antagonists have been identified to date, clinical studies with Hsp70-targeted drugs have yet to be initiated, and proof of principle for therapeutic benefits remains to be established. However, a large body of preclinical biological evidence suggests that this chaperone plays a key role in many human diseases associated with protein (un)folding and trafficking and that the continued development of Hsp70 modulators will yield novel therapeutic strategies.

Keywords 15-Deoxyspergualin, ADD70, Apoptozole, Hsc70, Hsp70, MAL3-101, VER-155008

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Contents

1 Introduction

Ten years after the first comprehensive report on the Human Genome Project, the number of protein encoding genes identified within the human genome was estimated to be 24,000 [1, 2]. However, this value underestimates the biodiversity encoded in the genome; a single gene can encode more than one protein, either due to posttranscriptional processes such as mRNA splicing or posttranslational modifications such as proteolytic processing, glycosylation, lipid modification, or acylation [3]. Although the number of functional proteins constitutively expressed within any given cell remains unknown, the Chromosome-Centric Human Proteome Project anticipates the identification of 17,000 distinct cellular proteins [4]. Other estimates suggest 50,000 commonly expressed proteins with a potential to generate ~2 million proteins in humans.

Normal cellular function relies on the successful expression folding and correct localization of proteins within a cell. Cellular stress can interrupt these biosynthetic pathways and can be highly deleterious, resulting in diseases, such as Alzheimer's, Parkinson's, and Huntington's disease, as well as cystic fibrosis and cancer [5, 6]. Cellular stress factors also include heat shock and nutrient deprivation, which result in irregular protein production and/or lack of protein stability [5]. Even "normal" cellular conditions, such as growth or differentiation, can trigger a stress response [7].

Due to the large number of cellular proteins that each have unique folding pathways and preferred conformations, it is not surprising that a sophisticated monitoring system exists to intercept folding and processing errors. In order to

minimize the impact of misfolded protein accumulation, newly synthesized proteins are monitored by a group of sentinel proteins known as molecular chaperones [8, 9].

1.1 Molecular Chaperones: Heat Shock Proteins as Anticancer Targets

Molecular chaperones have long been known to play critical roles in cellular processes, including protein folding, degradation, and transport [10–12]. As such, defects in molecular chaperone function have been identified as an underlying cause of pathogenesis in many human diseases [13]. The heat shock proteins (HSPs) are a family of molecular chaperones induced by cellular heat shock, oxidative stress, or in the presence of metal ions [5]. Research into HSP function led to a deeper understanding of molecular chaperone activity and regulation [8, 14]. The HSPs are now well recognized for their ability to prevent protein misfolding and protein aggregation. HSPs are cytoprotective and are able to counteract the induction of apoptosis and autophagy [15–17]. Therefore, it is not surprising that HSPs are commonly overexpressed in malignancies and their inhibition can increase the sensitivity of cancer cell lines to antitumor agents [18–21]. Due to the particularly stressful environment in cells with dysregulated signaling and growth pathways, HSPs are of significant interest in cancer research.

HSPs are grouped according to their molecular weights, and the most abundant members are Hsp110/Hsp105, Hsp104, Hsp90, Hsp70, Hsp60, Hsp40, the small heat shock proteins (<30,000), and Hsp10. HSPs are often recruited as multiprotein complexes with other cofactors and co-chaperones, and their expression levels are interdependent [22]. Among the HSPs, Hsp90 has garnered the most significant attention in the field of anticancer research, both by pharmaceutical companies and in academia, because of its established role in the maturation of oncogenic protein kinases and growth factor receptors, and based on its distinct ATP-binding pocket [23–26]. In contrast, Hsp70 has remained a secondary drug target to date, even though it also plays an important role in cancer cell proliferation and often compensates for decreased Hsp90 levels [20].

1.2 Hsp90 Inhibitor Design and Evaluation

Hsp90 function is critical to maintain cellular signaling pathways and is essential for cell survival even under nonstress conditions [27, 28]. The protein is made up of three structural domains: an ATP-binding N-terminal domain (NBD), a middle domain where client protein-binding occurs, and a C-terminal dimerization domain [29]. The ATPase function of the NBD was identified after a naturally occurring

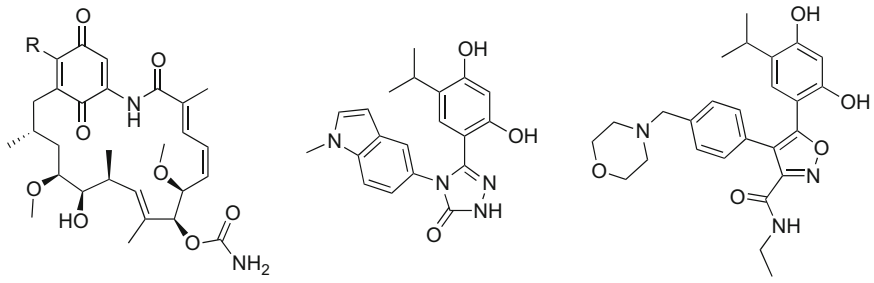


Fig. 1 Examples of small-molecule inhibitors of Hsp90

antitumor antibiotic, geldanamycin (GA, Fig. 1) [30], a member of the ansamycin family, was found to be a highly selective competitive inhibitor of Hsp90 through its association with the ATP-binding pocket [24, 31, 32]. The active form of Hsp90 is a homodimer. The two monomeric halves of Hsp90 make contact through the C-terminal domain, forming a cleft wherein a client protein can associate with the parallel-oriented middle domains [29]. When ATP is bound, the NBDs converge and trap client proteins so that they can undergo conformational alterations before their release [29].

Many cancer cell lines show heightened Hsp90 dependence and expression, thus encouraging the search for anticancer agents targeting this chaperone [24, 25]. In fact, numerous small molecular probes that inhibit Hsp90 have been developed, and several of these have proceeded to clinical trials [33, 34]. One of the earliest Hsp90 inhibitors was a synthetic GA derivative, 17-AAG (Fig. 1), which reached Phase III clinical trials. The results of these trials strongly supported the concept that HSP inhibitors can selectively impact the survival of cancer cells while leaving healthy cells relatively unscathed [35–37].

Off-target effects and associated toxicities of the ansamycin-type inhibitors have hampered their use in the clinic, but they have inspired the search for novel synthetic scaffolds for potential use as Hsp90 inhibitors. Two compounds of particular note are STA-9090 and AUY922, which are currently in Phase I–III trials (Fig. 1) [33, 34]. STA-9090 is a resorcinol-containing triazolone that binds to the ATPase domain of Hsp90 and has been shown to arrest cell proliferation and trigger apoptosis in a number of cancer lines, including acute myeloid leukemia and erlotinib-resistant lung adenocarcinomas [38]. AUY922 was discovered from a screen of a library of 4,5-diarylisoxazoles and has a high affinity for the NBD in Hsp90 [39, 40]. AUY922 shows activity against many different cancer lines [33] and was recently demonstrated to inhibit the growth of non-small cell lung carcinoma, a tumor that is often accompanied by high Hsp90 levels and poor patient prognosis [41, 42].

During the testing of the GA-based inhibitors, Hsp70 induction was observed as a consequence of Hsp90 inhibition. For example, although it is a potent inhibitor of Hsp90, 17-DMAG, a 17-AAG derivative with improved pharmacokinetic properties, resulted in a significant increase in Hsp70 expression [43]. This allowed cancer cells to compensate for Hsp90 inactivation, thus preventing apoptosis [44]. In fact, unless Hsp90 inhibitors that bypass Hsp70 induction can be developed, it may prove necessary to use Hsp90 and Hsp70 inhibitors in concert for clinical efficacy, and pilot studies have provided support for this hypothesis [45]. In addition to combination therapies, the use of dual Hsp90/Hsp70 inhibitors could lead to a synergistic regulation of oncogenic protein folding. The growing need for Hsp70 inhibitors for anticancer treatment, along with an increased knowledge of Hsp70 function, has begun to shift attention from Hsp90 to Hsp70 [46, 47]. The challenge, then, has been to identify effective chemical and biological tool compounds and drug candidates to modulate this chaperone.

1.3 Hsp70 Function

Hsp70 family members facilitate DNA replication, stabilize membranes, correct defects during protein folding, enable substrate stabilization and the formation of multiprotein complexes, and identify proteins for ubiquitination and subsequent degradation [48–51]. Hsp70s play a role in a variety of pathological conditions in addition to cancer. For example, Hsp70 is necessary for the survival of cells infected by parasites, such as *Trypanosoma* or *Plasmodium falciparum*, and viruses, such as hepatitis B, papillomavirus, HSV, or polyomavirus, while inhibition of Hsp70 function in turn inhibits the spread of these infections [47]. Hsp70 function also plays a role in neurological disorders, such as Parkinson's, Huntington's, and Alzheimer's disease, various ataxias, and in other protein folding disorders, such as cystic fibrosis and Niemann–Pick disease [52–56]. For example, increased Hsp70 expression reduces the accumulation of tau protein aggregates, which are linked to Alzheimer's disease [57]. Studies of Huntington's disease, caused by polyglutamine-repeat containing protein aggregates, suggest a similar reduction of aggregates with increased Hsp70 expression [58, 59]. In addition, Hsp70 can solubilize and promote the degradation of α-synuclein, a neuronal protein associated with Parkinson's disease that may arise from proteasome dysfunction [60]. Furthermore, cystic fibrosis, the most common lethal inherited disease in Caucasians in North America, often results from the premature degradation of mutated cystic fibrosis transmembrane conductance regulator (CFTR), a process that is modulated by Hsp70 [55, 61].

Hsp70 proteins are expressed in all organisms and their activity is highly conserved [62, 63]. This is demonstrated in part by the homology observed between prokaryotic Hsp70s and their human counterparts [64, 65]. For example, the *E. coli* Hsp70, DnaK, shares over 45% amino acid identity with eukaryotic Hsp70s [66, 67].

It is even possible to express heterologous Hsp70s in different organisms and rescue associated defects when the native Hsp70 is dysfunctional [68].

2 Structure and Mechanism

The high homology among Hsp70 family members suggests significant structural and mechanistic similarities in their functions. All Hsp70s contain two major domains: a ~45 kDa N-terminal nucleotide binding domain (NBD) containing an ATPase active site and a C-terminal substrate binding domain (SBD) of ~25 kDa, joined by a flexible linker (Fig. 2) [49]. The NBD is highly conserved in all identified protein sequences, with the ATPase binding site sharing up to 70% pairwise identity [69]. By comparison, the SBD shows greater sequence variation, perhaps due to its ability to recognize a myriad of protein substrates depending on the organism, subcellular residence, and/or regulation. Cytosolic eukaryotic Hsp70s are known to contain a conserved EEVD motif at the C-terminus, which allows interaction with a range of tetratricopeptide repeat (TPR) containing proteins [70, 71]. In one example, co-chaperone HOP (Hsp-organizing protein) contains three TPR motifs, which in turn bind to the EEVD motifs of Hsp70 and Hsp90, leading to the formation of an active Hsp70–Hsp90 multichaperone complex [72]. The Hsp70 SBD also contains a flexible lid, which rotates to hold or release a given substrate (Figs. 2 and 3) [74]. The NBD and SBD participate jointly in an allosteric catalytic mechanism, promoted by ATP hydrolysis [75].

Hsp70s switch between two distinct conformations during their catalytic cycle, an open (ATP-bound) and closed (ADP-bound) form. When ATP resides in the NBD, protein substrates are weakly bound in the SBD in an intermediate state (Fig. 3) [73, 76]. It is only upon hydrolysis of ATP to ADP that the lid portion of the SBD closes and locks the substrate in place, a process facilitated by a major

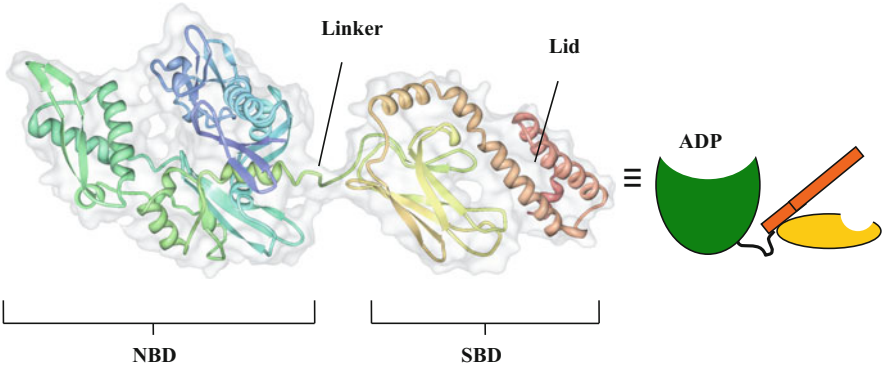


Fig. 2 Structure of Hsp70 with ADP bound, revealing the NBD and SBD. PDB Protein Workshop 4.1.0, protein data bank code 2KHO with corresponding cartoon representation

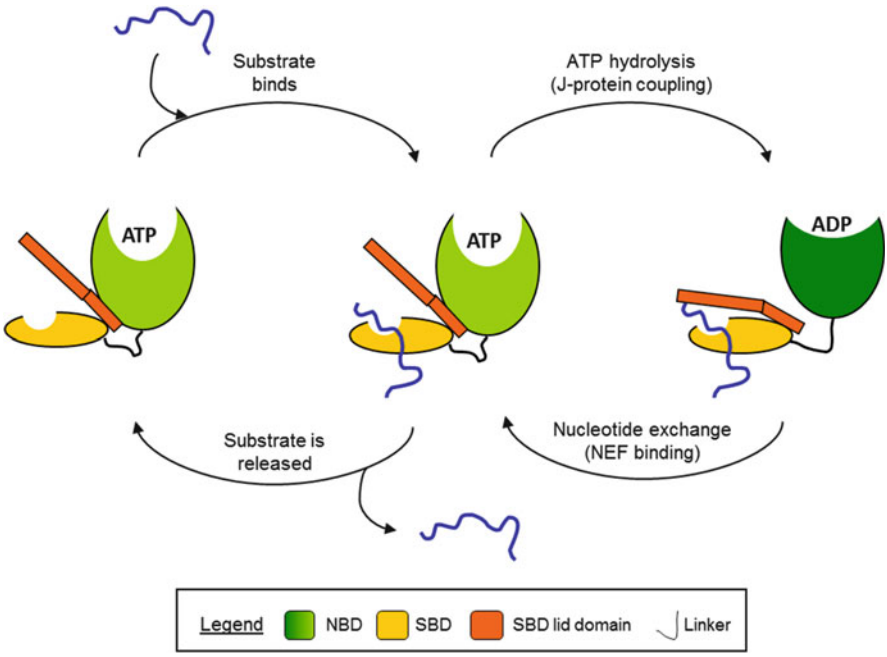


Fig. 3 The Hsp70 catalytic cycle, the cycle depicts the proposed intermediate state of the enzyme complex as proposed by Gierasch and co-workers [73]

allosteric rearrangement of the enzyme conformation [75]. In this closed form, Hsp70 may recruit additional cellular machinery to manipulate the fate of the trapped substrate. The substrate is only released when the lid swings open following ADP displacement. The higher affinity of the NBD site for ATP over ADP and the higher concentration of cellular ATP over ADP favor a return to the ATP-bound form and thus the open Hsp70 conformation [51].

2.1 NBD and SBD Components

As evident from Fig. 3, nucleotide exchange and substrate recognition are critical features of Hsp70 activity. The molecular features underlying Hsp70 nucleotide binding are now well documented by several ADP-Hsp70 co-crystal structures and NMR studies. The crystal structure of the ATPase domain from a constitutively expressed bovine Hsp70 homolog (bHsc70) led to our first understanding of NBD composition [67]. The NBD is composed of two lobes, I and II, which can be further split into four alpha–beta subdomains, known as IA, IB, IIA, and IIB (Fig. 4). IA contains the C-terminal end of the NBD and is joined to the SBD. The four

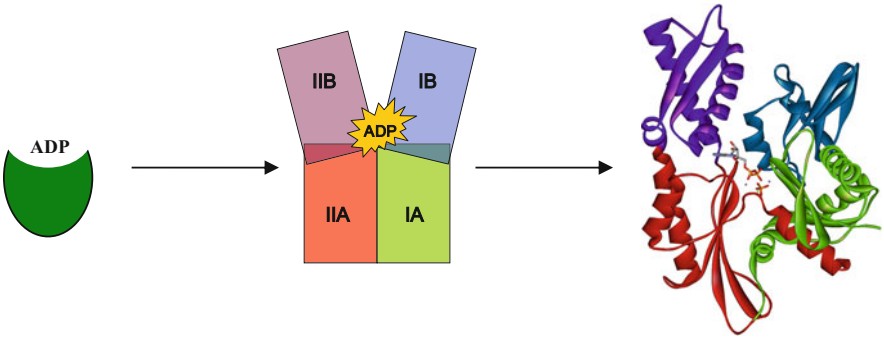


Fig. 4 Model showing the components of the NBD of Hsp70. The image shown is bHsc70 crystal structure, protein data bank code: 3HSC, viewerlite

subdomains are arranged to create a cleft such that each subdomain contacts the adenosine nucleotide.

Three central β-strands of IA and IIA are highly conserved across diverse eukaryotic Hsp70 sequences, a fact attributed to the need for high-affinity nucleotide binding in all Hsp70s [67]. This study also revealed conserved portions of the IB, IIB, and IA domains, which lie closest to the N-terminus of the SBD and have long been associated with the allosteric interaction between these sections. Until recently, the subdomains were considered to be highly rigid, regardless of nucleotide occupancy, but NMR studies carried out on ADP-bound bHsc70 revealed the possibility of substantial conformational alterations [77]. In particular, subdomains IA and IIA were shown to be capable of a shearing motion associated with cofactor binding, as discussed in Sect. 2 [77].

The structure of the SBD was first determined in 1996 from partial co-crystal structures of the *E. coli* Hsp70 homolog, DnaK, bound to synthetic peptide substrates [69]. These structures revealed the SBD to possess a β-sheet sandwich portion that creates a central hydrophobic pocket and a lid composed of a range of α-helices (Fig. 5). The β-sheet sandwich pocket, which is essential for substrate binding, was shown in NMR studies to be highly flexible and allows a range of substrates to be accommodated [78]. This portion of the SBD β-sandwich contains many hydrophobic amino acid residues, which correlates with Hsp70's high affinity for hydrophobic peptides. Given that the preferred Hsp70 substrates are misfolded proteins, this is not unexpected, as a common consequence of protein misfolding is the exposure of hydrophobic regions of peptides which would otherwise be hidden from solution [47]. When the β-sandwich region of DnaK is compared with that of common baker's yeast, *S. cerevisiae*, and bovine Hsc70, the residues in this region are well conserved, with 65–76% pairwise identity [69]. In comparison, the lid-encoding portions of the amino acid sequence of these proteins show conservation as low as 18%, suggesting that differences in Hsp70s substrate recognition may be lid dependent and confirming the important role of the lid and the conserved EEVD motif at the C-terminus in co-chaperone and substrate binding [51, 69].

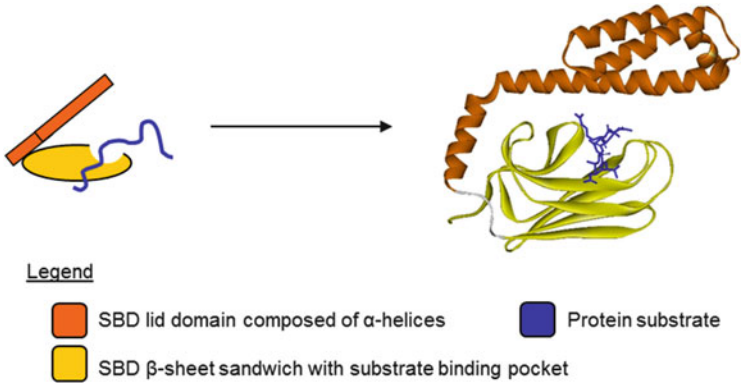


Fig. 5 Model showing the ability of the SBD of Hsp70 to bind a peptide substrate DnaK crystal structure, protein data bank code: 1DKX, viewerlite

Indeed, removal of the lid portion of the SBD results in increased substrate dissociation rates and lower substrate affinities ($k = 300–470$ s^{-1} and K_d (p5 peptide) = 222 μM for lidless DnaK variants, compared with $k = 2–8$ s^{-1} and K_d (p5 peptide) = 7.1 μM for wild-type DnaK), proving that it is needed for Hsp70 to exhibit high affinity for peptide substrates [78, 79].

2.2 *Allosteric Interactions*

Until recently, the majority of X-ray structural information available for Hsp70 was limited to its closed conformation. It had long been accepted that binding of ATP at the NBD induced an allosteric alteration in Hsp70 that results in the release of substrates held by the SBD (Fig. 6). However, the allosteric or open state remained elusive, mainly due to difficulties associated with Hsp70-ATP co-crystallization attempts. In fact, the well-documented *E. coli* DnaK crystal structures, obtained in the presence of ADP, revealed the NBD and SBD conformations to be entirely independent of one another [76, 81]. NMR solution studies by Gierasch and co-workers suggested that the SBD interacts with the NBD only when ATP was bound and that binding of a substrate to the SBD disrupted NBD–SBD interactions, such that the ATPase activity was stimulated via the action of the linker [76]. The involvement of the NBD–SBD linker in allostery was first proposed in 1996 by Hendrickson and co-workers [69], and in the aforementioned study, the linker was shown to bind in a hydrophobic groove between IA and IIA of the NBD only when a substrate was bound in the SBD [76].

In 2012, Mayer and co-workers confirmed the allosteric interaction of the NBD and SBD, supporting the hypothesis that the attachment of these domains directly impacts the rate of ATP cycling [75, 80]. These investigators were able to grow crystals of an open conformation form of DnaK, confirming that the linker region

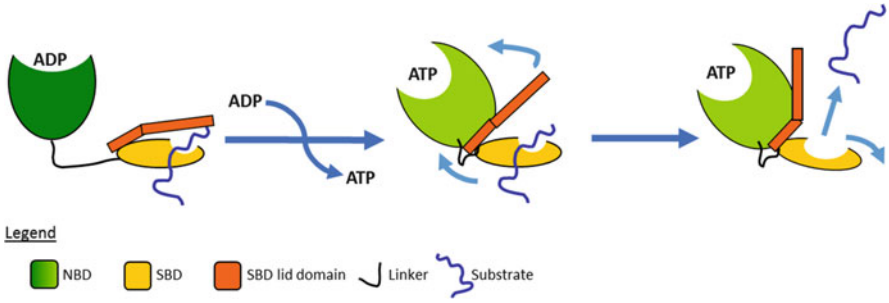


Fig. 6 Model Hsp70 system, depicting allosteric coupling of the SBD and NBD upon ATP binding at the NBD and subsequent lower substrate affinity. Adapted from Qinglian and co-workers [80]

binds to the NBD while the β-sandwich and α-helical portions of the SBD are detached from one another and linked to distinct elements in the NBD [75, 80]. The α-helices in the SBD will only contact the NBD when substrates are absent, revealing the influence of substrate binding on the return to the open conformation (Fig. 6). It was initially proposed that the frequency of transition between the open and closed forms of Hsp70 regulates substrate affinity rather than the conformational change itself [75]. Liu and co-workers now suggest that the transition relates directly to ATP displacement of ADP, as this appears to regulate the allosteric opening of the SBD [80].

2.3 *A Helping Hand: J-Domain-Containing Proteins and Nucleotide Exchange Factors*

The ATPase activity of Hsp70 is quite low, typically between 3×10^{-4} and 1.6×10^{-2} s^{-1}, and binding of a substrate at the SBD stimulates ATP hydrolysis only two- to tenfold [51]. In contrast, ATP hydrolysis is significantly accelerated through the binding of Hsp70 to a co-chaperone that belongs to the J-domain-containing protein, or "J-protein" family [82]. The best example of this stimulation was documented with *E. coli* DnaJ, which increases ATP hydrolysis of DnaK by over 1,000-fold [83].

Based on homology to DnaJ, J-proteins (also known as Hsp40s) all contain a conserved "J-domain" of ~70 kDa, which interacts with the Hsp70 NBD and possibly the linker portion of the enzyme to stimulate ATPase activity [84–86]. Co-crystal structures of the NBD and linker portions of Hsp70s with J-proteins have revealed dramatic alterations to the Hsp70 conformation following J-protein binding. In particular, J-proteins must displace Hsp70 SBD contacts with the NBD to allow for the J-domain to contact the NBD [84].

As a group, J-proteins generally do not share significant structural identity beyond the α-helical J-domain [87]. They are nevertheless classified into distinct subtypes (type I–III) based upon a mixture of functional and sequence similarities. Type I J-proteins closely resemble DnaJ and contain distinct motifs, including two zinc fingers and a C-terminal extension [88]. Type II J-proteins differ from Type I in that they lack the zinc finger motifs or a distinct Gly-Phe region adjacent to the J-domain [87]. Type III J-proteins contain only the conserved J-domain of DnaJ [88]. As more than one J-protein can interact with a single Hsp70, a number of mechanisms of action have been identified that are J-protein dependent [87]. For example, Type I and II J-proteins are able to bind misfolded proteins and present them to the Hsp70 SBD, before in turn binding the NBD [89]. In this model, the J-protein will transfer its cargo to the SBD of Hsp70 and then stimulate its ATPase activity to ensure the substrate is trapped [87]. Other J-proteins may only bind at the NBD after a substrate weakly associates with the Hsp70. Regardless of the J-protein mechanism, stimulation of the ATP hydrolysis activity of the Hsp70 results in hastened allosteric closing of the lid portion of the SBD over associated substrates.

Although the rate of ADP dissociation from Hsp70 is often faster than the endogenous rate of ATP hydrolysis, once a J-protein stimulates Hsp70, nucleotide dissociation may become a rate-limiting step in the Hsp70 catalytic cycle. Therefore, many Hsp70s are also regulated by nucleotide exchange factors (NEFs), such as the Hsp110s, BAG proteins, or GrpE in bacteria [90]. These co-chaperones alter the conformation of the NBD, facilitating ADP release from the NBD [91, 92].

Unlike the J-proteins, the four types of identified NEFs lack structural similarity and are believed to destabilize the NBD binding cleft through different mechanisms [74]. Hsp110s show high structural similarity to Hsp70, possessing both an NBD and SBD [93]. In the yeast Hsp110, Sse1, a significantly extended α-helical portion of the SBD interacts with large portions of the NBD II lobe of the corresponding Hsp70 [91, 93]. These interactions open the binding cleft in the Hsp70 NBD and allow ADP to exit [94]. Subsequent binding of ATP in the binding cleft then causes NEF dissociation, thereby initiating a new cycle.

3 Hsp70 Expression

The mechanistic and structural characteristics of Hsp70s are conserved among organisms, but it is important to realize that "Hsp70" is often used as an umbrella term. In fact, many different isoforms of Hsp70 can be found within a single cell and within a single organism. For example, sixteen Hsp70 genes along with ~30 pseudogenes have been identified through a genome analysis in humans [95]. Eight of these genes are bona fide chaperones and share 52–99% amino acid sequence homology (Table 1) [20, 64]. A clear demarcation between these related isoforms can be drawn through an analysis of their expression patterns. Some Hsp70s are stress induced, while others are constitutively expressed (the "Hsc" family), and both types are commonly present in eukaryotes [64]. Both stress-induced and

Table 1 The established members of the human Hsp70 family

Human Hsp70 Gene	Protein product (including alternative names)	% Homology (to HSPA1A)	Stress induced	Expression (subcellular or tissue specific)
HSPA1A	Hsp70-1a	100	+	Cytosol, nucleus, and lysosome
HSPA1B	Hsp70-1b	99	+	Cytosol, nucleus, and lysosome
HSPA1L	Hsp70-1t	91	–	Testis
HSPA2	Hsp70-2	84	–	Testis and brain
HSPA5	BiP, Grp78, or Hsp70-5	64	–	Endoplasmic reticulum
HSPA6	Hsp70-6	85	+	Blood and dendritic cells
HSPA8	Hsc70 or Hsp70-8	86	–	Cytosol and nucleus
HSPA9	mtHsp75 or Hsp70-9	52	–	Mitochondria

Adapted from Jäättelä and co-workers [64]

constitutively expressed Hsp70s will be discussed below in the context of the human variants.

3.1 Heat Shock-Inducible Hsp70s

Heat shock-induced Hsp70 represents the isoform for which the family was first named. In humans, the major stress-induced proteins are Hsp70-1a and Hsp70-1b, and these were also the first isoforms identified [96]. These proteins are nearly identical (99% amino acid homology), encoded by the genes HSPA1A and HSPA1B, respectively, and are localized 12 kb apart on chromosome 6 [97]. Like all known major stress-induced Hsp70s, Hsp70-1a and Hsp70-1b are primarily expressed in the cytosol of most tissues [98] but also in nuclei under stress conditions [95]. Although Hsp70 mRNA is constitutively expressed at low concentrations [96], gene expression is strongly induced under heat shock conditions by heat shock factor 1 (HSF1). HSF1 forms an active trimer only when a cell is stressed and binds to the promoter region of HSPA1A/B, causing upregulation of mRNA synthesis [99, 100]. Hsp70-1 analogs, such as Hsp70.1 and Hsp70.3 in mice [101], SSA1-4 from yeast [102, 103], and DnaK from *E. coli*, have proved crucial to probe Hsp70 function. DnaK is only essential under stress conditions and singularly fulfills many of the roles of the multiple eukaryotic Hsp70 isoforms [104].

The only other Hsp70 gene that is stress induced in humans, HSPA6, is also expressed in the cytosol and nucleus, but only after severe stress [105, 106]. While the function of the encoded protein, Hsp70-6, has been questioned due to an absence of expression in most tissues, some analyses suggest that it is present in the blood, in dendritic cells, and in monocytes [64, 107].

3.2 *Constitutively Expressed Hsp70*

The stress-induced Hsp70s may aid in cellular survival during potentially lethal conditions, but the constitutive expression of Hsc70 suggests that errors in protein folding and aggregation occur regularly in the absence of exterior pressures [10]. Members of this protein family play many other roles in maintaining cell health (see Sect. 4). As such, a high level of constitutively expressed Hsp70 (i.e., Hsc70) is present in most cells [108].

In humans, the major constitutively expressed Hsp70, known as Hsc70 or Hsp70-8, is encoded by HSPA8 and is found on chromosome 11 [109]. Hsc70 is expressed in the cytosol and nucleus of all tissues and shares 85% sequence homology with Hsp70-1 [64, 98]. Some Hsc70s are essential for normal cellular growth, as determined in mouse knockout studies [64]. While HSPA1/B knockout mice were viable (although some genomic instability was recognized) [101, 110], HSPA8 knockouts were embryonically lethal [111].

Two other constitutively expressed Hsp70s are also found in the cytosol and nucleus, namely, Hsp70-1t and Hsp70-2, encoded by the genes HSPA1L and HSPA2, respectively [64, 112]. These isoforms are present in most tissues, but both of the proteins are found in significantly higher concentrations in the testis and also in the brain in the case of Hsp70-2 [95]. Although the function of Hsp70-1t remains elusive [64], Hsp70-2 is believed to play a role in germ cell meiosis, perhaps through DNA repair and recombination [113].

The two remaining constitutively expressed genes, HSPA5 and HSPA9, are unique among the human Hsp70s in that their encoded proteins, Hsp70-5 and Hsp70-9, are constitutively expressed within specific organelles. The expression of Hsp70-5, or Grp78, is confined to the lumen of the endoplasmic reticulum (ER) [114] due to an ER associated C-terminal KDEL sequence, which prevents secretion into the extracellular space [115]. ER Hsp70s are present in all eukaryotes and are known collectively as BiP [116]. In fact, the homology of BiP isoforms between organisms commonly exceeds the similarity of Hsp70s within an organism [116]. For example, the amino acid identity between human BiP and mouse BiP is 99%, whereas that of human BiP and human Hsp70-1 is only ~60% [117]. The nature of this difference is unknown, but it might have to do with an expansion in the number and activities of cytoplasmic Hsp70s compared to the ER lumenal Hsp70s.

A similar trend is seen with Hsp70-9, also known as mtHsp75, which is solely expressed in the mitochondria [118, 119]. The amino acid identity between this protein and Hsp70-1 is the lowest of the human Hsp70s, at just 52%, while mitochondrial homologs from other organisms show higher similarity [64, 118]. The mRNA encoding Hsp70-9 adds a 46 amino acid hydrophobic/basic polypeptide motif at the N-terminus that targets the protein to the mitochondria [118, 120, 121]. Mitochondrial Hsp70 is critical for cell function, as deletion of the yeast homolog, SCC1, results in cell death [64, 122].

4 Hsp70 Function

Given the multitude of Hsp70 isoforms, and their expression in all organisms, the functions attributable to these proteins are unsurprisingly diverse. The constitutively expressed Hsc70s are considered housekeeping proteins that often work in concert with co-chaperones, transporting proteins to their final target, refolding proteins, aiding in the formation of protein oligomers, and identifying proteins for ubiquitination and subsequent degradation [108]. The stress-induced Hsp70s often facilitate the same processes, but only under extreme conditions, when the likelihood of protein aggregation and misfolding is increased [64].

4.1 Protein Folding

Some proteins require co- and posttranslational folding assistance by Hsp70, either alone or aided by co-chaperones. The importance of this process is evident in *E. coli* where DnaK associates with 5–18% of all newly synthesized proteins [123]. The evidence for Hsp70 involvement in protein folding is also well documented [51].

Proteins are unable to adopt the majority of their final tertiary structural elements during translation due to spatial constraints conferred by the ribosome [124]. This increases the possibility that nascent proteins synthesized from different ribosomes will aggregate before adopting their native conformations [125, 126]. To counteract incorrect folding or aggregation in eukaryotes, Hsp70 binds nascent protein chains as they are being translated, thus facilitating the posttranslation folding of targeted domains [124].

Following translation, Hsp70 folding activity has been attributed to two distinct mechanisms [51]. In the first, Hsp70 assumes a passive role, whereby newly synthesized proteins which have yet to attain their final tertiary structure are held by Hsp70s in an intermediate phase [51]. This prevents aggregation by reducing the concentration of unfolded proteins in the cell [124]. A single substrate can undergo multiple binding and release cycles with Hsp70 prior to attaining its final structure. However, due to the short lifetime of Hsp70-substrate complexes, the second mechanism holds that Hsp70 acts as an unfoldase, binding to misfolded regions of proteins and actively facilitating refolding through removal of kinetic barriers [127].

Regardless of the exact mechanism, it is important to note that Hsp70 interacts with a range of co-chaperones to fulfill its function, some of which were discussed in Sect. 2. These co-chaperones dictate the manner in which proteins are targeted for folding and heighten the specificity of Hsp70 for a particular substrate and/or accelerate the ATP hydrolytic cycle [82]. As noted in Sect. 2.3, some misfolded substrates are initially identified by Hsp40s, also known as J-proteins, and escorted to Hsp70 [128]. Other J-proteins are associated with select subcellular compartments or locations, such as the exit site of the ribosome [129]. Other co-chaperones

associate with the Hsp70/Hsp40 complex and alter Hsp70 activity. For example, Hip (Hsc70-interacting protein) improves Hsp70 substrate affinity through binding at the NBD and stabilizing the ADP-bound conformation [130]. For the removal of conferred substrate affinity and accelerated ADP release, the co-chaperone BAG-1 competes with Hip and acts as a NEF [131].

The interacting co-chaperones do need not to be Hsp70 specific, as seen by the interaction between Hsp70s and the Hsp60 chaperonins [132]. Hsp60s are heptameric chaperones comprised of ~60 kDa subunits arranged in a ring, creating a central binding cavity that houses nonnative proteins and actively facilitates folding concomitant with ATP hydrolysis [133]. Recently, eukaryote cytosolic chaperonin-containing TCP-1 (CCT), an octomeric Hsp60 with a specialized role in folding cytoskeletal elements, was shown to bind to the NBD of Hsc70 [134]. It is believed that following the formation of the Hsc70–Hsp60 complex, unfolded proteins bound at the SBD of Hsp70 are directed into a cavity of Hsp60, removing them from solvent and allowing for more efficient folding [134].

Hsp70 and its co-chaperones do not only prevent aggregation, but can solubilize and fold aggregated proteins as well [135]. Large heat shock proteins, such as Hsp104 from yeast or Hsp100 in bacteria, bind protein aggregates, but in the absence of Hsp70 and Hsp40 homologs, they cannot initiate protein refolding [49, 135–137]. However, these chaperones couple ATP hydrolysis to expose misfolded, hydrophobic patches on aggregates which then recruit Hsp70s, the corresponding J-proteins, and NEFs [136, 138]. Multiple Hsp70 molecules associated at different regions on a misfolded peptide may also allow local conformational changes, which, through multiple iterations of this process, solubilize aggregates [137, 139]. In the case of smaller aggregates, Hsp70-like proteins may facilitate protein solubilization without the aid of Hsp100, thereby preventing the formation of larger protein aggregates [140]. Such a process was recently observed with the yeast Hsp110, Sse1 [141].

4.2 Protein Transport and Degradation

Another key role played by Hsp70 is in facilitating protein transport. Hsc70 in particular controls the translocation of various proteins into the ER and mitochondria [108]. The ER-specific Hsp70, BiP, is involved in the recognition of ER-targeted proteins at the ER membrane and their subsequent entry into the ER lumen [50]. Mitochondrial Hsp70 is required for the translocation and folding of mitochondrial targeted proteins, which are unfolded in the cytosol so that they can pass through translocation pores [120]. Transport of proteins from the cytosol to the nucleus is also highly dependent on Hsp70. For example, removal of both Hsc70 and Hsp70 in HeLa cell extracts prevented the localization of nuclear resident proteins [142]. Hsp70s are also needed for nuclear receptor and kinase protein folding and regulation, although the mature forms of these proteins are only obtained when Hsp70 works in concert with Hsp90 [143, 144]. Through association

with HOP, which binds the TPR domain of the SBD, Hsp70 presents substrates to Hsp90 for further manipulation [72, 145, 146].

Hsp70 also plays a role in protein targeting to the lysosome and to the proteasome for degradation [144, 147]. Some proteins bound by Hsp70 cannot be returned to their native state, at which point they need to be identified for degradation. Although Hsp70 itself does not usually fulfill this role, it presents substrates to different co-chaperones, including chaperone-associated ubiquitin ligases such as CHIP (carboxyl terminus of Hsc70-interacting protein), which converts Hsp70 into a degradation-catalyzing chaperone [148]. CHIP associates with the C-terminal EEVD motif through its C-terminal TPR domain, whereas at the N-terminus CHIP has a U-box with ubiquitin-ligase activity, which leads to substrate ubiquitination [149, 150]. Subsequent recruitment of the proteasome to the ubiquitinated substrate/chaperone complex results in substrate degradation [151, 152].

4.3 Apoptosis

Another notable Hsp70 function lies in the regulation of cellular apoptotic processes, which can be induced by stress [153]. Hsp70 inhibits apoptosis by blocking the functions of the apoptosome, caspases, and apoptosis-inducing factor (AIF) [154]. The diverse inhibitory functions of Hsp70 during apoptosis are summarized in Fig. 7.

Apoptosis extrinsically occurs through the activation of cell surface death receptors such as Fas, which results in binding of related proteins (e.g., FADD and DAXX) and activates apoptosis pathways associated with caspase-8 and c-Jun N-terminal kinase (JNK), respectively [155, 156]. Some of the activities of Hsp70 before mitochondrial signaling include the inhibition of the JNK apoptotic signaling pathway. Phosphorylation of JNK results in the cleavage of cytosolic Bid to give truncated tBid, a mitochondria-targeted death ligand [157]. tBid also signals a conformational shift in cytosolic BAX (proapoptotic protein from the Bcl-2 family), which triggers association with the mitochondrial membrane [158]. In the presence of Hsp70, JNK phosphorylation, Bid cleavage, and BAX membrane association can all be inhibited, each in turn stopping the release of cytochrome *c* from the mitochondria [159–161].

At the level of the mitochondria, Hsp70 inhibition of the release of apoptotic factors from this organelle, specifically cytochrome *c* or AIF, is also important for cell survival [153, 162]. Finally, downstream in the caspase-9 cascade, Hsp70 prevents caspase-9 from binding to the apoptosome [163], inhibiting the formation of the apoptosome itself [164], and arresting caspase-3-induced apoptosis [165, 166]. In addition, Hsp70 inhibits apoptosis by stabilizing the lysosomal membrane, which would otherwise become permeable and release proapoptotic factors (such as lysosomal hydrolases) into the cytosol [167, 168]. It is apparent that the many antiapoptotic roles attributable to Hsp70 promote cell survival.

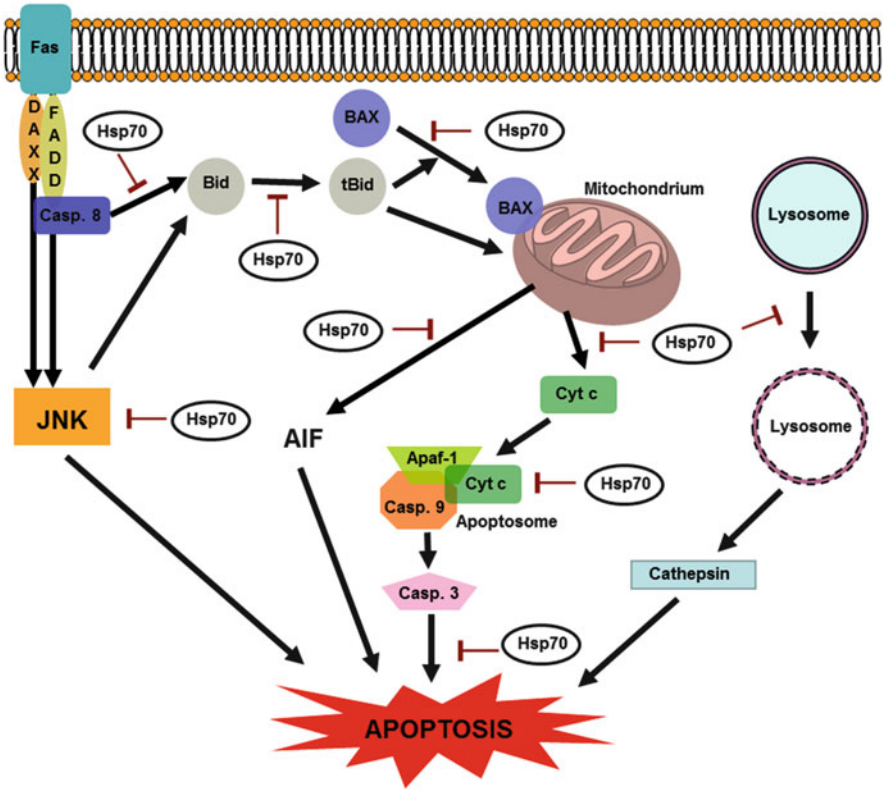


Fig. 7 Apoptotic pathways inhibited by Hsp70. Adapted from Gestwicki and co-workers [154]

5 Therapeutic Relevance

Given the numerous essential roles fulfilled by Hsp70, irregular expression and/or function of this chaperone can have serious consequences, resulting in or contributing to numerous diseases.

Cancer is particularly well known for exploiting Hsp70 function, with heightened levels often correlating with poor prognosis [18, 19, 169, 170]. This observation can be explained by the ability of Hsp70 to direct folding and prevent aggregation of nonnative proteins, a common by-product of cancer cell genomic instability, coupled with the inherent protection conferred through inhibition of apoptosis [169]. As a result, cancer cells are able to persist despite being subjected to permanent stress conditions [20]. Indeed, three Hsp70 isoforms facilitate tumorigenesis, namely, Hsc70, Hsp70-1, and Hsp70-2 [171–174]. Jäättelä and co-workers established that when Hsp70-1 and Hsp70-2 were jointly suppressed using RNA interference, cancer cell death was significant (>80%), while non-tumorigenic epithelial cells survived [171]. Accordingly, the inhibition of these Hsp70s is of

particular interest in the search for novel anticancer agents, particularly as many current anticancer treatments rely upon activation of apoptotic pathways, which are blocked by Hsp70 activity [21, 47]. Moreover, cancer cell lines may be resensitized to chemotherapeutics in the presence of an Hsp70 inhibitor, which would help to restore the susceptibility of the apoptotic pathways, counteracting acquired resistance seen with many cancer types during and/or following traditional treatment regimes [46].

While inhibition of Hsp70 is expected to provide a viable new option for anticancer treatment, other diseases, such as the neurological disorders Parkinson's, Alzheimer's, and Huntington's disease, would benefit from increased Hsp70 expression [56]. These diseases are caused by the proliferation of aberrant protein aggregates or pre-aggregates, resulting in reduced neuron viability and function [175]. Specific examples of aggregated proteins include α-synuclein in Parkinson's disease [176], amyloid β-peptides and tau protein in the case of Alzheimer's disease [177], and genetic expansion of the polyglutamine-containing protein huntingtin, the cause of heritable Huntington's disease [178]. The importance of Hsp70 in the etiology of these diseases is well documented [56]. For example, a decrease in Hsp70 levels correlates with disease progression in the case of Huntington's disease [179], while overexpression of Hsp70 in models of Alzheimer's and Parkinson's disease prevents protein aggregation [53, 60]. Interestingly, Hsc70 was recently identified as a potential cause of tau aggregation, as it may prevent protein degradation [57]. However, when coupled with an allosteric inhibitor (YM-01, an analog of MKT-077; see below), the function of Hsc70 can be switched from being a pro-folding to a pro-degradation chaperone, resulting in aggregate dispersal [57]. The multiple facets by which Hsp70 influences aberrant protein accumulation make it both a promising and a very challenging target in the search for novel neurological disease therapies [56].

6 Hsp70 Modulators

The increasing interest in disease-related Hsp70 functions has inspired the discovery of specific antagonists as well as agonists. The chemical modulators of Hsp70 identified to date can be grouped into two categories: (1) those that directly affect Hsp70 function and (2) those that indirectly modulate Hsp70. Both are discussed in detail below.

6.1 Direct Hsp70 Modulators

6.1.1 15-Deoxyspergualin and Analogs

15-Deoxyspergualin (15-DSG) was originally discovered in 1982 by Umezawa and co-workers during chemical modification of the antibiotic spergualin (Fig. 8). Both compounds contain spermidine, 7-guanidinoheptanoic acid, and α-hydroxyglycine moieties [180].

Spergualin sparked significant interest when it was first shown to prolong the lifespan of mice in a leukemia model and exhibited low cumulative toxicity in spite of high-dose administration (up to 80 mg/kg via intravenous injection) [181, 182]. It was later found that 15-DSG, which differs from spergualin only in the absence of the C-15 hydroxyl group, exhibited eightfold more effective antitumor activity in mice (although a greater LD_{50} of 35–40 mg/kg was observed) [180]. In subsequent experiments, 15-DSG displayed immunosuppressive activity at higher doses than those which induced antitumor activity [183]. Indeed, the immunosuppressant activity of 15-DSG (see below) leads to successful reversal of acute graft-versus-host disease and prolonged survival following xenograft and allograft transplants [184].

Based on these data and the greater ease of synthesizing a compound with fewer stereogenic carbons [185], 15-DSG became the new lead compound in the spergualin-based series. Although many synthetic routes to 15-DSG have been achieved, large-scale enantioselective synthesis remains difficult [180, 186, 187]. As a result, the majority of biological data associated with 15-DSG has been obtained with its racemate, despite some information that only (−)-15-DSG has anticancer and immunosuppressant activity [187–189].

In 1992, the biological target of 15-DSG was identified as Hsc70 [190]. Mazzucco and co-workers used a methylated DSG-Sepharose column to isolate 15-DSG-binding proteins in human T-cell lysates. The resulting peptide hits were purified and sequenced, and in all cases, they corresponded to Hsc70 [190]. Following this finding, a small collection of 15-DSG analogs was synthesized and tested alongside 15-DSG for inhibitory effects against Hsc70 and Hsp90. Interestingly, 15-DSG was found to have equal affinity for both molecular chaperones, with K_D values of 4 and 5 μM, respectively [191].

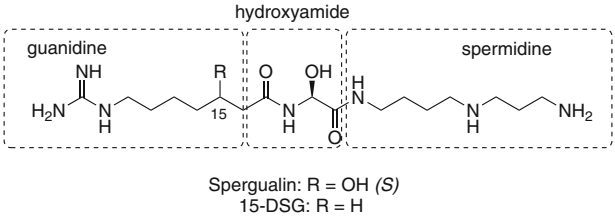


Fig. 8 The chemical structures of spergualin and 15-DSG

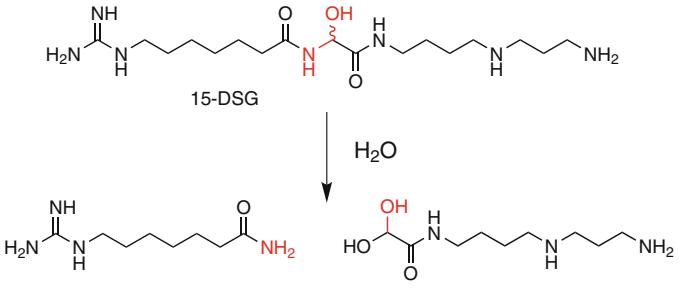
In 1998, Marquardt and co-workers explained the correlation between Hsc70 and Hsp90 affinity through their discovery that 15-DSG bound to the SBD of both proteins [192]. In fact, it appeared that 15-DSG bound to the C-terminal EEVD motif, which is present in both Hsc70 and Hsp90 and is used to dock proteins with TPR motifs (see Sect. 2). As described above, TPR motif-containing proteins represent an important class of cytoplasmic Hsp70 and Hsp90 regulators [71, 146]. Given the importance of the EEVD motif, it is unsurprising that 15-DSG disrupts the activity of these chaperones. In contrast, substrate binding to Hsc70 is unaffected by 15-DSG [70, 192].

Over the years, 15-DSG has been shown to affect a number of other biological processes, including the transport of transcription factors into the nucleus [192], cytochrome *c* oxidase activity [193], polyamine biosynthesis in tumor cells [194], and antibody production [195]. The broad effects of 15-DSG are consistent with interference of Hsp70/Hsp90 binding to TPR domain-containing proteins, since – as noted above – these co-chaperones modulate the HSPs [196].

More recently, it was suggested that 15-DSG might hinder protein substrate binding to the Hsp70 SBD. However, this hypothesis was based on studies with the Hsp70 from the parasite *Plasmodium falciparum*, which leads to the most common form of malaria [197]. The SBD of Hsp70 appears to be blocked upon 15-DSG addition as nuclear-encoded and apicoplast-targeted proteins are now unable to bind to the chaperone [197]. Whether this phenomenon is universal among other Hsp70s is unclear.

15-DSG has been approved for clinical use to treat allograft rejection [198, 199], but it has proven less effective when used to treat cancer [200]. Factors limiting the utility of 15-DSG are the inherent instability of the molecule at varying pHs in vitro and its rapid metabolic degradation in vivo; 15-DSG also exhibits low oral bioavailability (<5%) [198, 201]. Like the parent compound, spergualin, 15-DSG undergoes facile hydrolysis of the hydroxyamide moiety, with a half-life of 48 h at pH 7 and only 2 h at pH 10 (Scheme 1) [201].

Despite these setbacks, the initial promising activities associated with 15-DSG led to the synthesis of a large number of analogs with the hope of finding compounds that are more stable and efficacious and that show higher selectivity and reduced toxicity [188, 202]. More than 400 variants were synthesized by Saino and



Scheme 1 Hydrolytic products of 15-DSG

co-workers in 1993. An analysis of the resulting structure–activity relationship (SAR) suggested that the guanidylated alkyl group and hydroxyl amide core were necessary for bioactivity, confirming earlier findings [187, 203]. In 1999, Renaut and co-workers increased the stability of the core hydroxyl amide against hydrolysis through the substitution of the hemiaminal group with a carbamate [201]. The resulting product, known as tresperimus (Fig. 9), exhibited a half-life of >50 days at both acidic and neutral pH and a half-life of 24 h at pH 10 [201]. Most importantly, this compound was as active as 15-DSG in a graft-versus-host disease model in mice [201]. Subsequently, Gestwicki and colleagues used the Ugi multicomponent reaction to resolve difficulties generally associated with the synthesis of 15-DSG-based compounds and identified analogs with at least twofold greater chemical stability than 15-DSG at neutral pH (Fig. 9) [188]. Unfortunately, these compounds (and 15-DSG) still undergo metabolism of the spermidine moiety in vivo. In turn, altering the spermidine-derived portion of 15-DSG is detrimental to activity, and as a result, this motif must likely be maintained [187, 202, 204].

Subsequent to the correlation between the immunosuppressive effect of certain 15-DSG analogs and their binding to Hsc70, the discovery of other compounds was reported [191, 202]. In a search of the National Cancer Institute Library for compounds bearing structural similarity to 15-DSG [205], NSC 630668-R/1 was identified (Fig. 9). This symmetrical bis-carbamate inhibited the ATPase activity of a yeast Hsp70 both alone and in the presence of an Hsp40 co-chaperone, leading to a ~50% decrease in ATPase activity at 300 μM [205].

Unlike 15-DSG, NSC 630668-R/1 also inhibited the ATPase activity of a second yeast Hsp70, the ER resident BiP, which is required to translocate polypeptide

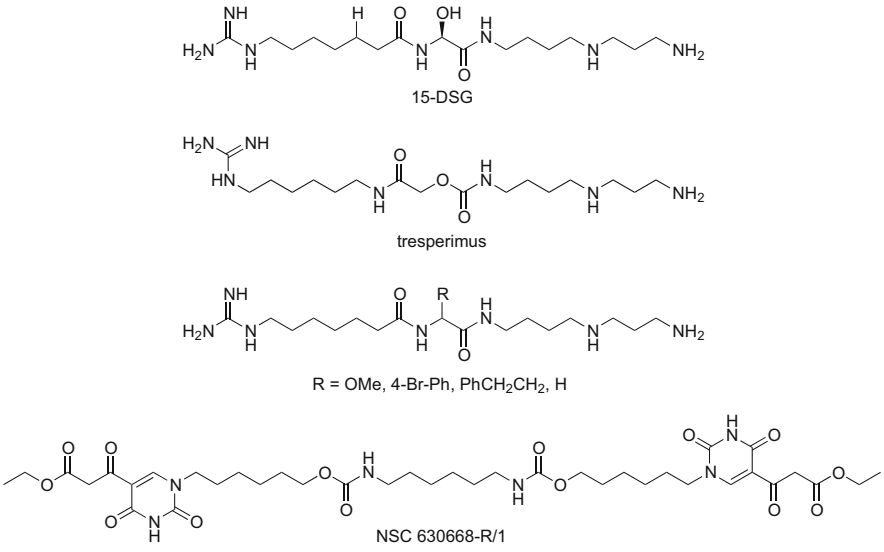


Fig. 9 Examples of 15-DSG derivatives

substrates into this organelle (see also Sect. 3.2). Consistent with this inhibitory activity, translocation was inhibited by NSC 630668-R/1 in an in vitro assay.

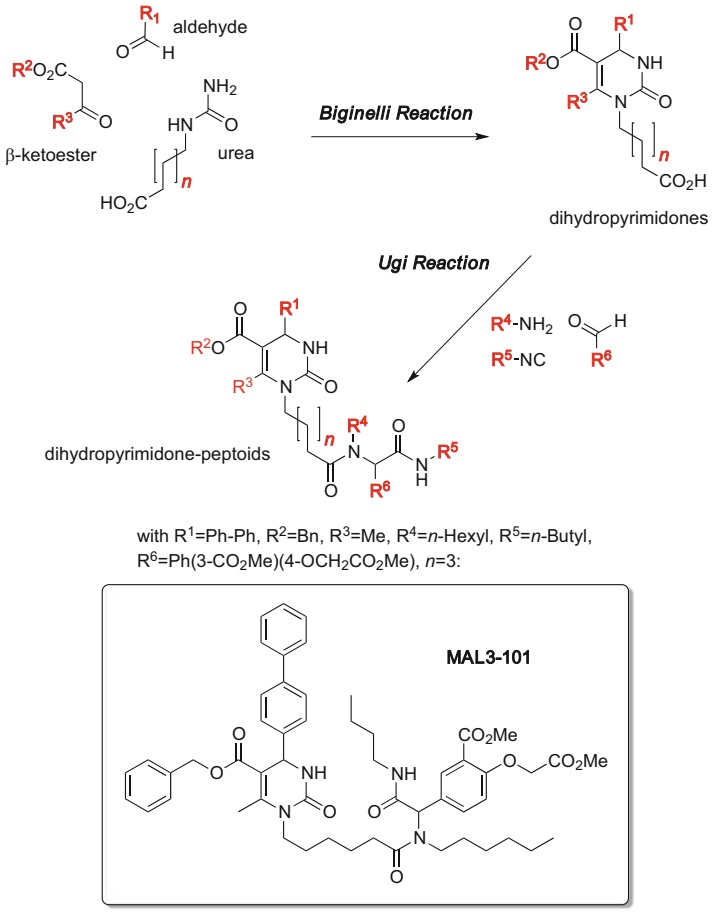
6.1.2 MAL3-101 and Analogs

The work inspired by the discovery of spergualin and 15-DSG led to the analysis of a series of compounds which were structurally related to the original NSC 630668-R/1 lead [206]. By maintaining the pyrimidinone core, while varying the substituents through multicomponent and cascade reactions, we envisaged that targeted small-molecule libraries could be used to identify a superior Hsp70 modulator. Indeed, through the consecutive use of a Biginelli dihydropyrimidinone synthesis and an Ugi four-component condensation, a collection of β-ketoesters, aldehydes, and ureas were combined to first generate dihydropyrimidone heterocycles, followed by side chain extensions to generate the desired dihydropyrimidinone peptoids (Scheme 2) [207]. Many of the compounds synthesized in this manner exhibited varying effects on Hsp70 ATPase activity, either alone or when combined with a co-chaperone [208]. Based on these data, a SAR analysis could be tailored to different biological target systems.

MAL3-101 (Scheme 2) was one member of a ~360 compound library and was identified as a novel modulator of Hsp70 activity in 2004 [206]. Specifically, we established that MAL3-101 had a minor stimulatory effect on the K_{cat} for ATP hydrolysis (see also [209]), but more intriguingly, it significantly reduced the ability of Hsp40 co-chaperones to enhance the ATPase activity of Hsp70 in single-turnover studies [210]. Specifically, MAL3-101 at 300 μM decreased the rate of T antigen-stimulated Hsp70 ATPase hydrolysis approximately fourfold, from 13×10^3 to 3×10^3 %ATP hydrolyzed/s [196]. MAL3-101 also impaired the transport of a nascent polypeptide into the ER, which requires not only the activity of Hsp70s in the ER and cytoplasm (see above) but also associated Hsp40s [50].

These findings suggested that the active MAL compounds might bind to the surface underlying the Hsp70 NBD that mediates contact with the Hsp40 J-domain. This hypothesis was later confirmed by NMR and by multiplex expression profiling studies, which measured distinct cellular responses to the model dihydropyrimidinone 115-7c, originally known as UPCMLD00WMAL1-271 (PubChem CID 5461551, Fig. 10) [211, 212]. Pyrimidinone 115-7c interacts with the IA and IIA domains at the underside of the Hsp70 NBD, regions commonly attributed to Hsp40 binding [85, 86, 212]. However, the 115-7c and J-domain binding sites probably do not completely overlap because 115-7c acts as an agonist of Hsp70 (DnaK)-Hsp40 (DnaJ) ATPase activity [212]. Interestingly, a 115-7c derivative that more closely resembles MAL3-101 (116-9e, Fig. 10) was an antagonist when examined in the presence of DnaK and DnaJ, indicating that relatively small changes in the "fit" of the dihydropyrimidinones can be tailored to differentially modulate Hsp70 activity [212].

Dihydropyrimidinone-based inhibitors of the Hsp40-stimulated activity of Hsp70 inhibit breast cancer cell proliferation [210, 213], possess synergistic effects



Scheme 2 Preparation of Hsp70 modulators by consecutive Biginelli–Ugi multicomponent reactions and structure of MAL3-101

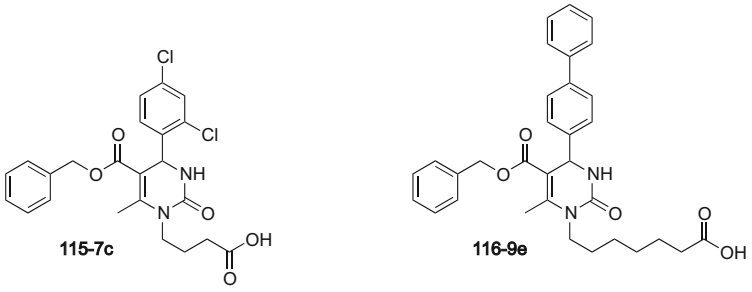
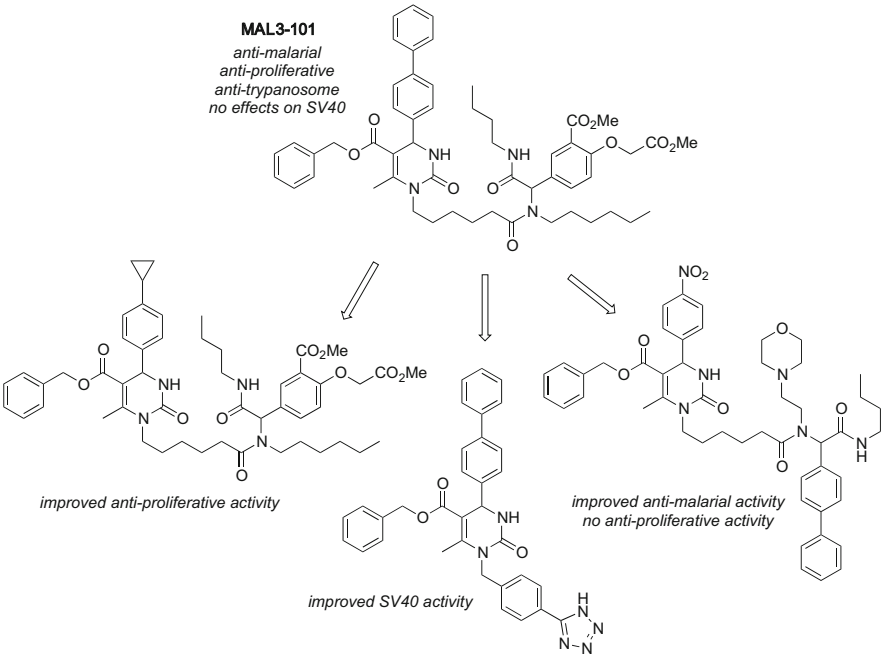


Fig. 10 MAL3-101 analogs 115-7c and 116-9e. These analogs were used to map the Hsp70 binding site of these dihydropyrimidinones

on multiple myeloma cell growth when examined with Hsp90 or proteasome inhibitors [214], and prevent the replication of polyomaviruses [209, 215], which recruit Hsp70 to an oncogenic protein that is encoded by the viral genome. MAL3-101 and its structural analogs have also served as in vitro probes for Hsp70 function. Specific pyrimidinones inhibited the insertion of a tail-anchor protein into ER membranes and prevented protein translocation into the ER and the replication of a *Trypanosoma*, a parasite that is the cause of African sleeping sickness [216]. Other members of the MAL3-101 family prevented *P. falciparum* replication in infected red blood cells, which requires Hsp70 and Hsp40 co-chaperones [217, 218].

Current work on the MAL analogs is focused on improving the physicochemical attributes of the structure, through reductions in lipophilicity (*c*logP), molecular weight and flexibility of the side chains, and substituents on the core heterocycle of the molecule. MAL3-101 does not pass two of Lipinski's "rule of five" recommendations for orally active pharmaceuticals [219], but is a valuable probe molecule and is broadly used as a selective Hsp70 modulator in cancer, viral, and parasite research [208]. Indeed, members of MAL3-101-based chemical libraries continue to be used as a starting point for the discovery of more selective leads to combat Hsp70-linked diseases (Scheme 3) [208, 215, 220].



Scheme 3 Structural modifications of the Hsp70 probe molecule MAL3-101 can lead to changes in the biological profile [208]

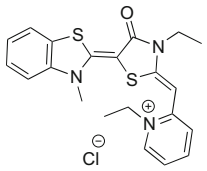


Fig. 11 Structure of the Hsp70 inhibitor MKT-007

6.1.3 MKT-077

MKT-007 (Fig. 11) was identified as an inhibitor of Hsp70 function in 2000 [221, 222], but this rhodacyanine dye first garnered interest in 1996 when it was shown to have anticancer activity and preferentially accumulated in mitochondria of tumor cells [223]. Clinical trials commenced in 1999, but were abandoned after severe renal toxicity was noted [224]. Gestwicki and co-workers recently renewed interest in the scaffold when they identified MKT-077 as an allosteric inhibitor of the Hsp70 NBD [225]. Further information on the function of this inhibitor and synthetic modifications of the MKT-077 scaffold [226] can be found in the chapter from Gestwicki and colleagues in this volume.

6.1.4 Adamantyl Sulfogalactosyl Ceramide, AdaSGC

In 2003, adamantyl sulfogalactosyl ceramide (adaSGC, Fig. 12) was found to inhibit Hsp70 ATPase activity [227]. The design of adaSGC was based upon knowledge of the binding of sulfogalactolipids (SGL), such as sulfogalactosylglycerolipid (SGG), to a putative sulfatide-binding site on a variety of Hsp70s [228–230]. This sulfatide SGL-binding site lies at the base of the ATPase domain of Hsp70, and SGL binding is known to decrease ATPase activity [231]. This information was exploited in the synthesis of water-soluble SGL derivatives, where an SGC core was substituted with bulkier adamantyl and norbornane groups in place of the original acyl chain (Fig. 12) [227, 232].

AdaSGC not only inhibited Hsc70/SGL binding (IC_{50} values of 50–75 μM) but also acted as a noncompetitive ATP inhibitor and decreased Hsc70 ATPase activity by 76% at 300 μM [227, 232]. Because inhibition of the ATPase activity was delayed when kinetic analyses were performed, it was proposed that adaSGC might have a greater affinity for the ADP-bound conformation of Hsc70, a hypothesis that was later supported by mutagenesis studies [227, 233]. AdaSGC has also been shown to disrupt the binding of peptides to a yeast Hsp70, Ssa1 [233]. Based on these activities, the effect of adaSGC on the maturation of CFTR was assessed, as Hsp70–Hsp40 complexes target misfolded, disease-causing forms of the protein for premature degradation [233]. As hoped, adaSGC partially rescued maturation of a disease associated mutant [233], suggesting that adaSGC and SGL derivatives might have therapeutic potential for cystic fibrosis [234].

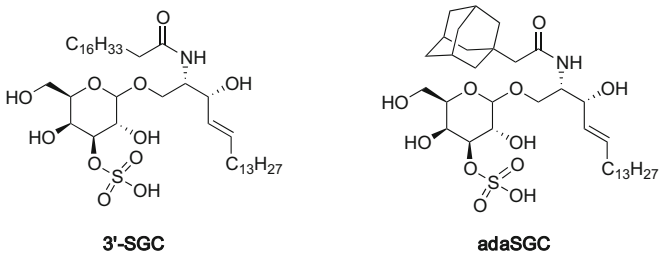


Fig. 12 Structures of 3′-sulfogalactosyl ceramide (3′-SGC) and adaSGC

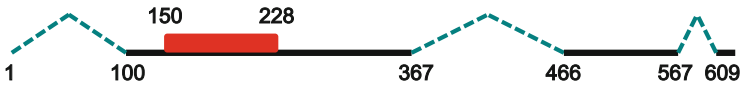


Fig. 13 Schematic of the ADD70 protein. The deleted AIF regions are depicted by *dashed lines* (in *blue*), and the Hsp70 binding region, amino acid residues 150–228 (in *red*)

6.1.5 ADD70

The first Hsp70 inhibitor to be synthesized by rational design was ADD70 [235]. In 2003, Garrido and co-workers reported that the activity of apoptosis-inducing factor (AIF), a 57-kDa mitochondrial flavoprotein that triggers apoptosis upon entering the nucleus (also see Sect. 4.3), is nullified by Hsp70 sequestration in the cytosol [236]. AIF was then shown to bind the Hsp70 SBD, and by deletion analysis amino acids 150–228 were found to be essential for efficient Hsp70 binding [235]. This information was used to synthesize a "AIF derived decoy for HSP70," or ADD70 (Fig. 13). The goal was to retain the Hsp70 binding activity of AIF without the AIF-associated cytotoxicity. Subsequent tests in mouse embryonic fibroblast cells confirmed that Hsp70 bound ADD70 without inducing Hsp70 expression [235]. Furthermore, ADD70 treatment not only activated AIF but also resensitized the cells to several apoptosis inducers [235]. Specifically, ADD70 resensitized a range of human cancer cell lines (including cervical, breast, and colon cancers) to cisplatin and staurosporine treatment [235].

In vivo testing of ADD70 in rat colon carcinoma and mice melanoma models confirmed its utility for neutralizing Hsp70 activity, as tumor growth was inhibited ~2-fold in some cases [237]. The peptide also showed additive inhibitory effects when paired with the Hsp90 inhibitor 17-AAG and with cisplatin. However, ADD70 was ineffective in immune compromised animals, suggesting that the treatment elicits an antitumor immune response [237]. Indeed, further immunohistochemical analysis of tumor cells in ADD70-treated mice revealed increased cytotoxic T-cell ($CD8^+$ T cell) infiltration (40–60%) when compared to tumors in untreated mice (0–10%) [237]. As such, the suppression of Hsp70 by ADD70 not only inhibits tumor growth but also results in tumor regression by eliciting an immune response [237, 238]. This antitumor response has also been observed

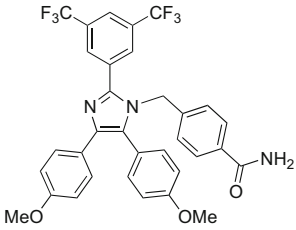


Fig. 14 The chemical structure of the imidazole derivative apoptozole

with alternate Hsp70-binding peptide aptamers that were synthesized following the success of ADD70 [239]. Together, these data highlight the potential use of peptide therapies to target Hsp70 and the utility of this approach to potentiate other anticancer therapies [237, 240].

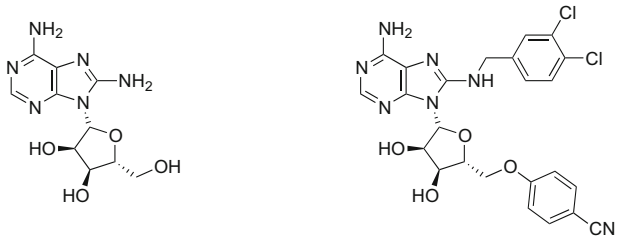
6.1.6 Apoptozole

Apoptozole (Fig. 14) was identified in 2008 as an inducer of apoptosis in a screen of 216 imidazole derivatives in a P19 embryonic carcinoma cell line [241]. Affinity chromatography was then used to uncover the apoptozole target, and cytosolic Hsc70 was identified [241]. It was further demonstrated that apoptozole exhibited high binding affinity for both stress-inducible Hsp70 and the constitutively expressed Hsc70 (K_d of 0.14 and 0.21 μM, respectively). Moreover, the pro-apoptotic activity of the compound arose from the well-established role of Hsp70 as a pro-survival factor (see Sect. 4.3). In 2010, a patent was issued that protected the use of apoptozole and encompassed the development of additional derivatives and other potential uses [242], including the treatment of Alzheimer's and Parkinson's disease, which are linked to proapoptotic phenomena (see Sect. 5) [243, 244].

Through its ability to inhibit Hsp70 activity, it was envisaged that apoptozole could also be used to treat cystic fibrosis. As noted in Sect. 1.3, the most common form of this disease results from the premature degradation of mutated CFTR, a process that requires Hsp70 [55, 245, 246]. As hypothesized, nanomolar concentrations of apoptozole rescued the defective trafficking and premature degradation of mutant CFTR by disrupting association with Hsp70 and suppressing ubiquitination, which is required for proteasome-mediated degradation [61]. Further analyses elucidated that apoptozole inhibits Hsp70 by binding to the NBD [61].

6.1.7 VER-155008, an Adenosine Analog

Since Hsp70's chaperone activity requires ATP binding and hydrolysis, Massey and co-workers examined whether small-molecule inhibitors of ATP binding could be developed as Hsp70-selective therapeutics [247]. To this end, a range of commercially available analogs was screened as ATP competitors for cytosolic Hsp70 and



8-aminoadenosine derivative
IC_{50} (Hsp70-ATP affinity) = 9.4 μM
GI_{50} (HCT116) = 0.05 μM

VER-155008
IC_{50} (Hsp70-ATP affinity) = 0.5 μM
GI_{50} (HCT116) = 5 μM

Fig. 15 Structures of adenosine-derived ATPase inhibitors of Hsp70

for activity against a human colon tumor line, HCT116. An 8-aminoadenosine derivative (Fig. 15) inhibited ATP binding with an IC_{50} of 9.4 μM, but exhibited more potent cytostatic activity against HCT116 cells (GI_{50} of 0.05 μM) [247]. The latter activity was due to alternate cytotoxic mechanisms in addition to those attributable to Hsp70 inhibition, which is consistent with the fact that the structure of the ATP-binding domain of Hsp70 is mimicked in other ATPases [67]. Based on this fact, it might be unusually difficult to develop selective inhibitors of Hsp70 based on active site ATP mimicry [22]. Nevertheless, synthetic modifications of the adenosine analog led to the identification of VER-155008 (Fig. 15), which was a more potent Hsp70 ATP/ADP binding inhibitor (IC_{50} of 0.5 μM), and had a more moderate GI_{50} (5 μM) against HCT116 cells [247].

Details of the binding interaction of these ATP-competitive inhibitors emerged when X-ray co-crystal structures of Hsc70 and the BAG1 exchange factor were obtained with four of the fifteen derivatives, including an 8-aminoadenosine derivative and VER-155008 [247]. Increased Hsp70 inhibition correlated with greater inter- and intramolecular π-stacking and H-bond interactions between aromatic substituents on the compounds and amino acids in the NBD. Later, VER-155008 and an expanded library of compounds based on the ATP-inhibitory activity of the first derivatives were tested against BiP [248] due to correlations seen between the overexpression of this chaperone and tumor cell survival [249, 250]. VER-155008 was the most potent analog in the series, suggesting that this compound is a nonspecific pan-inhibitor for several Hsp70 homologs [248]. An X-ray crystal structure obtained with the NBD of BiP revealed the ATP binding site to be more hydrophobic than in its cytosolic cousin, information that could prove valuable in the synthesis of BiP-specific inhibitors [248].

Despite its promiscuity and rapid elimination in vivo, VER-155008 represents another valuable tool compound to examine anticancer activities when Hsp70 and Hsp90 inhibitors are combined. In fact, when HCT116 colon carcinoma cells were exposed to 10 or 20 μM VER-155008 and 0.5 μM of the Hsp90 inhibitor 17-AAG, cell survival was reduced by 70 and 92%, respectively, compared to a negative control [251]. Enhanced apoptosis was reported in two multiple myeloma cell lines

when VER-155008 was combined with an Hsp90 inhibitor, NVP-AUY922 [252]. Most recently, VER-155008 was tested alone and in combination with another Hsp90 inhibitor, 17-DMAG, and as expected, synergistic proapoptotic effects were evident in an acute myeloid leukemia (AML) model [253]. These findings offer further evidence of the utility of combining Hsp70/Hsp90-targeted anticancer therapies.

6.1.8 PES and Analogs

In 2006, Gudkov and co-workers reported that 2-phenylethynesulfonamide (PES, Fig. 16) reduced the amount of p53 tumor suppressor protein bound to the mitochondrial membrane in vitro; this resulted in radioprotection of normal cells from apoptosis [254]. PES activity was attributed to the prevention of p53 accumulation in mitochondria, which would otherwise induce apoptosis [254, 255]. In contrast, PES was later found to be cytotoxic to tumor cells by targeting Hsp70 [256]. PES bound specifically to stress-induced Hsp70 in two cancer cell lines, but did not interact with Hsc70, BiP, or Hsp90. Further, tumor cell death was caused by an increase in protein aggregation and disrupted autophagy, regardless of p53 status [256]. As such, the increased production of Hsp70 targets PES to cancer cells, while its ability to reduce p53 function helps protect healthy cells from apoptotic pathways [257]. This combined effect may explain its low cytotoxicity in nontumor cells [256].

The Hsp70 inhibitory activity of PES was ultimately ascribed to the inability of PES-bound Hsp70 to interact with a number of key Hsp40 and NEF co-chaperones [256]. The Hsp70 binding site was narrowed to the SBD through a series of deletion analyses, and more recently it was ascertained that the C-terminal α-helical lid was required for PES binding; through the use of in silico docking programs, a binding pocket was also proposed [258]. The structure–activity relationship of PES was then probed using synthetic derivatives, leading to the discovery of PES-Cl (Fig. 16). PES-Cl is a more potent compound against cancer cells, and in vivo testing showed improved survival rates in a lymphoma mouse model (72% vs. 35% after 210 days, at a dose of 20 mg/kg once a week for 20 weeks) [258]. These results, paired with a growing number of positive preclinical in vitro and in vivo studies [259–261], suggest that PES and its derivatives could form the basis of novel anticancer therapies. However, the electrophilic nature of the alkynylsulfone in PES, as well as its low molecular weight, might indicate off-target effects.

Recently, PES-Cl and two unrelated compounds (MKT-077 and VER-155008) were compared for their ability to inhibit autophagy, influence the levels of Hsp90

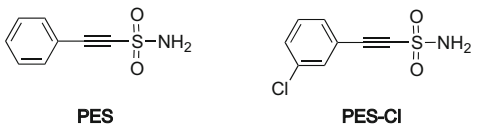


Fig. 16 Chemical structures of PES and PES-Cl

client proteins, induce cell cycle arrest, and inhibit the enzymatic activity of the anaphase-promoting complex/cyclosome (APC/C) [262]. All three were found to inhibit autophagy and cause reduced levels of Hsp90 client proteins. However, only PES-Cl was able to inhibit the APC/C and induce G2/M arrest [262].

6.1.9 Azure C, Methylene Blue, and Myricetin

In 2009, a new high-throughput screen was performed by Dickey and co-workers, which led to the identification of other modulators of Hsp70 ATPase activity [211]. In addition to the Hsp70 activator, 115-7c (discussed in Sect. 6.1.2), three inhibitors, azure c, methylene blue, and myricetin (Fig. 17) were confirmed as hits in a library of 2,800 bioactive compounds. The FDA-approved compounds inhibited human Hsp70 ATPase activity by >80% when used at a concentration of 10 μM and reduced levels of tau aggregates both in human embryonic kidney (HEK) cells and in rTg4510 transgenic mice [211].

The discovery that azure c, methylene blue, and myricetin reduced the levels of protein aggregates was surprising, as these data suggested that Hsp70 inhibitors might also temper the severity of diseases associated with protein aggregation. This phenomenon was rationalized by considering that lower ATPase activity could reduce the ability of Hsp70 to refold tau, marking Hsp70-tau complexes for degradation [211, 263]. Although the specificity of these compounds for other cellular targets is an open question, all three may serve as molecular probes. Of particular interest is the use of these compounds to study the effect of Hsp70 inhibition on an Hsp70-regulated pro-survival kinase, Akt [264].

In later work, the flavonoid myricetin was shown to bind the IB and IIB segments in the NBD of Hsp70 [265]. By binding to these domains, myricetin prevents Hsp40 binding to Hsp70, but it does so indirectly, through allostery (IC_{50} (DnaK-DnaJ) = 15 μM, 75% inhibition of DnaK–DnaJ activity at 200 μM). Therefore, myricetin is another example of an allosteric Hsp70 modulator [265], and given the profound conformational changes exhibited by Hsp70 during its reaction cycle, it is likely that many more allosteric modulators of this chaperone can be defined. In contrast, the mechanism of action of the two benzothiazines, azure c and methylene blue, remains unknown. NMR studies revealed that methylene blue

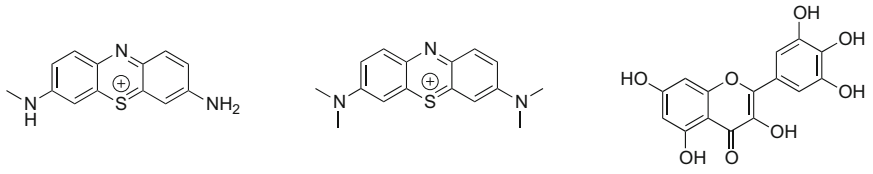


Fig. 17 Chemical structures of Hsp70 inhibitors identified in an ATPase HTS assay, with EC_{50} values

binds to the NBD of Hsc70 and its demethylated analog, azure C, is anticipated to bind in the same region [211]. Interestingly, methylene blue is currently in Phase III clinical trials for Alzheimer's disease following positive in vitro and in vivo Phase II results [266].

6.1.10 2,5′-Thiodipyrimidine Acrylamides and Related Derivatives

As our understanding of the correlation between allosteric conformational changes and the function of Hsp70 has increased, so too has the interest in potential Hsp70 disruption through allosteric inhibition. Recent work in 2013 by Chiosis and co-workers used in silico homology modeling of human Hsp70 to identify and score five previously unknown allosteric pockets [267]. They then focused on the site calculated to have highest druggability, situated in a cleft region close to the ATP binding site and surrounded by IB and IIB subregions [267, 268]. Knowledge of available binding site interactions and the presence of a free cysteine was then used to engineer compound YK5 (17a), based on the 2,5′-thiodipyrimidine scaffold (Fig. 18) [267].

Although the reactive cysteine residue (Cys267) appears to be hidden in available crystal structures, it was hypothesized that it would become accessible during Hsp70 conformational changes, enabling covalent bond formation with the acrylamide of YK5 [267]. Indeed, YK5 derivatives and 5-(phenylthio)pyrimidine acrylamides proved to be specific irreversible binders of the targeted allosteric pocket [267, 268]. The most potent inhibitor remained YK5, with IC_{50} growth inhibition of Kasumi-1 acute myeloid leukemia cells of 0.9 μM, and IC_{50} caspase-3,7 activation of 1.2 μM [268]. Further analyses suggested that downstream effects of the Hsp70 allosteric inhibition include disruption of Hsp70-HOP-Hsp90 interactions, destabilizing oncoproteins and leading to apoptosis [268].

Although treatment of cells with high concentrations of YK5 revealed no unspecific oxidation or undesired labeling of alternate cysteines [267], the presence of the reactive acrylamide moiety in these derivatives may still result in off-target effects. However, further investigations revealed that substitution of the acrylamide moiety could occur through improvement of overall enthalpy of binding of

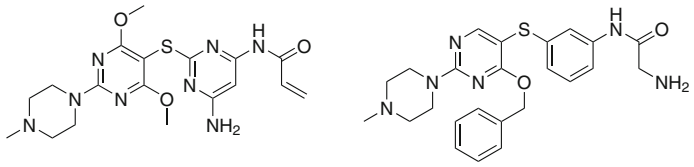


Fig. 18 Chemical structures of potent irreversible (YK5) and reversible (27c) Hsp70 allosteric inhibitors. IC_{50} values reported are against Kasumi-1 acute myeloid leukemia cancer cells

derivatives [268]. Based on resultant SAR studies, the reversible 5-(phenylthio) pyrimidine inhibitor 27c bearing an α-amino amide in place of the acrylamide was identified (Fig. 18), with Kasumi-1 acute myeloid leukemia growth inhibition ($IC_{50} = 2.3$ μM) and caspase-3,7 activation ($IC_{50} = 1.9$ μM) comparable with that of YK5 [269].

As the possibility of irreversible and reversible inhibition of Hsp70 allosteric pockets has been validated, it is not surprising that further novel interrupters of the allosteric regulation of Hsp70 are being identified. In 2014, a piperidine carboxamide compound known as HS-72 was shown to bind in an allosteric pocket in the NBD of Hsp70 [270], while more recently, the natural product Novolactone was shown to covalently modify a glutamic acid residue of the SBD of Hsp70 [271]. Given the range of allosteric pockets available, allosteric inhibition is an area of Hsp70 anticancer research that will undoubtedly continue to grow.

6.2 Indirect Hsp70 Modulation

The Hsp70 modulators discussed above are related to each other by their mechanism of action; i.e., they are known to interact directly with Hsp70, causing alterations in protein function. However, this mechanism is not the only method available to modulate Hsp70: Small molecules that affect the production of Hsp70 have also been developed in order to manipulate Hsp70 levels and thus its function in the cell [272, 273]. In cancer, inhibiting Hsp70 expression is advantageous, whereas in some neurodegenerative diseases, increasing Hsp70 levels by activating the heat shock response is of benefit. This is evidenced, for example, when the heat shock activator arimoclomol, an amyotrophic lateral sclerosis (ALS) drug candidate, is administered to transgenic mice with motor neuron disease, improving muscle function and survival of these animals [274, 275].

As described in Sect. 3.1, stress-induced heat shock protein transcription in cells is controlled by HSF1, which when activated binds to a promoter upstream of each of the genes that encode the major HSPs. This allows for the rapid transcription and translation of the gene products [99, 100]. Although the mechanism of HSF1 gene activation remains uncertain [100], HSF1 activation has been linked to cancer and fulfills seven of eight cancer hallmarks considered necessary for tumorigenesis [272]. The inhibition or activation of HSF1 could be considered a dubious target, as it affects more than one HSP, but alternatively this could prove advantageous given the redundant functions exhibited among many of the HSPs, especially with regard to disease onset [272].

6.2.1 Quercetin

In 1990, quercetin (Fig. 19), a flavonoid closely related to myricetin, became one of the first compounds shown to inhibit HSP synthesis under heat shock conditions,

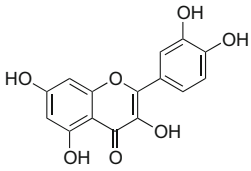


Fig. 19 Structure of the HSF1 modulator quercetin

albeit at a high concentration (~100 μM) [276]. The observed suppression was explained in 1992, when quercetin was revealed to act upon HSF1, thereby inhibiting thermotolerance in a human colon carcinoma-derived cell line [277, 278].

The effect of quercetin on Hsp70 was defined by Santoro and co-workers when it was shown to block both transcription and translation of Hsp70 in human erythroleukemia cells that were pretreated with prostaglandin A1 to induce thermotolerance [279]. Despite these positive results, quercetin is not selective in its suppression of HSF1 cellular levels [280] as inhibition of many other pathways, such as glycolysis and protein kinase activity, has also been reported for this compound and related analogs [281, 282]. More recently, quercetin was shown to bind IRE1 [283], which is an ER resident kinase that induces the unfolded protein response (UPR) and is also associated with cancer cell survival [284]. The wide range of activities of this molecule may be further related to the rapid oxidative conversion in vivo, a factor which decreases its biological utility [285]. Regardless, quercetin remains a useful tool to uncover the effects of HSF1 modulation in cell lines [286].

6.2.2 KNK437

KNK437 (Fig. 20) was identified as a dose-dependent inhibitor of the induction of various HSPs, including Hsp70, in 2000 [287]. Its ability to block the acquisition of thermotolerance was greater than that observed with quercetin, and in agreement with this result, a more substantial inhibition of HSP mRNA synthesis was observed [287]. KNK437 is also considered to be less promiscuous than quercetin but could possibly be a formylating agent [287].

Pretreatment of murine tumors with KNK437 inhibited Hsp70 induction upon heat stress and modulated thermotolerance (200 mg/kg administered by intraperitoneal injection 6 h before heat treatment) [288]. This, in turn, led to apoptosis in carcinoma cell lines [289]. The mechanism of action of KNK437 is believed to differ from that of quercetin in that the binding between HSF1 and a transcriptional enhancer upstream of most HSPs is compromised [289]. KNK437 and its derivatives could also be of particular value in sensitizing cancer cells to Hsp90 inhibitors, as it sensitizes leukemia cells to apoptosis in the presence of 17-AAG ([KNK437] = 400 μmol/L, [17-AAG] = 2 μmol/L led to 65% apoptosis of HL-60 cells compared to 40% with 17-AAG alone), downregulating 17-AAG induction of Hsp70 (by 56%). [44].

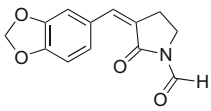


Fig. 20 Structure of KNK437, an inhibitor of HSF1 function

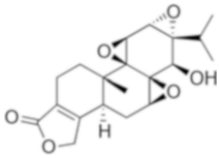


Fig. 21 Structure of triptolide

6.2.3 Triptolide

Triptolide, a diterpene triepoxide (Fig. 21), is a bioactive compound found in *Tripterygium*, a plant used in Chinese herbal remedies for the treatment of immune inflammatory diseases such as asthma and arthritis [290]. In 2006, triptolide was reported to reversibly block HSF1 activation and inhibit the heat shock response and the cytoprotective response in HeLa cells, which ultimately led to cell death [291]. These findings suggested that triptolide might sensitize cancer cells to natural antitumor responses. Consistent with this model, administration of triptolide (0.2 mg/kg for 60 days) decreased pancreatic tumor growth (tumor volume <0.2 cm^3 compared to ~1.0 cm^3 for the control mice) and metastasis (seen in 88% of control mice vs. 13% in triptolide treated) by inhibiting Hsp70 induction [292]. Hsp70 inhibition was also shown to play a key role in triptolide's ability to initiate neuroblastoma cell death in mice models [293].

A recent study on experimental autoimmune encephalomyelitis, the animal model for multiple sclerosis, revealed that disease onset was delayed following oral triptolide treatment and that inflammation of central nervous system (CNS) tissue was suppressed [294]. Ironically, there was an increase in Hsp70 mRNA and protein levels in the CNS. It is unclear whether this anomaly reflects a unique feature of the animal model, a peculiarity of how the CNS responds to the compound, or a consequence of the dosing regimen. It should be noted that the three epoxide functions in triptolide constitute potentially powerful electrophiles in biological environments, even though their high degree of substitution undoubtedly serves to moderate their reactivity.

6.2.4 Triazole Nucleosides

A recent example of a new class of HSF1 modulators was provided by Peng and co-workers who modified a triazole nucleoside scaffold that was previously found to downregulate the expression of another HSF1 target, Hsp27 [295, 296]. After synthesizing variants, a compound with greater antiproliferative activity was obtained (Fig. 22). The modest antiproliferative activity seen against two drug-

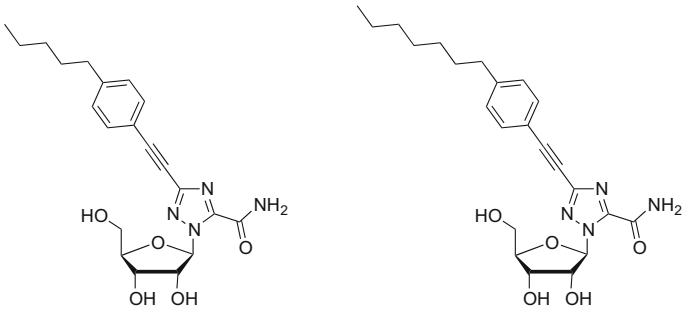


Fig. 22 Structures of original triazole nucleoside lead and a subsequent, more potent analog

resistant pancreatic cancer cell lines, MiaPaCa-2 and Panc-1, was attributed to HSF1 inhibition [295]. Further evidence for HSF1 as a target was obtained from downregulation of Hsp27, Hsp70, and Hsp90 in the same cell lines [295]. This was confirmed in vivo, as apoptosis of human pancreatic cancer cells in tumor-xenografted mice and a reduced tumor burden were observed [295].

As in the examples provided above, it remains unclear if the coordinated inhibition of the heat shock response will prove superior to the inhibition of a single chaperone, such as Hsp70. However, it is worth noting that the downstream targets of HSF1 are significantly more diverse in some cancers, and this response pathway is particularly important when considering several cancer malignancies [297]. Therefore, inhibition of a "master regulator" of the heat shock response may prove quite valuable in some anticancer therapies.

7 Conclusions

The available biological data clearly demonstrate that Hsp70 is an important therapeutic target for a range of human diseases [298]. In this chapter, we have provided an overview of the structure and function of this ubiquitous chaperone. Hsp70 participates in a complex reaction cycle and interacts with a range of co-chaperones. Many of the mechanistic details underlying Hsp70's action have only been realized in recent years, but an increasing number of chemical probe molecules are now at our disposal to interrogate in vitro and in vivo pathways involving this chaperone. The complexity of the reaction cycle and the breadth of dynamic partners provide a wealth of targets that can be coopted to either compromise or enhance Hsp70 function. Small-molecule Hsp70 modulators exhibit a variety of effects: Inhibitors can bind to different domains or motifs in Hsp70 and directly block function and catalytic cycle, or they can thwart the association of co-chaperones. They can even act as allosteric modulators, and some enhance the catalytic turnover activity of the chaperone. Other compounds act by regulating the

inducible expression of Hsp70. As a result of this emerging toolbox, we anticipate that there will be a significant portfolio of new Hsp70-directed therapies emerging in the coming years.

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References

1. Ensembl (2013) Human assembly and gene annotation. http://Jan2013.archive.ensembl.org/Homo_sapiens/Info/Annotation/. Accessed 20 Feb 2013
2. Flicek P, Amode MR, Barrell D et al (2012) Ensembl 2012. Nucleic Acids Res 40:D84–D90
3. Walsh CT, Garneau-Tsodikova S, Gatto GJ (2005) Protein posttranslational modifications: the chemistry of proteome diversifications. Angew Chem Int Ed 44:7342–7372
4. Marko-Varga G, Omenn GS, Paik Y-K et al (2013) A first step toward completion of a genome-wide characterization of the human proteome. J Proteome Res 12:1–5
5. Fulda S, Gorman AM, Hori O et al (2010) Cellular stress responses: cell survival and cell death. Int J Cell Biol 2010:214074. doi:10.1155/2010/214074
6. Chaudhuri TK, Paul S (2006) Protein-misfolding diseases and chaperone-based therapeutic approaches. FEBS J 273:1331–1349
7. Rutkowski DT, Hegde RS (2010) Regulation of basal cellular physiology by the homeostatic unfolded protein response. J Cell Biol 189:783–794
8. Ellis J (1987) Proteins as molecular chaperones. Nature 328:378–379
9. Lee AS (1987) Coordinated regulation of a set of genes by glucose and calcium ionophores in mammalian cells. Trends Biochem Sci 12:20–23
10. Frydman J (2001) Folding of newly translated proteins *in vivo*: the role of molecular chaperones. Annu Rev Biochem 70:603
11. Hartl FU, Hayer-Hartl M (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 295:1852–1858
12. McClellan AJ, Frydman J (2001) Molecular chaperones and the art of recognizing a lost cause. Nat Cell Biol 3:E51
13. Jolly C, Morimoto RI (2000) Role of the heat shock response and molecular chaperones in oncogenesis and cell death. J Natl Cancer Inst 92:1564–1572
14. Pelham HRB (1986) Speculations on the functions of the major heat shock and glucose-regulated proteins. Cell 46:959–961
15. Yao T-P (2010) The role of ubiquitin in autophagy-dependent protein aggregate processing. Genes Cancer 1:779–786
16. Gamerdinger M, Carra S, Behl C (2011) Emerging roles of molecular chaperones and co-chaperones in selective autophagy: focus on BAG proteins. J Mol Med 89:1175–1182
17. Kaushik S, Bandyopadhyay U, Sridhar S et al (2011) Chaperone-mediated autophagy at a glance. J Cell Sci 124:495–499
18. Powers MV, Workman P (2007) Inhibitors of the heat shock response: biology and pharmacology. FEBS Lett 581:3758–3769
19. Calderwood SK, Stevenson MA, Murshid A (2012) Heat shock proteins, autoimmunity, and cancer treatment. Autoimmune Dis 2012:10
20. Murphy ME (2013) The HSP70 family and cancer. Carcinogenesis 34:1181–1188
21. Sherman MY, Gabai VL (2014) Hsp70 in cancer: back to the future. Oncogene. doi:10.1038/onc.2014.349

22. Massey AJ (2010) ATPases as drug targets: insights from heat shock proteins 70 and 90. J Med Chem 53:7280–7286
23. Yamaki H, Nakajima M, Shimotohno KW et al (2011) Molecular basis for the actions of Hsp90 inhibitors and cancer therapy. J Antibiot (Tokyo) 64:635–644
24. Whitesell L, Lindquist SL (2005) HSP90 and the chaperoning of cancer. Nat Rev Cancer 5: 761–772
25. Neckers L (2007) Heat shock protein 90: the cancer chaperone. J Biosci 32:517–530
26. Patel HJ, Modi S, Chiosis G et al (2011) Advances in the discovery and development of heat-shock protein 90 inhibitors for cancer treatment. Expert Opin Drug Discov 6:559–587
27. Zhang H, Burrows F (2004) Targeting multiple signal transduction pathways through inhibition of Hsp90. J Mol Med 82:488–499
28. Wayne N, Mishra P, Bolon DN (2011) Hsp90 and client protein maturation. In: Calderwood SK, Prince TL (eds) Molecular chaperones: methods and protocols, vol 787, Methods in molecular biology. Springer, New York, pp 33–44
29. Pearl LH, Prodromou C (2006) Structure and mechanism of the Hsp90 molecular chaperone machinery. Annu Rev Biochem 75:271–294
30. DeBoer C, Meulman PA, Wnuk RJ et al (1970) Geldanamycin, a new antibiotic. J Antibiot (Tokyo) 23:442–447
31. Stebbins CE, Russo AA, Schneider C et al (1997) Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. Cell 89:239–250
32. Whitesell L, Mimnaugh EG, De Costa B et al (1994) Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. Proc Natl Acad Sci U S A 91:8324–8328
33. Den RB, Lu B (2012) Heat shock protein 90 inhibition: rationale and clinical potential. Ther Adv Med Oncol 4:211–218
34. Whitesell L, Lin NU (2012) HSP90 as a platform for the assembly of more effective cancer chemotherapy. BBA Mol Cell Res 1823:756–766
35. Solit DB, Basso AD, Olshen AB et al (2003) Inhibition of heat shock protein 90 function down-regulates Akt kinase and sensitizes tumors to taxol. Cancer Res 63:2139–2144
36. Kamal A, Thao L, Sensintaffar J et al (2003) A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. Nature 425:407–410
37. García-Morales P, Carrasco-García E, Ruiz-Rico P et al (2007) Inhibition of Hsp90 function by ansamycins causes downregulation of cdc2 and cdc25c and G2/M arrest in glioblastoma cell lines. Oncogene 26:7185–7193
38. Wang Y, Trepel JB, Neckers LM et al (2010) STA-9090, a small-molecule Hsp90 inhibitor for the potential treatment of cancer. Curr Opin Investig Drugs 11:1466–1476
39. Brough PA, Aherne W, Barril X et al (2007) 4,5-Diarylisoxazole Hsp90 chaperone inhibitors: potential therapeutic agents for the treatment of cancer. J Med Chem 51:196–218
40. Eccles SA, Massey A, Raynaud FI et al (2008) NVP-AUY922: a novel heat shock protein 90 inhibitor active against xenograft tumor growth, angiogenesis, and metastasis. Cancer Res 68:2850–2860
41. Garon EB, Finn RS, Hamidi H et al (2013) The HSP90 inhibitor NVP-AUY922 potently inhibits non–small cell lung cancer growth. Mol Cancer Ther 12:890–900
42. Gallegos Ruiz MI, Floor K, Roepman P et al (2008) Integration of gene dosage and gene expression in non-small cell lung cancer, identification of HSP90 as potential target. PLoS One 3:e0001722
43. Pacey S, Wilson RH, Walton M et al (2011) A phase I study of the heat shock protein 90 inhibitor alvespimycin (17-DMAG) given intravenously to patients with advanced solid tumors. Clin Cancer Res 17:1561–1570
44. Guo F, Rocha K, Bali P et al (2005) Abrogation of heat shock protein 70 induction as a strategy to increase antileukemia activity of heat shock protein 90 inhibitor 17-allylamino-demethoxy geldanamycin. Cancer Res 65:10536–10544

45. Cui XB, Yu ZY, Wang W et al (2012) Co-inhibition of HSP70/HSP90 synergistically sensitizes nasopharyngeal carcinoma cells to thermotherapy. Integr Cancer Ther 11:61–67
46. Goloudina AR, Demidov ON, Garrido C (2012) Inhibition of HSP70: a challenging anti-cancer strategy. Cancer Lett 325:117–124
47. Brodsky JL, Chiosis G (2006) Hsp70 molecular chaperones: emerging roles in human disease and identification of small molecule modulators. Curr Top Med Chem 6:1215–1225
48. Buck TM, Wright CM, Brodsky JL (2007) The activities and function of molecular chaperones in the endoplasmic reticulum. Semin Cell Dev Biol 18:751–761
49. Bukau B, Weissman J, Horwich A (2006) Molecular chaperones and protein quality control. Cell 125:443–451
50. Fewell SW, Travers KJ, Weissman JS et al (2001) The action of molecular chaperones in the early secretory pathway. Annu Rev Genet 35:149–191
51. Mayer MP, Bukau B (2005) Hsp70 chaperones: cellular functions and molecular mechanism. Cell Mol Life Sci 62:670–684
52. Massey AC, Zhang C, Cuervo AM (2006) Chaperone-mediated autophagy in aging and disease. Curr Top Dev Biol 73:205–235
53. Evans CG, Wisen S, Gestwicki JE (2006) Heat shock proteins 70 and 90 inhibit early stages of amyloid beta-(1-42) aggregation *in vitro*. J Biol Chem 281:33182–33191
54. Kirkegaard T, Roth AG, Petersen NH et al (2010) Hsp70 stabilizes lysosomes and reverts Niemann-Pick disease-associated lysosomal pathology. Nature 463:549–553
55. Brodsky JL (2001) Chaperoning the maturation of the cystic fibrosis transmembrane conductance regulator. Am J Physiol Lung Cell Mol Physiol 281:L39–L42
56. Turturici G, Sconzo G, Geraci F (2011) Hsp70 and its molecular role in nervous system diseases. Biochem Res Int. doi:10.1155/2011/618127
57. Abisambra J, Jinwal UK, Miyata Y et al (2013) Allosteric heat shock protein 70 inhibitors rapidly rescue synaptic plasticity deficits by reducing aberrant tau. Biol Psychiatry. doi:10.1016/j.biopsych.2013.02.027
58. Carmichael J, Chatellier J, Woolfson A et al (2000) Bacterial and yeast chaperones reduce both aggregate formation and cell death in mammalian cell models of Huntington's disease. Proc Natl Acad Sci U S A 97:9701–9705
59. Jana NR, Tanaka M, Wang G-h et al (2000) Polyglutamine length-dependent interaction of Hsp40 and Hsp70 family chaperones with truncated N-terminal huntingtin: their role in suppression of aggregation and cellular toxicity. Hum Mol Genet 9:2009–2018
60. Witt SN (2010) Hsp70 molecular chaperones and Parkinson's disease. Biopolymers 93:218–228
61. Cho HJ, Gee HY, Baek K-H et al (2011) A small molecule that binds to an ATPase domain of Hsc70 promotes membrane trafficking of mutant cystic fibrosis transmembrane conductance regulator. J Am Chem Soc 133:20267–20276
62. Boorstein WR, Ziegelhoffer T, Craig EA (1994) Molecular evolution of the HSP70 multigene family. J Mol Evol 38:1–17
63. Rensing SA, Maier UG (1994) Phylogenetic analysis of the stress-70 protein family. J Mol Evol 39:80–86
64. Daugaard M, Rohde M, Jäättelä M (2007) The heat shock protein 70 family: highly homologous proteins with overlapping and distinct functions. FEBS Lett 581:3702–3710
65. Hunt C, Morimoto RI (1985) Conserved features of eukaryotic hsp70 genes revealed by comparison with the nucleotide sequence of human hsp70. Proc Natl Acad Sci U S A 82:6455–6459
66. Bardwell JC, Craig EA (1984) Major heat shock gene of Drosophila and the *Escherichia coli* heat-inducible dnaK gene are homologous. Proc Natl Acad Sci U S A 81:848–852
67. Flaherty KM, DeLuca-Flaherty C, McKay DB (1990) Three-dimensional structure of the ATPase fragment of a 70k heat-shock cognate protein. Nature 346:623–628
68. Normington K, Kohno K, Kozutsumi Y et al (1989) *S. cerevisiae* encodes an essential protein homologous in sequence and function to mammalian BiP. Cell 57:1223–1236

69. Zhu X, Zhao X, Furkholder WF et al (1996) Structural analysis of substrate binding by the molecular chaperone DnaK. Science 272:1606–1614
70. Freeman BC, Myers MP, Schumacher R et al (1995) Identification of a regulatory motif in Hsp70 that affects ATPase activity, substrate binding and interaction with HDJ-1. EMBO J 14:2281–2292
71. Allan RK, Ratajczak T (2011) Versatile TPR domains accommodate different modes of target protein recognition and function. Cell Stress Chaperones 16:353–367
72. Scheufler C, Brinker A, Bourenkov G et al (2000) Structure of TPR domain–peptide complexes: critical elements in the assembly of the Hsp70–Hsp90 multichaperone machine. Cell 101:199–210
73. Zhuravleva A, Clerico EM, Gierasch LM (2012) An interdomain energetic tug-of-war creates the allosterically active state in Hsp70 molecular chaperones. Cell 151:1296–1307
74. Cyr DM (2008) Swapping nucleotides, tuning Hsp70. Cell 133:945–947
75. Kityk R, Kopp J, Sinning I et al (2012) Structure and dynamics of the ATP-bound open conformation of Hsp70 chaperones. Mol Cell 48:863–874
76. Swain JF, Dinler G, Sivendran R et al (2007) Hsp70 chaperone ligands control domain association via an allosteric mechanism mediated by the interdomain linker. Mol Cell 26: 27–39
77. Zhang Y, Zuiderweg ERP (2004) The 70-kDa heat shock protein chaperone nucleotide-binding domain in solution unveiled as a molecular machine that can reorient its functional subdomains. Proc Natl Acad Sci U S A 101:10272–10277
78. Pellecchia M, Montgomery DL, Stevens SY et al (2000) Structural insights into substrate binding by the molecular chaperone DnaK. Nat Struct Biol 7:298–303
79. Slepenkov SV, Witt SN (2002) Kinetic analysis of interdomain coupling in a lidless variant of the molecular chaperone DnaK: DnaK's lid inhibits transition to the low affinity state. Biochemistry 41:12224–12235
80. Qi R, Sarbeng EB, Liu Q et al (2013) Allosteric opening of the polypeptide-binding site when an Hsp70 binds ATP. Nat Struct Mol Biol 20:900–907
81. Bertelsen EB, Chang L, Gestwicki JE et al (2009) Solution conformation of wild-type *E. coli* Hsp70 (DnaK) chaperone complexed with ADP and substrate. Proc Natl Acad Sci U S A 106: 8471–8476
82. Kelley WL (1998) The J-domain family and the recruitment of chaperone power. Trends Biochem Sci 23:222–227
83. Laufen T, Mayer MP, Beisel C et al (1999) Mechanism of regulation of hsp70 chaperones by DnaJ cochaperones. Proc Natl Acad Sci U S A 96:5452–5457
84. Jiang J, Maes EG, Taylor AB et al (2007) Structural basis of J cochaperone binding and regulation of Hsp70. Mol Cell 28:422–433
85. Suh W-C, Burkholder WF, Lu CZ et al (1998) Interaction of the Hsp70 molecular chaperone, DnaK, with its cochaperone DnaJ. Proc Natl Acad Sci U S A 95:15223–15228
86. Gässler CS, Buchberger A, Laufen T et al (1998) Mutations in the DnaK chaperone affecting interaction with the DnaJ cochaperone. Proc Natl Acad Sci U S A 95:15229–15234
87. Kampinga HH, Craig EA (2010) The HSP70 chaperone machinery: J proteins as drivers of functional specificity. Nat Rev Mol Cell Biol 11:579–592
88. Kota P, Summers DW, Ren HY et al (2009) Identification of a consensus motif in substrates bound by a Type I Hsp40. Proc Natl Acad Sci U S A 106:11073–11078
89. Craig EA, Huang P, Aron R et al (2006) The diverse roles of J-proteins, the obligate Hsp70 co-chaperone. In: Amara SG, Bamberg E, Grinstein S et al (eds) Reviews of physiology, biochemistry and pharmacology, vol 156. Springer, Berlin, pp 1–21. doi:10.1007/s10254-005-0001-0
90. Schroder H, Langer T, Hartl FU et al (1993) DnaK, DnaJ and GrpE form a cellular chaperone machinery capable of repairing heat-induced protein damage. EMBO J 12:4137–4144
91. Polier S, Dragovic Z, Hartl FU et al (2008) Structural basis for the cooperation of Hsp70 and Hsp110 chaperones in protein folding. Cell 133:1068–1079

92. Tzankov S, Wong MJH, Shi K et al (2008) Functional divergence between co-chaperones of Hsc70. J Biol Chem 283:27100–27109
93. Shaner L, Morano KA (2007) All in the family: atypical Hsp70 chaperones are conserved modulators of Hsp70 activity. Cell Stress Chaperones 12:1–8
94. Schuermann JP, Jiang J, Cuellar J et al (2008) Structure of the Hsp110:Hsc70 nucleotide exchange machine. Mol Cell 31:232–243
95. Brocchieri L, Conway dME, Macario AJL (2008) Hsp70 genes in the human genome: conservation and differentiation patterns predict a wide array of overlapping and specialized functions. BMC Evol Biol 8:19
96. Wu B, Hunt C, Morimoto R (1985) Structure and expression of the human gene encoding major heat shock protein HSP70. Mol Cell Biol 5:330–341
97. Tavaria M, Gabriele T, Kola I et al (1996) A hitchhiker's guide to the human Hsp70 family. Cell Stress Chaperones 1:23–28
98. Hageman J, Kampinga HH (2009) Computational analysis of the human HSPH/HSPA/DNAJ family and cloning of a human HSPH/HSPA/DNAJ expression library. Cell Stress Chaperones 14:1–21
99. Wu C (1995) Heat shock transcription factors: structure and regulation. Annu Rev Cell Dev Biol 11:441–469
100. Calderwood SK, Xie Y, Wang X et al (2010) Signal transduction pathways leading to heat shock transcription. Sign Transduct Insights 2:13–24
101. Hunt CR, Dix DJ, Sharma GG et al (2004) Genomic instability and enhanced radiosensitivity in Hsp70.1- and Hsp70.3-deficient mice. Mol Cell Biol 24:899–911
102. Werner-Washburne M, Becker J, Kosic-Smithers J et al (1989) Yeast Hsp70 RNA levels vary in response to the physiological status of the cell. J Bacteriol 171:2680–2688
103. Werner-Washburne M, Stone DE, Craig EA (1987) Complex interactions among members of an essential subfamily of hsp70 genes in Saccharomyces cerevisiae. Mol Cell Biol 7: 2568–2577
104. Calloni G, Chen T, Schermann Sonya M et al (2012) DnaK functions as a central hub in the *E. coli* chaperone network. Cell Rep 1:251–264
105. Leung TK, Rajendran MY, Monfries C et al (1990) The human heat-shock protein family. Expression of a novel heat-inducible HSP70 (HSP70B') and isolation of its cDNA and genomic DNA. Biochem J 267:125–132
106. Parsian AJ, Sheren JE, Tao TY et al (2000) The human Hsp70B gene at the HSPA7 locus of chromosome 1 is transcribed but non-functional. BBA Gene Struct Exp 1494:201–205
107. Su AI, Wiltshire T, Batalov S et al (2004) A gene atlas of the mouse and human protein-encoding transcriptomes. Proc Natl Acad Sci U S A 101:6062–6067
108. Liu T, Daniels CK, Cao S (2012) Comprehensive review on the HSC70 functions, interactions with related molecules and involvement in clinical diseases and therapeutic potential. Pharmacol Ther 136:354–374
109. Dworniczak B, Mirault ME (1987) Structure and expression of a human gene coding for a 71 kd heat shock 'cognate' protein. Nucleic Acids Res 15:5181–5197
110. Huang L, Mivechi NF, Moskophidis D (2001) Insights into regulation and function of the major stress-induced hsp70 molecular chaperone *in vivo*: analysis of mice with targeted gene disruption of the hsp70.1 or hsp70.3 gene. Mol Cell Biol 21:8575–8591
111. Florin L, Becker KA, Sapp C et al (2004) Nuclear translocation of papillomavirus minor capsid protein L2 requires Hsc70. J Virol 78:5546–5553
112. Bonnycastle LLC, Yu C-E, Hunt CR et al (1994) Cloning, sequencing, and mapping of the human chromosome 14 heat shock protein gene (HSPA2). Genomics 23:85–93
113. Dix DJ, Allen JW, Collins BW et al (1996) Targeted gene disruption of Hsp70-2 results in failed meiosis, germ cell apoptosis, and male infertility. Proc Natl Acad Sci U S A 93: 3264–3268
114. Munro S, Pelham HRB (1986) An hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. Cell 46:291–300

115. Munro S, Pelham HRB (1987) A C-terminal signal prevents secretion of luminal ER proteins. Cell 48:899–907
116. Haas IG (1994) BiP (GRP78), an essential hsp70 resident protein in the endoplasmic reticulum. Experientia 50:1012–1020
117. Consortium TU (2012) Reorganizing the protein space at the Universal Protein Resource (UniProt). Nucleic Acids Res 40:D71–D75
118. Bhattacharyya T, Karnezis AN, Murphy SP et al (1995) Cloning and subcellular localization of human mitochondrial hsp70. J Biol Chem 270:1705–1710
119. Mizzen LA, Chang C, Garrels JI et al (1989) Identification, characterization, and purification of two mammalian stress proteins present in mitochondria, grp 75, a member of the hsp 70 family and hsp 58, a homolog of the bacterial groEL protein. J Biol Chem 264: 20664–20675
120. Deocaris CC, Kaul SC, Wadhwa R (2006) On the brotherhood of the mitochondrial chaperones mortalin and heat shock protein 60. Cell Stress Chaperones 11:116–128
121. Omura T (1998) Mitochondria-targeting sequence, a multi-role sorting sequence recognized at all steps of protein import into mitochondria. J Biochem 123:1010–1016
122. Craig EA, Kramer J, Shilling J et al (1989) SSC1, an essential member of the yeast HSP70 multigene family, encodes a mitochondrial protein. Mol Cell Biol 9:3000–3008
123. Bukau B, Deuerling E, Pfund C et al (2000) Getting newly synthesized proteins into shape. Cell 101:119–122
124. Hartl FU, Bracher A, Hayer-Hartl M (2011) Molecular chaperones in protein folding and proteostasis. Nature 475:324–332
125. Eichmann C, Preissler S, Riek R et al (2010) Cotranslational structure acquisition of nascent polypeptides monitored by NMR spectroscopy. Proc Natl Acad Sci U S A 107:9111–9116
126. Elcock AH (2006) Molecular simulations of cotranslational protein folding: fragment stabilities, folding cooperativity, and trapping in the ribosome. PLoS Comput Biol 2:e98
127. Slepenkov SV, Witt SN (2002) The unfolding story of the *Escherichia coli* Hsp70 DnaK: is DnaK a holdase or an unfoldase? Mol Microbiol 45:1197–1206
128. Lu Z, Cyr DM (1998) The conserved carboxyl terminus and zinc finger-like domain of the co-chaperone Ydj1 assist Hsp70 in protein folding. J Biol Chem 273:5970–5978
129. Yan W, Schilke B, Pfund C et al (1998) Zuotin, a ribosome-associated DnaJ molecular chaperone. EMBO J 17:4809–4817
130. Höhfeld J, Minami Y, Hartl F-U (1995) Hip, a novel cochaperone involved in the eukaryotic hsc70/hsp40 reaction cycle. Cell 83:589–598
131. Hohfeld J, Jentsch S (1997) GrpE-like regulation of the hsc70 chaperone by the anti-apoptotic protein BAG-1. EMBO J 16:6209–6216
132. Bukau B, Horwich AL (1998) The Hsp70 and Hsp60 chaperone machines. Cell 92:351–366
133. Sigler PB, Xu Z, Rye HS et al (1998) Structure and function in GroEL-mediated protein-folding. Annu Rev Biochem 67:581
134. Cuellar J, Martin-Benito J, Scheres SH et al (2008) The structure of CCT-Hsc70 NBD suggests a mechanism for Hsp70 delivery of substrates to the chaperonin. Nat Struct Mol Biol 15:858–864
135. Rosenzweig R, Moradi S, Zarrine-Afsar A et al (2013) Unraveling the mechanism of protein disaggregation through a ClpB-DnaK interaction. Science 339:1080–1083
136. Glover JR, Lindquist S (1998) Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. Cell 94:73–82
137. Seyffer F, Kummer E, Oguchi Y et al (2012) Hsp70 proteins bind Hsp100 regulatory M domains to activate AAA+ disaggregase at aggregate surfaces. Nat Struct Mol Biol 19: 1347–1355
138. Goloubinoff P, Mogk A, Zvi APB et al (1999) Sequential mechanism of solubilization and refolding of stable protein aggregates by a bichaperone network. Proc Natl Acad Sci U S A 96:13732–13737

139. Ben-Zvi A, De Los Rios P, Dietler G et al (2004) Active solubilization and refolding of stable protein aggregates by cooperative unfolding action of individual Hsp70 chaperones. J Biol Chem 279:37298–37303
140. Diamant S, Ben-Zvi AP, Bukau B et al (2000) Size-dependent disaggregation of stable protein aggregates by the DnaK chaperone machinery. J Biol Chem 275:21107–21113
141. Rampelt H, Kirstein-Miles J, Nillegoda NB et al (2012) Metazoan Hsp70 machines use Hsp110 to power protein disaggregation. EMBO J 31:4221
142. Shi Y, Thomas JO (1992) The transport of proteins into the nucleus requires the 70-kilodalton heat shock protein or its cytosolic cognate. Mol Cell Biol 12:2186–2192
143. Pratt WB, Toft DO (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr Rev 18:306–360
144. Patterson C, Höhfeld J (2008) Molecular chaperones and the ubiquitin–proteasome system. Protein science encyclopedia. Wiley-VCH, Weinheim, pp 1–30. doi:10.1002/9783527610754.dd03
145. Hernández MP, Sullivan WP, Toft DO (2002) The assembly and intermolecular properties of the hsp70-Hop-hsp90 molecular chaperone complex. J Biol Chem 277:38294–38304
146. Pratt WB, Toft DO (2003) Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. Exp Biol Med 228:111–133
147. Terlecky SR, Chiang HL, Olson TS et al (1992) Protein and peptide binding and stimulation of in vitro lysosomal proteolysis by the 73-kDa heat shock cognate protein. J Biol Chem 267:9202–9209
148. McDonough H, Patterson C (2003) CHIP: a link between the chaperone and proteasome systems. Cell Stress Chaperones 8:303–308
149. Ballinger CA, Connell P, Wu Y et al (1999) Identification of CHIP, a novel tetratricopeptide repeat-containing protein that interacts with heat shock proteins and negatively regulates chaperone functions. Mol Cell Biol 19:4535–4545
150. Jiang J, Ballinger CA, Wu Y et al (2001) CHIP Is a U-box-dependent E3 ubiquitin ligase: identification of Hsc70 as a target for ubiquitylation. J Biol Chem 276:42938–42944
151. Demand J, Alberti S, Patterson C et al (2001) Cooperation of a ubiquitin domain protein and an E3 ubiquitin ligase during chaperone/proteasome coupling. Curr Biol 11:1569–1577
152. Lüders J, Demand J, Höhfeld J (2000) The ubiquitin-related BAG-1 provides a link between the molecular chaperones Hsc70/Hsp70 and the proteasome. J Biol Chem 275:4613–4617
153. Mosser DD, Caron AW, Bourget L et al (2000) The chaperone function of hsp70 is required for protection against stress-induced apoptosis. Mol Cell Biol 20:7146–7159
154. Evans CG, Chang L, Gestwicki JE (2010) Heat shock protein 70 (Hsp70) as an emerging drug target. J Med Chem 53:4585–4602
155. Elmore S (2007) Apoptosis: a review of programmed cell death. Toxicol Pathol 35:495–516
156. Salomoni P, Khelifi AF (2006) Daxx: death or survival protein? Trends Cell Biol 16:97–104
157. Dhanasekaran DN, Reddy EP (2008) JNK signaling in apoptosis. Oncogene 27:6245–6251
158. Korsmeyer SJ, Wei MC, Saito M et al (2000) Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. Cell Death Differ 7:1166–1173
159. Schmitt E, Gehrmann M, Brunet M et al (2007) Intracellular and extracellular functions of heat shock proteins: repercussions in cancer therapy. J Leukoc Biol 81:15–27
160. Zorzi E, Bonvini P (2011) Inducible Hsp70 in the regulation of cancer cell survival: analysis of chaperone induction, expression and activity. Cancer 3:3921–3956
161. Stankiewicz AR, Lachapelle G, Foo CPZ et al (2005) Hsp70 inhibits heat-induced apoptosis upstream of mitochondria by preventing Bax translocation. J Biol Chem 280:38729–38739
162. Ruchalski K, Mao H, Li Z et al (2006) Distinct hsp70 domains mediate apoptosis-inducing factor release and nuclear accumulation. J Biol Chem 281:7873–7880
163. Beere HM, Wolf BB, Cain K et al (2000) Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. Nat Cell Biol 2:469–475

164. Saleh A, Srinivasula SM, Balkir L et al (2000) Negative regulation of the Apaf-1 apoptosome by Hsp70. Nat Cell Biol 2:476–483
165. Li C-Y, Lee J-S, Ko Y-G et al (2000) Heat shock protein 70 inhibits apoptosis downstream of cytochrome c release and upstream of caspase-3 activation. J Biol Chem 275:25665–25671
166. Jäättelä M, Wissing D, Kokholm K et al (1998) Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases. EMBO J 17(21):6124–6134. doi:10.1093/emboj/17.21.6124
167. Gyrd-Hansen M, Nylandsted J, Jäättelä M (2004) Heat shock protein 70 promotes cancer cell viability by safeguarding lysosomal integrity. Cell Cycle 3:1484–1485
168. Guicciardi ME, Leist M, Gores GJ (2004) Lysosomes in cell death. Oncogene 23:2881–2890
169. Hatfield MPD, Lovas S (2012) Role of Hsp70 in cancer growth and survival. Protein Pept Lett 19:616–624
170. Mosser DD, Morimoto RI (2004) Molecular chaperones and the stress of oncogenesis. Oncogene 23:2907–2918
171. Rohde M, Daugaard M, Jensen MH et al (2005) Members of the heat-shock protein 70 family promote cancer cell growth by distinct mechanisms. Genes Dev 19:570–582
172. Daugaard M, Jäättelä M, Rohde M (2005) Hsp70-2 is required for tumor cell growth and survival. Cell Cycle 4:877–880
173. Kastan MB, Bartek J (2004) Cell-cycle checkpoints and cancer. Nature 432:316–323
174. Daugaard M, Kirkegaard-Sørensen T, Ostenfeld MS et al (2007) Lens epithelium-derived growth factor is an Hsp70-2 regulated guardian of lysosomal stability in human cancer. Cancer Res 67:2559–2567
175. Lee S-J, Lim H-S, Masliah E et al (2011) Protein aggregate spreading in neurodegenerative diseases: problems and perspectives. Neurosci Res 70:339–348
176. Dauer W, Przedborski S (2003) Parkinson's disease: mechanisms and models. Neuron 39:889–909
177. Blennow K, de Leon MJ, Zetterberg H (2006) Alzheimer's disease. Lancet 368:387–403
178. Ross CA, Tabrizi SJ (2011) Huntington's disease: from molecular pathogenesis to clinical treatment. Lancet Neurol 10:83–98
179. Hay DG, Sathasivam K, Tobaben S et al (2004) Progressive decrease in chaperone protein levels in a mouse model of Huntington's disease and induction of stress proteins as a therapeutic approach. Hum Mol Genet 13:1389–1405
180. Iwasawa H, Kondo S, Ikeda D et al (1982) Synthesis of (-)-15-deoxyspergualin and (-)-spergualin-15-phosphate. J Antibiot (Tokyo) 35:1665–1669
181. Takeuchi T, Iinuma H, Kunimoto S et al (1981) A new antitumor antibiotic, spergualin: isolation and antitumor activity. J Antibiot (Tokyo) 34:1619–1621
182. Umezawa H, Kondo S, Iinuma H et al (1981) Structure of an antitumor antibiotic, spergualin. J Antibiot (Tokyo) 34:1622–1624
183. Umezawa H (1983) The Leeuwenhoek lecture, 1982: studies of microbial products in rising to the challenge of curing cancer. Proc R Soc Lond B Biol Sci 217:357–376
184. Holcombe H, Mellman I, Janeway CA et al (2002) The immunosuppressive agent 15-deoxyspergualin functions by inhibiting cell cycle progression and cytokine production following naive T cell activation. J Immunol 169:4982–4989
185. Muindi JF, Lee S-J, Baltzer L et al (1991) Clinical pharmacology of deoxyspergualin in patients with advanced cancer. Cancer Res 51:3096–3101
186. Bergeron RJ, McManis JS (1987) Total synthesis of (.+-.)-15-deoxyspergualin. J Org Chem 52:1700–1703
187. Maeda K, Umeda Y, Saino T (1993) Synthesis and background chemistry of 15-deoxyspergualin. Ann N Y Acad Sci 685:123–135
188. Evans CG, Smith MC, Carolan JP et al (2011) Improved synthesis of 15-deoxyspergualin analogs using the Ugi multi-component reaction. Bioorg Med Chem Lett 21:2587–2590

189. Umeda Y, Moriguchi M, Ikai K et al (1987) Synthesis and antitumor activity of spergualin analogues. III. Novel method for synthesis of optically active 15-deoxyspergualin and 15-deoxy-11-O-methylspergualin. J Antibiot (Tokyo) 40:1316–1324
190. Nadler SG, Tepper MA, Schacter B et al (1992) Interaction of the immunosuppressant deoxyspergualin with a member of the Hsp70 family of heat shock proteins. Science 258: 484–486
191. Nadeau K, Nadler SG, Saulnier M et al (1994) Quantitation of the interaction of the immunosuppressant deoxyspergualin and analogs with Hsc70 and Hsp90. Biochemistry 33: 2561–2567
192. Nadler SG, Dischino DD, Malacko AR et al (1998) Identification of a binding site on Hsc70 for the immunosuppressant 15-deoxyspergualin. Biochem Biophys Res Commun 253: 176–180
193. Nosaka C, Kunimoto S, Takeuchi T (1999) The decrease of cytochrome c oxidase activity by 15-deoxyspergualin results in enhancement of XTT reduction in cultured cells. J Antibiot (Tokyo) 52:803
194. Hibasami H, Tsukada T, Suzuki R et al (1991) 15-Deoxyspergualin, an antiproliferative agent for human and mouse leukemia cells shows inhibitory effects on the synthetic pathway of polyamines. Anticancer Res 11:325–330
195. Tepper MA, Petty B, Bursuker I et al (1991) Inhibition of antibody production by the immunosuppressive agent, 15-deoxyspergualin. Transplant Proc 23:328–331
196. Ramya TN, Surolia N, Surolia A (2007) 15-deoxyspergualin inhibits eukaryotic protein synthesis through eIF2alpha phosphorylation. Biochem J 401:411–420
197. Banerjee T, Singh RR, Gupta S et al (2012) 15-Deoxyspergualin hinders physical interaction between basic residues of transit peptide in PfENR and Hsp70-1. IUBMB Life 64:99–107
198. Ohlman S, Zilg H, Schindel F et al (1994) Pharmacokinetics of 15-deoxyspergualin studied in renal transplant patients receiving the drug during graft rejection. Transpl Int 7:5–10
199. Kaufman DB, Gores PF, Kelley S et al (1996) 15-deoxyspergualin: immunotherapy in solid organ and cellular transplantation. Transplant Rev 10:160–174
200. Dhingra K, Valero V, Gutierrez L et al (1994) Phase II study of deoxyspergualin in metastatic breast cancer. Invest New Drugs 12:235–241
201. Lebreton L, Annat J, Derrepas P et al (1999) Structure – immunosuppressive activity relationships of new analogues of 15-deoxyspergualin. 1. Structural modifications of the hydroxyglycine moiety. J Med Chem 42:277–290
202. Lebreton L, Jost E, Carboni B et al (1999) Structure – immunosuppressive activity relationships of new analogues of 15-deoxyspergualin. 2. Structural modifications of the spermidine moiety. J Med Chem 42:4749–4763
203. Umeda Y, Moriguchi M, Kuroda H et al (1985) Synthesis and antitumor activity of spergualin analogues. I. Chemical modification of 7-guanidino-3-hydroxyacyl moiety. J Antibiot (Tokyo) 38:886–898
204. Umeda Y, Moriguchi M, Kuroda H et al (1987) Synthesis and antitumor activity of spergualin analogues. II. Chemical modification of the spermidine moiety. J Antibiot (Tokyo) 40: 1303–1315
205. Fewell SW, Day BW, Brodsky JL (2001) Identification of an inhibitor of hsc70-mediated protein translocation and ATP hydrolysis. J Biol Chem 276:910–914
206. Fewell SW, Smith CM, Lyon MA et al (2004) Small molecule modulators of endogenous and co-chaperone-stimulated Hsp70 ATPase activity. J Biol Chem 279:51131–51140
207. Werner S, Turner DM, Lyon MA et al (2006) A focused library of tetrahydropyrimidinone amides via a tandem Biginelli-Ugi multi-component process. Synlett 2006:2334–2338
208. Huryn DM, Resnick LO, Wipf P (2013) Contributions of academic laboratories to the discovery and development of chemical biology tools. J Med Chem. doi:10.1021/jm400132d
209. Wright CM, Seguin SP, Fewell SW et al (2009) Inhibition of Simian virus 40 replication by targeting the molecular chaperone function and ATPase activity of T antigen. Virus Res 141: 71–80

210. Wright CM, Chovatiya RJ, Jameson NE et al (2008) Pyrimidinone-peptoid hybrid molecules with distinct effects on molecular chaperone function and cell proliferation. Bioorg Med Chem 16:3291–3301
211. Jinwal UK, Miyata Y, Koren J 3rd et al (2009) Chemical manipulation of hsp70 ATPase activity regulates tau stability. J Neurosci 29:12079–12088
212. Wisén S, Bertelsen EB, Thompson AD et al (2010) Binding of a small molecule at a protein–protein interface regulates the chaperone activity of Hsp70–Hsp40. ACS Chem Biol 5:611–622
213. Anna R, Yuhong S, Nian W et al (2007) Selective compounds define Hsp90 as a major inhibitor of apoptosis in small-cell lung cancer. Nat Chem Biol 3:498–507
214. Braunstein MJ, Scott SS, Scott CM et al (2011) Antimyeloma effects of the heat shock protein 70 molecular chaperone inhibitor MAL3-101. J Oncol. doi:10.1155/2011/232037:232037, 232011 pp
215. Ireland AW, Gobillot TA, Gupta T et al (2014) Synthesis and structure–activity relationships of small molecule inhibitors of the simian virus 40 T antigen oncoprotein, an anti-polyomaviral target. Bioorg Med Chem 22:6490–6502
216. Rabu C, Wipf P, Brodsky JL et al (2008) A precursor-specific role for Hsp40/Hsc70 during tail-anchored protein integration at the endoplasmic reticulum. J Biol Chem 283: 27504–27513
217. Botha M, Chiang A, Needham P et al (2011) Plasmodium falciparum encodes a single cytosolic type I Hsp40 that functionally interacts with Hsp70 and is upregulated by heat shock. Cell Stress Chaperones 16:389–401
218. Chiang AN, Valderramos J-C, Balachandran R et al (2009) Select pyrimidinones inhibit the propagation of the malarial parasite, *Plasmodium falciparum*. Bioorg Med Chem 17: 1527–1533
219. Lipinski CA, Lombardo F, Dominy BW et al (2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev 46:3–26
220. Huryn DM, Brodsky JL, Brummond KM et al (2011) Chemical methodology as a source of small-molecule checkpoint inhibitors and heat shock protein 70 (Hsp70) modulators. Proc Natl Acad Sci U S A 108:6757–6762
221. Wadhwa R, Sugihara T, Yoshida A et al (2000) Selective toxicity of MKT-077 to cancer cells is mediated by its binding to the hsp70 family protein mot-2 and reactivation of p53 function. Cancer Res 60:6818–6821
222. Tikoo A, Shakri R, Connolly L et al (2000) Treatment of ras-induced cancers by the F-actin-bundling drug MKT-077. Cancer J 6:162–168
223. Koya K, Li Y, Wang H et al (1996) MKT-077, a novel rhodacyanine dye in clinical trials, exhibits anticarcinoma activity in preclinical studies based on selective mitochondrial accumulation. Cancer Res 56:538–543
224. Propper DJ, Braybrooke JP, Taylor DJ et al (1999) Phase I trial of the selective mitochondrial toxin MKT077 in chemo-resistant solid tumours. Ann Oncol 10:923–927
225. Rousaki A, Miyata Y, Jinwal UK et al (2011) Allosteric drugs: the interaction of antitumor compound MKT-077 with human Hsp70 chaperones. J Mol Biol 411:614–632
226. Li X, Srinivasan SR, Connarn J et al (2013) Analogues of the allosteric heat shock protein 70 (Hsp70) inhibitor, MKT-077, as anti-cancer agents. ACS Med Chem Lett 4:1042–1047
227. Whetstone H, Lingwood C (2003) 3′Sulfogalactolipid binding specifically inhibits Hsp70 ATPase activity *in vitro*. Biochemistry 42:1611–1617
228. Boulanger J, Faulds D, Eddy EM et al (1995) Members of the 70 kDa heat shock protein family specifically recognize sulfoglycolipids: role in gamete recognition and mycoplasma-related infertility. J Cell Physiol 165:7–17
229. Mamelak D, Lingood C (1997) Expression and sulfogalactolipid binding specificity of the recombinant testis-specific cognate heat shock protein 70. Glycoconj J 14:715–722

230. Mamelak D, Mylvaganam M, Whetstone H et al (2001) Hsp70s contain a specific sulfogalactolipid binding site. Differential aglycone influence on sulfogalactosyl ceramide binding by recombinant prokaryotic and eukaryotic Hsp70 family members. Biochemistry 40: 3572–3582
231. Mamelak D, Lingwood C (2001) The ATPase domain of hsp70 possesses a unique binding specificity for 3′-sulfogalactolipids. J Biol Chem 276:449–456
232. Mamelak D, Mylvaganam M, Tanahashi E et al (2001) The aglycone of sulfogalactolipids can alter the sulfate ester substitution position required for hsc70 recognition. Carbohydr Res 335:91–100
233. Park H-J, Mylvaganum M, McPherson A et al (2009) A soluble sulfogalactosyl ceramide mimic promotes ΔF508 CFTR escape from endoplasmic reticulum associated degradation. Chem Biol 16:461–470
234. De Rosa M, Park HJ, Mylvaganum M et al (2008) The medium is the message: glycosphingolipids and their soluble analogues. BBA Gen Sub 1780:347–352
235. Schmitt E, Parcellier A, Gurbuxani S et al (2003) Chemosensitization by a non-apoptogenic heat shock protein 70-binding apoptosis-inducing factor mutant. Cancer Res 63:8233–8240
236. Gurbuxani S, Schmitt E, Cande C et al (2003) Heat shock protein 70 binding inhibits the nuclear import of apoptosis-inducing factor. Oncogene 22:6669–6678
237. Schmitt E, Maingret L, Puig P-E et al (2006) Heat shock protein 70 neutralization exerts potent antitumor effects in animal models of colon cancer and melanoma. Cancer Res 66: 4191–4197
238. Chalmin F, Ladoire S, Mignot G et al (2010) Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells. J Clin Invest 120:457–471
239. Rérole A-L, Gobbo J, De Thonel A et al (2011) Peptides and aptamers targeting HSP70: a novel approach for anticancer chemotherapy. Cancer Res 71:484–495
240. Jego G, Hazoumé A, Seigneuric R et al (2013) Targeting heat shock proteins in cancer. Cancer Lett 332:275–285
241. Williams DR, Ko S-K, Park S et al (2008) An apoptosis-inducing small molecule that binds to heat shock protein 70. Angew Chem Int Ed 47:7466–7469
242. Shin I-j, Lee M-r, Williams D (2010) Imidazole derivatives that induce apoptosis and their therapeutic uses. United States Patent
243. Lev N, Melamed E, Offen D (2003) Apoptosis and Parkinson's disease. Prog Neuropsychopharmacol 27:245–250
244. Behl C (2000) Apoptosis and Alzheimer's disease. J Neural Transm 107:1325–1344
245. Meacham GC, Lu Z, King S et al (1999) The Hdj-2/Hsc70 chaperone pair facilitates early steps in CFTR biogenesis. EMBO J 18:1492–1505
246. Zhang Y, Nijbroek G, Sullivan ML et al (2001) Hsp70 molecular chaperone facilitates endoplasmic reticulum-associated protein degradation of cystic fibrosis transmembrane conductance regulator in yeast. Mol Biol Cell 12:1303–1314
247. Williamson DS, Borgognoni J, Clay A et al (2009) Novel adenosine-derived inhibitors of 70 kDa heat shock protein, discovered through structure-based design. J Med Chem 52: 1510–1513
248. Macias AT, Williamson DS, Allen N et al (2011) Adenosine-derived inhibitors of 78 kDa glucose regulated protein (Grp78) ATPase: insights into isoform selectivity. J Med Chem 54:4034–4041
249. Dong D, Ni M, Li J et al (2008) Critical role of the stress chaperone GRP78/BiP in tumor proliferation, survival, and tumor angiogenesis in transgene-induced mammary tumor development. Cancer Res 68:498–505
250. Li J, Lee AS (2006) Stress induction of GRP78/BiP and its role in cancer. Curr Mol Med 6: 45–54

251. Massey AJ, Dopson M, Lavan P et al (2010) A novel, small molecule inhibitor of Hsc70/Hsp70 potentiates Hsp90 inhibitor induced apoptosis in HCT116 colon carcinoma cells. Cancer Chemother Pharmacol 66:535–545
252. Chatterjee M, Andrulis M, Stuhmer T et al (2012) The PI3K/Akt signalling pathway regulates the expression of Hsp70, which critically contributes to Hsp90-chaperone function and tumor cell survival in multiple myeloma. Haematologica. doi:10.3324/haematol.2012.066175
253. Reikvam H, Nepstad I, Sulen A et al (2013) Increased antileukemic effects in human acute myeloid leukemia by combining HSP70 and HSP90 inhibitors. Expert Opin Invest Drugs 22:551–563
254. Strom E, Sathe S, Komarov PG et al (2006) Small-molecule inhibitor of p53 binding to mitochondria protects mice from gamma radiation. Nat Chem Biol 2:474–479
255. Leu JI-J, George DL (2007) Hepatic IGFBP1 is a prosurvival factor that binds to BAK, protects the liver from apoptosis, and antagonizes the proapoptotic actions of p53 at mitochondria. Genes Dev 21:3095–3109
256. Leu JIJ, Pimkina J, Frank A et al (2009) A small molecule inhibitor of inducible heat shock protein 70. Mol Cell 36:15–27
257. Liu EY, Ryan KM (2012) Autophagy and cancer–issues we need to digest. J Cell Sci 125:2349–2358
258. Balaburski GM, Leu JI, Beeharry N et al (2013) A modified HSP70 inhibitor shows broad activity as an anticancer agent. Mol Cancer Res 11:219–229
259. Leu JI-J, Pimkina J, Pandey P et al (2011) HSP70 inhibition by the small-molecule 2-phenylethynesulfonamide impairs protein clearance pathways in tumor cells. Mol Cancer Res 9:936–947
260. Kaiser M, Kuhnl A, Reins J et al (2011) Antileukemic activity of the HSP70 inhibitor pifithrin-mu in acute leukemia. Blood Cancer J 1:e28
261. Pimkina JS, Murphy ME (2011) Interaction of the ARF tumor suppressor with cytosolic HSP70 contributes to its autophagy function. Cancer Biol Ther 12:503–509
262. Budina-Kolomets A, Balaburski GM, Bondar A et al (2014) Comparison of the activity of three different HSP70 inhibitors on apoptosis, cell cycle arrest, autophagy inhibition, and HSP90 inhibition. Cancer Biol Ther 15:194–199
263. Jinwal UK, Koren J, O'Leary JC et al (2010) Hsp70 ATPase modulators as therapeutics for Alzheimer's and other neurodegenerative diseases. Mol Cell Pharmacol 2:43–46
264. Koren J, Jinwal UK, Jin Y et al (2010) Facilitating Akt clearance via manipulation of Hsp70 activity and levels. J Biol Chem 285:2498–2505
265. Chang L, Miyata Y, Ung PMU et al (2011) Chemical screens against a reconstituted multiprotein complex: myricetin blocks DnaJ regulation of DnaK through an allosteric mechanism. Chem Biol 18:210–221
266. Medina DX, Caccamo A, Oddo S (2011) Methylene blue reduces Aβ levels and rescues early cognitive deficit by increasing proteasome activity. Brain Pathol 21:140–149
267. Rodina A, Patel Pallav D, Kang Y et al (2013) Identification of an allosteric pocket on human Hsp70 reveals a mode of inhibition of this therapeutically important protein. Chem Biol 20:1469–1480
268. Kang Y, Taldone T, Patel HJ et al (2014) Heat shock protein 70 inhibitors. 1. 2,5-′-thiodipyrimidine and 5-(phenylthio)pyrimidine acrylamides as irreversible binders to an allosteric site on heat shock protein 70. J Med Chem 57:1188–1207
269. Taldone T, Kang Y, Patel HJ et al (2014) Heat shock protein 70 inhibitors. 2. 2,5-′-thiodipyrimidines, 5-(phenylthio)pyrimidines, 2-(pyridin-3-ylthio)pyrimidines, and 3-(phenylthio)pyridines as reversible binders to an allosteric site on heat shock protein 70. J Med Chem 57:1208–1224
270. Howe Matthew K, Bodoor K, Carlson David A et al (2014) Identification of an allosteric small-molecule inhibitor selective for the inducible form of heat shock protein 70. Chem Biol 21:1648–1659

271. Hassan AQ, Kirby Christina A, Zhou W et al (2015) The novolactone natural product disrupts the allosteric regulation of Hsp70. Chem Biol 22:87–97
272. Ciocca DR, Arrigo AP, Calderwood SK (2013) Heat shock proteins and heat shock factor 1 in carcinogenesis and tumor development: an update. Arch Toxicol 87:19–48
273. Heimberger T, Andrulis M, Riedel S et al (2013) The heat shock transcription factor 1 as a potential new therapeutic target in multiple myeloma. Br J Haematol 160:465–476
274. Kieran D, Kalmar B, Dick JR et al (2004) Treatment with arimoclomol, a coinducer of heat shock proteins, delays disease progression in ALS mice. Nat Med 10:402–405
275. Lanka V, Wieland S, Barber J et al (2009) Arimoclomol: a potential therapy under development for ALS. Expert Opin Invest Drugs 18:1907–1918
276. Hosokawa N, Hirayoshi K, Nakai A et al (1990) Flavonoids inhibit the expression of heat shock proteins. Cell Struct Funct 15:393–401
277. Koishi M, Hosokawa N, Sato M et al (1992) Quercetin, an inhibitor of heat shock protein synthesis, inhibits the acquisition of thermotolerance in a human colon carcinoma cell line. Jpn J Cancer Res 83:1216–1222
278. Hosokawa N, Hirayoshi K, Kudo H et al (1992) Inhibition of the activation of heat shock factor in vivo and in vitro by flavonoids. Mol Cell Biol 12:3490–3498
279. Elia G, Amici C, Rossi A et al (1996) Modulation of prostaglandin A1-induced thermotolerance by quercetin in human leukemic cells: role of heat shock protein 70. Cancer Res 56:210–217
280. Hansen RK, Oesterreich S, Lemieux P et al (1997) Quercetin inhibits heat shock protein induction but not heat shock factor DNA-binding in human breast carcinoma cells. Biochem Biophys Res Commun 239:851–856
281. Suolinna EM, Buchsbaum RN, Racker E (1975) The effect of flavonoids on aerobic glycolysis and growth of tumor cells. Cancer Res 35:1865–1872
282. Graziani Y, Chayoth R, Karny N et al (1982) Regulation of protein kinases activity by quercetin in Ehrlich ascites tumor cells. Biochim Biophys Acta 714:415–421
283. Wiseman RL, Zhang Y, Lee KPK et al (2010) Flavonol activation defines an unanticipated ligand-binding site in the kinase-RNase domain of IRE1. Mol Cell 38:291–304
284. Ma Y, Hendershot LM (2004) The role of the unfolded protein response in tumour development: friend or foe? Nat Rev Cancer 4:966–977
285. Boulton DW, Walle UK, Walle T (1999) Fate of the flavonoid quercetin in human cell lines: chemical instability and metabolism. J Pharm Pharmacol 51:353–359
286. Manwell LA, Heikkila JJ (2007) Examination of KNK437- and quercetin-mediated inhibition of heat shock-induced heat shock protein gene expression in Xenopus laevis cultured cells. Comp Biochem Physiol A Mol Integr Physiol 148:521–530
287. Yokota S-i, Kitahara M, Nagata K (2000) Benzylidene lactam compound, KNK437, a novel inhibitor of acquisition of thermotolerance and heat shock protein induction in human colon carcinoma cells. Cancer Res 60:2942–2948
288. Koishi M, Yokota S, Mae T et al (2001) The effects of KNK437, a novel inhibitor of heat shock protein synthesis, on the acquisition of thermotolerance in a murine transplantable tumor *in vivo*. Clin Cancer Res 7:215–219
289. Ohnishi K, Takahashi A, Yokota S et al (2004) Effects of a heat shock protein inhibitor KNK437 on heat sensitivity and heat tolerance in human squamous cell carcinoma cell lines differing in p53 status. Int J Radiat Biol 80:607–614
290. Qiu D, Kao PN (2003) Immunosuppressive and anti-inflammatory mechanisms of triptolide, the principal active diterpenoid from the Chinese medicinal herb *Tripterygium wilfordii* Hook. f. Drugs R D 4:1–18
291. Westerheide SD, Kawahara TLA, Orton K et al (2006) Triptolide, an inhibitor of the human heat shock response that enhances stress-induced cell death. J Biol Chem 281:9616–9622
292. Phillips PA, Dudeja V, McCarroll JA et al (2007) Triptolide induces pancreatic cancer cell death via inhibition of heat shock protein 70. Cancer Res 67:9407–9416

293. Antonoff MB, Chugh R, Skube SJ et al (2010) Role of Hsp-70 in triptolide-mediated cell death of neuroblastoma. J Surg Res 163:72–78
294. Kizelsztein P, Komarnytsky S, Raskin I (2009) Oral administration of triptolide ameliorates the clinical signs of experimental autoimmune encephalomyelitis (EAE) by induction of HSP70 and stabilization of NF-κB/IκBα transcriptional complex. J Neuroimmunol 217: 28–37
295. Xia Y, Liu Y, Rocchi P et al (2012) Targeting heat shock factor 1 with a triazole nucleoside analog to elicit potent anticancer activity on drug-resistant pancreatic cancer. Cancer Lett 318:145–153
296. Xia Y, Liu Y, Wan J et al (2009) Novel triazole ribonucleoside down-regulates heat shock protein 27 and induces potent anticancer activity on drug-resistant pancreatic cancer. J Med Chem 52:6083–6096
297. Mendillo Marc L, Santagata S, Koeva M et al (2012) HSF1 drives a transcriptional program distinct from heat shock to support highly malignant human cancers. Cell 150:549–562
298. van der Putten H, Lotz G (2013) Opportunities and challenges for molecular chaperone modulation to treat protein-conformational brain diseases. Neurotherapeutics 10:416–428

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Allosteric Inhibitors of Hsp70: Drugging the Second Chaperone of Tumorigenesis

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Abstract Cancer cells survive in the presence of stresses that would normally cause cell death. To accomplish this feat, they express elevated levels of the molecular chaperones: heat shock protein 70 (Hsp70) and heat shock protein 90 (Hsp90). Knockdown of these chaperones is selectively toxic to cancer cells, suggesting that they might be promising nodes for anticancer therapy. However, while inhibitors of Hsp90 are well known, progress in the development of Hsp70 inhibitors has proven more difficult. Hsp70 binds tightly to ATP through a highly conserved domain of the actin/hexokinase superfamily, making it challenging to identify selective, competitive inhibitors. Despite this obstacle, progress has been made and first-generation molecules are being deployed. To supplement these efforts, compounds that target important allosteric sites on the chaperone have also been discovered. In some of these cases, the molecules have been shown to control key protein–protein interactions between Hsp70 and its co-chaperones. In other cases, allosteric sites have been used to gain unexpected selectivity for members of the Hsp70 family. Here, we review recent progress in the development of Hsp70 inhibitors.

Keywords Allosteric inhibitors, Chaperones, Dihydropyrimidines, MKT-077, Protein folding, Protein–protein interactions, Spergualin

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Contents

1 Chaperones as Cell Survival Factors and Permissive Oncogenes

Tumorigenesis is so dependent on the activity of Hsp70 and Hsp90 that cancer cells have been described as being "addicted" to them [1, 2]. This conclusion comes from observations that chaperones protect against the cell death that would normally occur in response to the stresses encountered in tumors, such as oxidative damage, hypoxia, and/or proteotoxicity [3, 4]. Moreover, many oncogenes contain destabilizing mutations or aberrant chromosomal translocations that would normally render the proteins prone to rapid turnover. High levels of Hsp70 and Hsp90 seem to protect these "client" oncogenes from degradation [5–11], allowing cancer cells to thrive. Through these activities, Hsp90 and Hsp70 are often described as being "permissive oncogenes."

Because Hsp70 and Hsp90 are hubs for many cell survival and oncogenesis pathways, they have emerged as promising targets for anticancer drug discovery. Hsp90 and its inhibitors have been expertly reviewed in the recent literature [12, 13]; however, it is likely helpful to briefly summarize the key features of this system before engaging in a discussion of the up-and-coming area of Hsp70 inhibitors. As these two fields develop, similarities and differences are becoming increasingly clear.

2 Hsp90, "the Cancer Chaperone"

Hsp90 is an ATPase composed of an N-terminal domain that binds ATP, a middle domain that interacts with client proteins and a C-terminal domain that mediates homodimerization (reviewed in [14]). Recent structural studies have provided snapshots of Hsp90 dimers in the apo-, ATP-, and ADP-bound states [15–18]. These structures show that nucleotide cycling causes Hsp90's monomers to move in asymmetric "scissor-like" motions, which is thought to favor client binding.

Hsp90 interacts with at least 1,000 clients, a list that includes many pro-survival kinases, oncogenes, and transcription factors, including Akt, Raf-1, Her2, and Bcr-Abl [19–22]. A common feature of Hsp90 clients is that they contain metastable domains, such as steroid-binding clefts, nucleotide-binding cassettes, and latent protein–protein interaction motifs [23]. Hsp90 is thought to mask these regions,

protecting the proteins from degradation [24, 25]. Accordingly, a diagnostic feature of Hsp90 inhibitors is that they destabilize its clients [19–22]. More specifically, it is thought that interrupting Hsp90's ATPase cycle with a small molecule leads to premature release of the client, after which it is degraded by the proteasome and/or autophagy systems. Non-transformed cells appear to be less reliant on Hsp90 activity as these inhibitors typically have less dramatic effects on the viability of healthy cells [2, 26, 27].

There are three major categories of Hsp90 inhibitors, which are segregated based on their binding sites. The N-terminal domain is the site of binding for geldanamycin, its analogs, and many other synthetic inhibitors, such as PU-H71, STA-9090, and NVP-AUY922. These molecules compete with ATP [28–31], and their binding is presumed to stabilize an "open" state of the Hsp90 dimer. More recently, compounds related to novobiocin, such as KU-174, have been found to interact near the C-terminus of Hsp90 [32, 33]. Interestingly, these compounds also destabilize Hsp90 clients in cancer cells, despite the fact that they engage a distinct domain and don't directly compete for binding to nucleotide. Finally, a third category of Hsp90 inhibitors are those that impede interactions with co-chaperones [34–37]. A variety of co-chaperones, including immunophilins, p23, Aha1, Hop, and cdc37, associate with Hsp90 and assist in chaperone functions [38, 39]. Complexes between Hsp90 and its co-chaperones are enriched in cancer cells [40], suggesting that the cancer-specific roles of Hsp90 may be linked to cooperation with these factors [41]. Indeed, sansalvamide A [42, 43] and gedunin [34] are inhibitors of co-chaperone binding, and they are selectively toxic to cancer cells.

It is interesting that so many different classes of molecules, operating at nonoverlapping sites, share the ability to disrupt the stability of Hsp90 clients and kill cancer cells. These observations suggest that (a) Hsp90 is a sensitive "nano-machine" and (b) disturbing its ATP cycling or co-chaperone interactions has the potential to perturb chaperone activity. As discussed below, a similar relationship is becoming clear in the Hsp70 system.

3 Hsp70: The Second Chaperone of Tumorigenesis

Hsp70 is a two-domain chaperone that is composed of an N-terminal nucleotide-binding domain (NBD) and a C-terminal substrate-binding domain (SBD) (Fig. 1) [44]. The NBD has intrinsic ATPase activity [45], and it binds to nucleotide with a K_d of approximately 0.1–0.5 μM [46]. The NBD is further subdivided into two major lobes (A and B), with the ATP-binding cleft located between them. Likewise, the SBD is further subdivided into two major regions: a β-sandwich subdomain and an α-helical "lid." The β-sandwich subdomain contains the hydrophobic groove that binds client proteins, while the lid regulates the kinetics of client binding [47]. The NBD and SBD are connected by a short, hydrophobic linker, which mediates inter-domain allostery [48, 49].

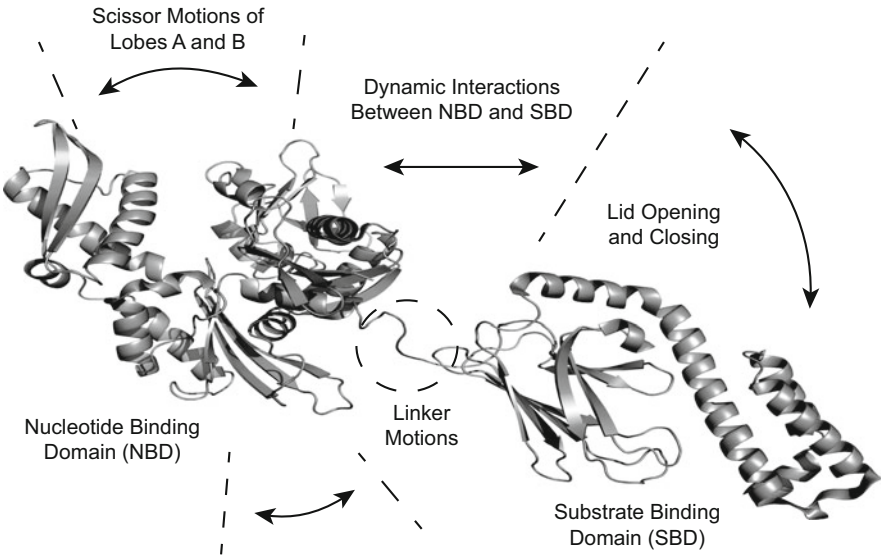

Fig. 1 Architecture and dynamics of heat shock protein 70 (Hsp70), highlighting the allosteric changes that occur during ATP hydrolysis

Structural studies have shown that Hsp70 is a highly flexible protein (Fig. 1) [50]. For example, lobes A and B of the NBD change their position in response to binding and hydrolysis of ATP [51]. This "scissor" motion helps communicate nucleotide status to the remainder of the protein through a number of allosteric networks. For example, when ATP binds, a structural rearrangement brings the NBD into close association with the SBD. Conversely, when ATP is hydrolyzed, this inter-domain contact is released, and the two domains now move independently in solution [52–55]. A further consequence of this hydrolysis-linked motion is that the helical lid moves from an "open" configuration to a "closed" state (Fig 2). Lid opening allows clients to enter, while its closing enhances the apparent affinity for clients by reducing the off rate (k_{off}). It is striking that these coordinated motions occur over such large distances (often greater than 20 Å) and involve rearrangements of nearly every subdomain. In this way, Hsp70 often serves a model for allosteric motions in multi-domain proteins, and it is a veritable playground for the discovery of allosteric inhibitors.

It is important to note that the allosteric motions in Hsp70 are further regulated by binding to co-chaperones [56]. There are three major families of co-chaperones in the mammalian Hsp70 system: J proteins, nucleotide exchange factors (NEFs), and tetratricopeptide repeat (TPR) domain-containing proteins. J proteins bind to Hsp70 and stimulate ATP hydrolysis [52]. These co-chaperones all contain a conserved J domain of about 70 amino acids, which interacts between the NBD and SBD of Hsp70s [52]. This protein–protein interaction favors the conversion of

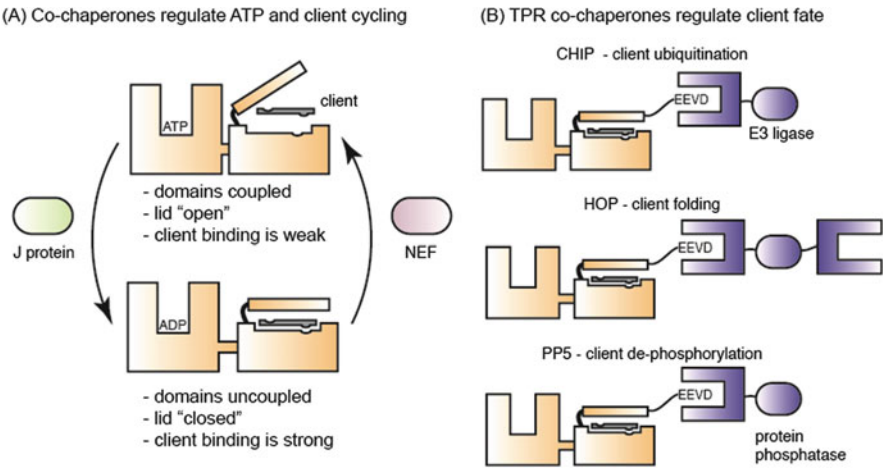

Fig. 2 Summary of the Hsp70 ATPase cycle and co-chaperone interactions. (**a**) Hsp70 binds tightly to ATP and adopts a conformation with the NBD and SBD being coupled. Hydrolysis of ATP, promoted by J proteins, leads the NBD and SBD to move independently. NEFs promote release of ADP and rebinding of ATP. During this cycle, clients bind tightly in the ADP-bound state and weakly in the ATP-bound state. (**b**) Various TPR co-chaperones compete for binding to the EEVD motif of Hsp70 and are thought to regulate the fate of bound clients

Hsp70 from the ATP-bound to ADP-bound state (Fig. 2a). The NEFs, which include Bag1, Bag2, Bag3, and Hsp105 [57], then replace ADP with ATP by opening the NBD [58]. Through these actions, the J proteins and NEFs coordinate ATP cycling and client loading (Fig. 2a). Hsp70 by itself has a slow ATPase rate that is likely not physiologically significant, such that it is only the combination of Hsp70, J proteins, and NEFs that provides meaningful turnover. The last category of Hsp70 co-chaperones encompasses those with a TPR domain (Fig. 2b). These proteins, including protein phosphatase 5 (PP5), Hsc70-organizing protein (HOP), and the C-terminal Hsc70-interacting protein (CHIP), bind to the C-terminal EEVD motif that is located at the end of Hsp70's lid [59–62]. Rather than impacting ATP cycling, the TPR co-chaperones appear to coordinate "hand-off" of Hsp70's clients to other pathways. For example, HOP binds to both Hsp70 and Hsp90, and it has been shown to facilitate transfer of clients between the chaperones [63, 64]. CHIP couples Hsp70 to the ubiquitin–proteasome system by specifically transferring poly-ubiquitin chains to Hsp70 clients [61, 65]. Because HOP and CHIP compete for the same site, competition between them balances whether Hsp70 clients are folded or degraded [66]. What controls these triage decisions? The full answer is not known yet phosphorylation of the C-terminus of Hsp70 appears to regulate TPR co-chaperone binding [67]. Thus, the enzymology and biology of Hsp70 are best regarded as being dependent on the coordinated motions of the chaperone in complex with its co-chaperones. This aspect of the system is critically important when considering strategies for drug discovery.

Hsp70 is thought to have a large number of possible clients. Indeed, studies using peptide arrays have suggested that the clients of Hsp70 might include nearly any protein that contains exposed hydrophobic regions [68]. NMR and crystallography studies have supported this idea by showing that Hsp70 makes most of its contacts with the amide backbone of client peptides [44, 69]. This mode of binding would be predicted to allow interactions with most, if not all, unfolded proteins [70]. Thus, the theoretical clients of Hsp70 would appear to encompass the same pool that is governed by Hsp90. Indeed, several studies have shown that knockdown of Hsp70 leads to degradation of Hsp90 clients, such as Akt and Her2, in cancer cells [46, 71–75]. In addition, Hsp70 would be expected to have additional clients that are not shared with Hsp90. For example, Hsp70 binds to mediators of endocytosis [76] that do not appear to be Hsp90 clients. However, the full scope of Hsp70-reliant clients is not yet clear.

There are 13 different Hsp70 genes in humans, and members of the Hsp70 family are found in every subcellular compartment [77]. These proteins are highly conserved, with amino acid identities typically around 80%. The major cytosolic family members are Hsc70 (HSPA8), which is constitutively expressed, and Hsp72 (HSPA1), which is expressed in response to stress. Cancer cells appear to have elevated Hsp72 levels [78, 79], consistent with a basal level of stress. Other Hsp70 family members include Grp75 (HSPA9; mortalin) in the mitochondria and Grp78 (HSPA5; BiP) in the ER. Recently, Grp75 has also been found in the cytosol and proposed to play an important cytoplasmic role in cancer [80, 81]. BiP plays a key role in protein folding and secretion in the ER lumen [82], which is an activity that is especially important in leukemia cells. Unless otherwise noted, we will use "Hsp70" when referring to general properties of the family members.

Seminal studies in the Workman laboratory have shown that siRNA knockdowns of *both* Hsc70 and Hsp72 are required to arrest tumor cell growth [73]. Likewise, Grp75 has been specifically implicated in preventing apoptosis and senescence in MCF7, U2OS, and COS7 cells [83, 84]. Thus, it isn't yet clear which Hsp70(s) might be the best target for anticancer therapy, or whether specific cancers might rely on distinct family members or whether pan-Hsp70 inhibitors might be preferred. As discussed below, progress has been made in the discovery of isoform-selective inhibitors, providing potential probes to ask these pressing questions.

3.1 Evidence Linking Hsp70 to Cancer

Hsp70 levels are constitutively elevated in both solid and liquid tumors when compared to normal or immortalized cells [85, 86]. Further, its expression levels are correlated with disease progression in multiple cancers [87], and Hsp70 expression directly predicts resistance to an array of treatments, including chemotherapeutics, radiation [7, 9, 10, 73, 88], and immune-mediated destruction [89,

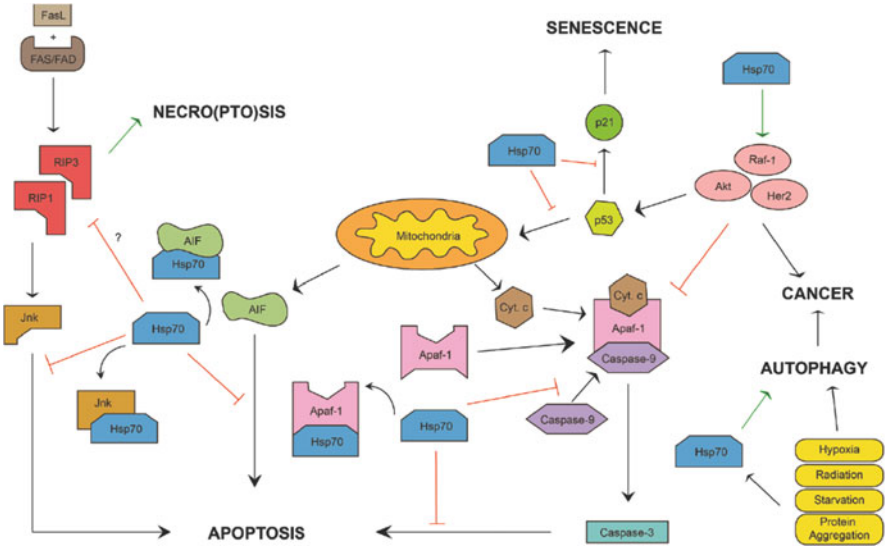

Fig. 3 Hsp70 promotes cell survival and oncogenic proliferation through multiple pathways. Although Hsp70 has been shown to inhibit apoptosis by interacting with several key effectors of the programed cell death pathway, its chaperone effects have also been noted to promote autophagy, inhibit senescence, and potentially influence necroptosis

90]. These observations have driven interest in better understanding the roles of Hsp70 in cancer.

The expression of Hsp70 in such a diverse set of tumors (with different oncogene and non-oncogene addictions) suggests that it may act broadly to support cancer cell survival. Indeed, Hsp70 has been shown to bind c-jun [91], APAF-1 [92], and AIF [93], inhibiting the formation of the death-inducing signaling complex [94]. However, it also prevents Bcl-2 translocation to the mitochondria [95, 96] and prevents caspase-9 recruitment to the apoptosome [92] (Fig. 3). Thus Hsp70 is thought to guard against both extrinsic and intrinsic death triggers, as well as caspase-dependent and caspase-independent pathways of apoptosis. Finally, recent results also suggest that Hsp70 might be involved in tumor initiation [97], necrosis [98], senescence [7, 10], and autophagic cell death [99]. Indeed, a comparison between the pathways reported to be regulated by Hsp90 and those controlled by Hsp70 suggests that Hsp70 may have an even broader role in cell survival (Fig. 4). Together, these observations support the notion that Hsp70 serves as a hub of pro-survival signaling and that it might be a “weak link” in many types of cancer.

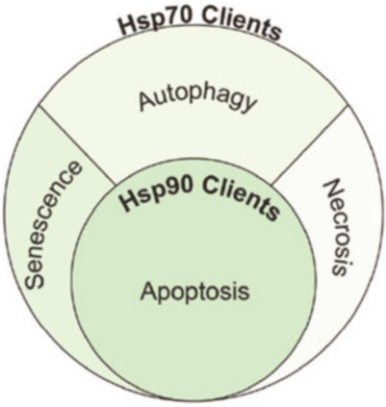

Fig. 4 Hsp70 regulates components belonging to multiple pathways that are important for cell survival. The "Hsp70-ome" is reported to consist of proteins involved in apoptosis, senescence, autophagy, and necrosis pathways. As indicated, the client of Hsp90 tends to be more restricted to the apoptosis pathway, including a subset of clients shared with Hsp70. Note that in the figure the relative sizes of the client pools are arbitrary

3.2 Pharmacological Targeting of Hsp70

Although there are no Hsp70 inhibitors in clinical trials, several groups are working toward that goal [100–103]. Some of their efforts will be discussed in the next sections. From this body of work, one striking theme is that there are multiple binding sites on Hsp70, distributed throughout the NBD and SBD. Figure 5 shows a summary of these sites and their spatial relationships. Some of the reported inhibitors directly compete for binding to ATP, while others compete with co-chaperones or bind at allosteric sites. Therefore, to organize this discussion, the inhibitors will be categorized by the location of their binding sites.

3.2.1 Compounds that Bind Hsp70's SBD

PES (or PFTμ)

2-Phenylethynesulfonamide (PES or PFTμ) was originally discovered out of a screen for molecules that activate p53-mediated apoptosis [99]. Subsequent work using a biotinylated PES analog showed that it binds Hsp70 in cell lysates, while a recent co-crystal structure of Hsp70's SBD with a bound PES analog, PET-16, has further refined our understanding of the interaction (Fig. 6a) [104]. PET-16 makes contacts with a number of residues in a hydrophobic groove in the SBD, which lies at the interface between the SBD and NBD. Importantly, this region has relatively low conservation among Hsp70 family members, and, consistent with this observation, PES has been shown to bind Hsp72, but not Grp75/Mortalin or Grp78/BiP.

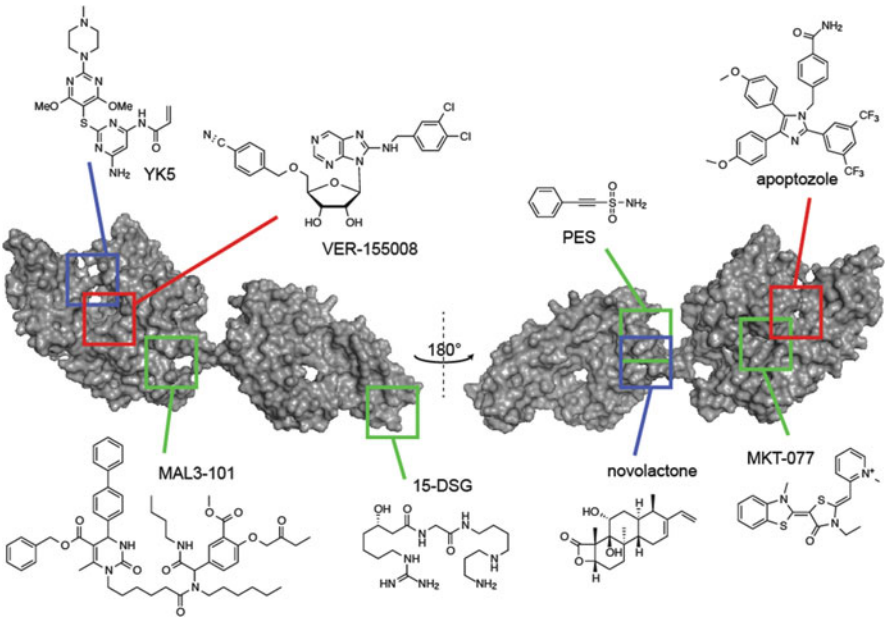

Fig. 5 Overview of the diversity of inhibitor binding sites on Hsp70. PDB code 2KHO. Binding sites are distributed throughout the NBD and SBD, including orthosteric molecules (VER-155008 and apoptozole) that compete with nucleotide and many different allosteric sites. ATP-competitive inhibitors are indicated by *red*, while covalent inhibitors are in *blue*, and other allosteric inhibitors are in *green*

As discussed above, this region is also heavily involved in allosteric motions (see Fig. 1), so ligands that bind to this site are well positioned to impact Hsp70 function.

PES is a simple, "fragment-like" molecule with a high ligand efficiency (LE), which might make it an attractive scaffold for further elaboration. Indeed, a search through a limited number of analogs produced PES-Cl and PET-16 [105], which are 2- to 10-fold more potent in antiproliferative assays (Fig. 6b). The improved potency of PET-16 can be rationalized by the contacts between the triphenylphosphine and the pocket, including P396 and I418 (Fig. 6a). Additional optimization efforts showed that the amide could be replaced with a pyrrolidine without a loss of activity, but that reducing the acetylene was not tolerated. However, beyond these observations, detailed structure–activity relationships (SAR) have not yet emerged.

What is the effect of PES and its analogs on protein–protein interactions (PPIs)? Although the binding site is not known to directly be involved in PPIs, analysis of PES-treated cell lysates showed that the compound disrupts binding of Hsp70 to p53, suggesting that some of the contacts between the chaperone and its clients may be disrupted by PES. Moreover, PES appears to block the interactions of Hsp70 with some of its co-chaperones, including CHIP, certain J proteins (e.g., DjB1), and Bag1. The structural basis of this effect is not yet clear, but it is consistent with the

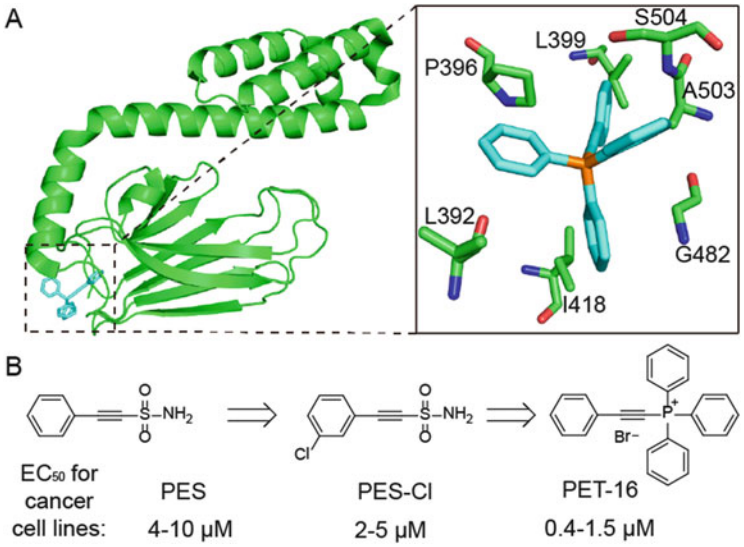

Fig. 6 PES and its analogs are allosteric inhibitors of Hsp90 and bind to the substrate-binding domain. (**a**) Crystal structure of PET-16 (carbon colored in *cyan*) with substrate-binding domain of Hsp90 (carbon colored in *green*); (**b**) optimization of PES led to more potent analogs PES-CI and PET-16

notion that Hsp70 is a dynamic, flexible machine – such that binding in one location can disrupt interactions at a distance.

PES-treated cells exhibit striking vacuolization of the cytosol, suggesting that the compound may impair lysosomal activity [99, 106]. Further, PES and its analogs elevate the levels of LC3-II in multiple cell lines, suggesting increased autophagic flux. This activity appears to be particularly toxic to acute myeloid leukemia (AML) and acute lymphoblastoid leukemia (ALL) cells [107], consistent with the notion that Hsp70 plays roles in lysosome stability [108, 109].

ADD70

Hsp70 counters apoptotic cell death, in part, by interacting with AIF and preventing its translocation from the mitochondria to the nucleus [110]. Schmitt et al. used deletion mutants of AIF to map the region of AIF that binds to Hsp70. They used this information to build a cytosolic AIF-mimetic peptide, named ADD70, which blocks binding of Hsp70 to AIF in cells when overexpressed. Although the mimetic itself is not cytotoxic, ADD70 does sensitize cancer cells to staurosporine, vinblastine, and other therapeutics. Based on analogy with other Hsp70 clients, one might assume that AIF binds in the SBD of Hsp70s; however, this hypothesis has not been tested. Further, membrane-permeable, synthetic analogs haven't yet been developed.

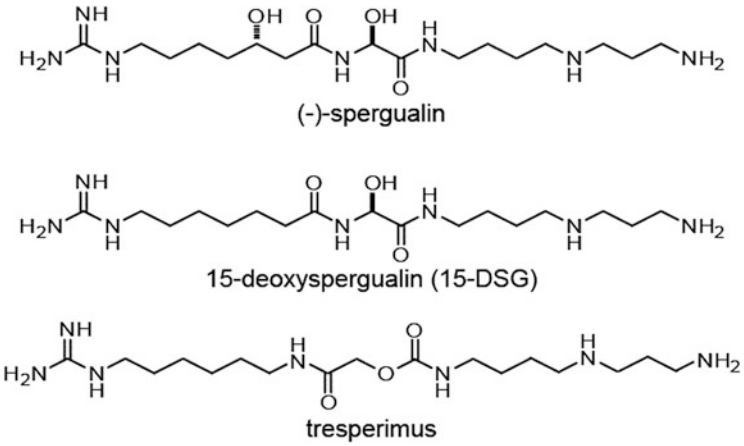

Fig. 7 Chemical structure of spergualin and some of its analogs

The lack of toxicity in response to ADD70 is interesting because most of the other molecules that bind Hsp70, such as PES, are cytotoxic as stand-alone agents. Thus, ADD70 may bind to the chaperone in a unique way, interrupting only select chaperone functions. Further mechanistic work will be required to address these issues.

Spergualin

The natural product, spergualin (Fig. 7), was originally identified as a potent antibacterial agent with broad-spectrum activity [111]. Subsequent work showed that this molecule has anticancer and immunosuppressive activities [112–114]. Synthetic efforts have removed some of the metabolic liabilities of spergualin, including the hydroxyl at carbon 15, which have greatly improved the physical properties of the chemical series [115, 116]. The most advanced of the spergualin analogs, 15-deoxyspergualin (15-DSG), is clinically approved in Japan for the treatment of acute allograft rejection, making this molecule the lone Hsp70 inhibitor approved for human use [117, 118]. Another close analog, tresperimus, was explored in phase III clinical trials by Laboratoires Fournier as an immunosuppressive [119].

15-DSG was found to be relatively selective for Hsp70 in cells, using mass spectrometry and pull-down studies [120, 121]. Further, this compound was shown to bind to the C-terminus of Hsp70s and chemical cross-linking studies suggested an interaction with the EEVD motif. As discussed above, this region of the SBD is interesting because it serves as a site for interactions with TPR domain co-chaperones. Thus, it is possible that spergualin analogs may disrupt co-chaperone interactions, although this has not been shown. Further, there is no crystal structure of 15-DSG bound to Hsp70, so the exact binding mode is uncertain, and, subsequently, SAR studies have not benefitted from structural knowledge.

Early SAR studies were focused on three key regions of 15-DSG, the central α-hydroxyglycine core, the guanidylated fatty chain, and the spermidine-derived polyamine. Nishizawa and coworkers replaced the central α-hydroxyglycine with various α- or ω-amino acids, and the antitumor activities of these analogs were evaluated against L1210 (IMC) mouse leukemia cells [115]. From the resulting SAR, the authors concluded that the central α-hydroxyglycine could be replaced with either glycine or an L-serine residue. The authors also altered the length and flexibility of the guanidinoheptanoic acid region to study its role in antitumor activity. It was discovered that a 4-guanidino-phenylbutyric acid shared similar antitumor activity with 15-DSG, suggesting that significant bulk could be tolerated in that region. Umeda and coworkers subsequently studied modifications of the spermidine moiety [122]. The SAR concluded that both the primary and secondary amino groups were important for antitumor activities. Together, these studies have shown a relatively narrow SAR around 15-DSG.

15-DSG was also explored as an anticancer agent in a phase I clinical trial against refractory solid tumors in 56 patients [123]. However, the average plasma concentration was only 0.07–7 μg/ml, largely because of rapid clearance (terminal half-life of 1.9 h). These concentrations are below the EC_{50} values in many cancer cell lines, suggesting that more potent and stable analogs are needed. Dose-limiting toxicity was reversible hypotension, which occurred in five of six patients with the highest dosage; however, it is not yet clear whether this toxicity arose from on- or off-target effects.

The mechanism by which spergualin analogs activate cell death is not yet clear. Also, additional medicinal chemistry efforts will likely be required to optimize potency and stability of these molecules. However, the relative safety of 15-DSG in humans makes this scaffold an attractive option for further mechanistic studies.

Novolactone

A group at Novartis recently described another inhibitor that binds in the SBD [124]. The authors started with a genomic approach to identify natural products with activity on Hsp70. Briefly, they measured the growth of ~6,000 *S. cerevisiae* deletion strains in the presence of 3,700 natural products, including novolactone. They found that novolactone sensitivity occurred in response to deletion of yeast Hsp70s, as well as an NEF and other chaperone cofactors. In parallel, they screened a randomly mutagenized set of haploid yeast strains, revealing yeast Hsp70 (known as SSA1) as a major potential target of novolactone. Interestingly, the point mutations in SSA1 that provided resistance were all located in the SBD, suggesting that this region could be the binding site.

In support of this idea, novolactone was found to inhibit the ATPase and luciferase refolding activity of Hsp70 complexes in vitro. Preincubation experiments clearly revealed an irreversible mechanism, which was confirmed by mass spectrometry and crystallography to occur via ring opening by Glu444 in the SBD

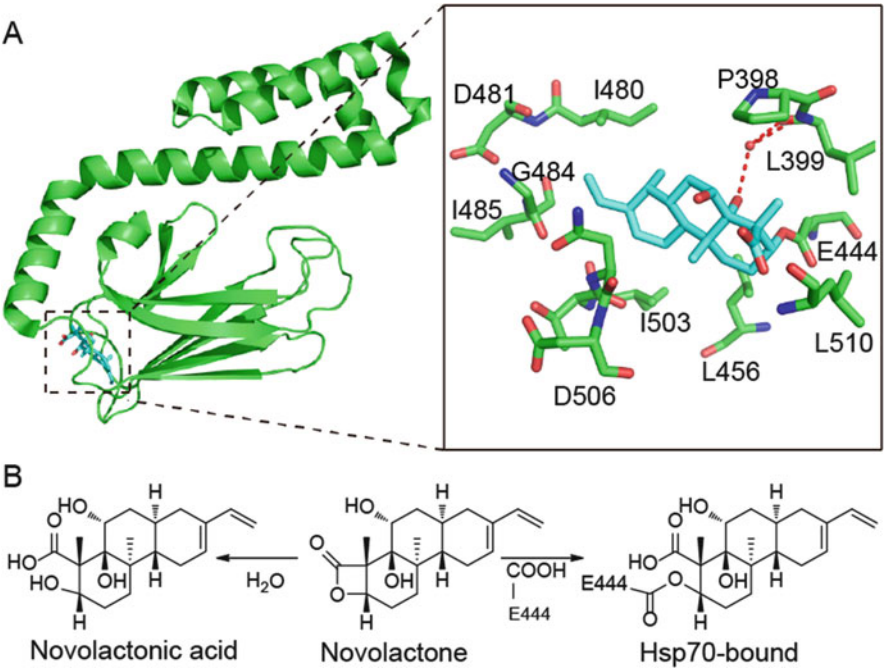

Fig. 8 Novolactone is a covalent inhibitor of Hsp70 that binds to the substrate-binding domain. (**a**) Crystal structure of Hsp70 (carbons colored in *green*) with bound novolactone (carbons colored in *cyan*; PDB 4WV7). Hydrogen bonds are indicated with *red dotted lines*. (**b**) Proposed mechanism of novolactone reactivity. Under physiological conditions, the lactone is hydrolyzed to an acid (*left*); however, when novolactone is close to E444 of Hsp70, a covalent bond is formed through transesterification

(Fig. 8a). Consistent with this idea, opening the lactone in the natural product with acidic methanol inactivated it (Fig. 8b), and replacing SSA1 in yeast with an E441Q mutant diminished sensitivity. Further, because Glu444 is only found in the mammalian cytosolic and ER-resident Hsp70 family members, novolactone is selective for them. This isoform selectivity is an example in which differential chemical reactivity between members of the Hsp70 family was used to gain selectivity, a theme that is emerging more broadly in the field (see below). Interestingly, novolactone significantly stabilized the Hsp70–client complex by 4000-fold. Thus, one would predict that it might inhibit binding of Hsp70 to its NEFs, which normally catalyze client release (see Fig. 2a). However, novolactone had no effect on these co-chaperones in an ATP release assay, so inhibition appears to involve interruption of the inter-domain communication required for client release, but not NEF-Hsp70 contacts per se. Novolactone treatment of HCT116 cells induced loss of chaperone clients, such as HER2 and CDK2, consistent with Hsp70 inhibition and suggestive of the potential use of this scaffold in oncology.

Interestingly, the binding site of novolactone is quite similar to the site bound by PET-16, as well as a handful of other, structurally unrelated molecules, including

low molecular mass thiophenes and antimicrobial peptides [125, 126]. This region appears to be a "hotspot" for interactions with small molecules.

3.2.2 Compounds that Bind in Hsp70's NBD

ATP/ADP Competitive Inhibitors

Hsp70 binds ATP with an affinity that is nearly 1000-fold tighter than binding of nucleotide to Hsp90 [46]. As such, it is more difficult for compounds to compete with ATP in Hsp70's nucleotide-binding pocket. Further, the key contacts in this cleft are polar, which creates hurdles in small molecule design. These challenges have been nicely summarized by Massey, who compared the hit rates from fragment screens performed against multiple nucleotide-binding targets, including Hsp70 and Hsp90. This analysis showed that Hsp70 had a hit rate of only 0.4%, while Hsp90 had a hit rate of 4.4%. High hit rates are typically associated with better "druggability," and they have been shown to be a predictor of future success in fragment elaboration campaigns. Thus, Massey concluded that the ATP-binding site of Hsp70 is an unusually poor drug target, having one of the worst hit rates yet described. Further, the ATP-binding cassette in Hsp70 is highly conserved among several abundant proteins, such as actin and hexokinase. Thus, it is predicted to be difficult to selectively inhibit Hsp70 with ATP mimetics. Despite these issues, a number of competitive inhibitors have been reported to bind in the ATP-binding cassette of Hsp70s, and these have been found to have antitumor activity.

Apoptozole

Apoptozole was discovered in a screen of imidazoles that trigger apoptosis [127]. A resin-linked version of apoptozole was used to show that the compound binds to the NBDs of both Hsc70 and Hsp72 and a fluorescent version of the molecule co-localized with anti-Hsp70 antibodies in cells. An analog, L8, was inactive in these assays (Fig. 9), suggesting some SAR. Using an analog of apoptozole with an installed electrophile, the binding site of the compound was further mapped in vitro to a region near the nucleotide-binding pocket [128]. Using this distance constraint, computational modeling results suggested that the compound might compete with nucleotide for binding to Hsp70 and that it might adopt a similar pose to ATP. Consistent with this model, apoptozole mildly inhibits Hsp70's ATPase activity (~60% reduction in ATPase rate at 200 μM) [127]. However, verification of this binding site by formal competition studies and/or structural studies has not yet been reported.

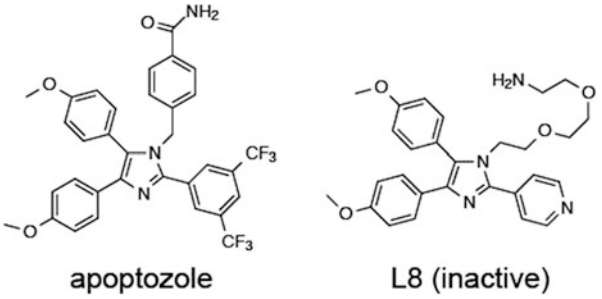

Fig. 9 Chemical structures of apoptozole and L8, an inactive analog

VER-155008

To identify competitive inhibitors of Hsp70, Williamson, Massey, and coworkers developed a collection of adenosine analogs and screened them in a fluorescence polarization (FP) assay. From the hit molecule, compound 4 (Fig. 10), multiple analogs with IC_{50} values in the low micromolar range were identified, and co-crystallization confirmed that two of these molecules bind in the ATP-binding cleft. From this starting point, structure-guided analogs were developed, and the most potent of these compounds, VER-155008, bound to Hsp70 with an affinity of approximately 0.5 μM by FP. The enhanced affinity of VER-155008 appears to originate from favorable contacts with key residues in the nucleotide-binding cleft, including Y15 (Fig. 10a). This compound also had promising antiproliferative activity in HCT116 cells, with an EC_{50} of 5 μM, and it destabilized some of the expected client proteins, including Raf1 and HER2 [129]. Subsequent work showed that VER-155008 induced both caspase-dependent and caspase-independent apoptosis in colon carcinoma cells [46]. These studies have demonstrated that inhibitors can be developed starting from structural knowledge of the Hsp70 nucleotide-binding site.

In a recent study, the same team has explored inhibitors of Grp78, the ER-resident member of the Hsp70 family. By comparing the crystal structures of Hsp70 and Grp78 in various nucleotide states, they proposed that the major contact that could be exploited was at position 37, which is an Ile in Grp78 and a Thr in Hsp70. To test this model, they synthesized additional analogs of VER-155008, ultimately co-crystallizing compound 10 with Grp78 [130]. They found that VER-155008 and compound 14 (Fig. 10c), which have hydrophobic cyano-benzyl groups in the key position, are not selective. However, compounds with more polar groups, such as alcohols and amides, at that position were relatively more selective for Grp78, as predicted by the structures and the presence of the Thr37. In the toolbox of available Hsp70 inhibitors, these active site competitors will likely serve an important role.

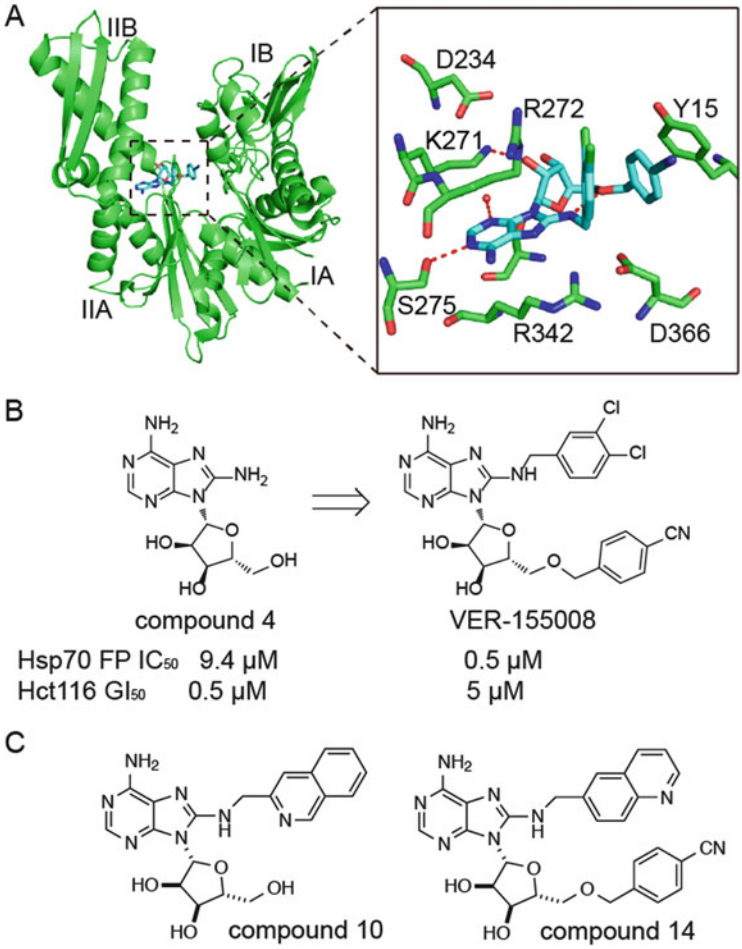

Fig. 10 VER-155008 is an ATP-competitive inhibitor of Hsp70 that binds to the nucleotide-binding domain (NBD). (**a**) Crystal structure of Hsp70 (carbons colored in *green*, PDB 3FZL) with VER-155008 (carbons colored in *cyan*). Hydrogen bonds are indicated with *red dotted lines*. (**b**) Structure-based optimization of hit compound 4 led to VER-155008. (**c**) Additional analogs used to probe selectivity for Grp78

3.2.3 Allosteric Modulators

One promising way to inhibit Hsp70's activity in cancer cells may be to target the chaperone's many allosteric sites [56, 100]. This approach might be expected to avoid some of the problems of selectivity and potency associated with active site inhibitors. Another potential advantage is that many allosteric sites in Hsp70 control binding to co-chaperones; thus, molecules that bind these regions might have profound effects on recruitment of co-chaperones.

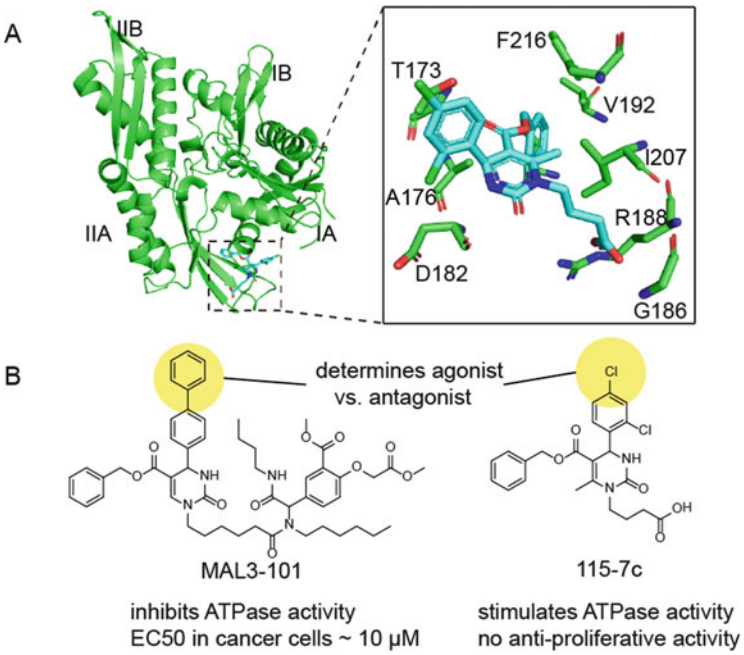

Fig. 11 MAL3-101 is an allosteric inhibitor of Hsp70 which disrupts its interaction with J protein co-chaperones, while 115-7c stimulates this interaction. (**a**) Model of 115-7c (carbons colored in *cyan*) binding to the NBD (carbons colored in *green*) based on NMR and mutagenesis. (**b**) Chemical structures of MAL3-101 and 115-7c. The region colored in *yellow* is directed at the J protein interface: diphenyl groups are inhibitors, while smaller chloro groups are activators

MAL3-101

As discussed above, members of the J protein family of co-chaperones are critical regulators of Hsp70 [52, 58, 59]. Fewell et al. screened for compounds that would preferentially act on the ability of J proteins to stimulate Hsp70's ATPase activity. In a panel of dihydropyrimidines, they discovered MAL3-101 [131] (Fig. 11).

Subsequent work showed that analogs of MAL3-101 bind to the NBD in a region previously implicated in J protein interactions [132]. This site is highly conserved, and members of this chemical series have activity against multiple human, yeast, and prokaryotic Hsp70s [133–135]. Interestingly, early analogs of MAL3-101 had displayed a puzzling SAR in which large, bulky groups appended to the 4 position of the dihydropyrimidine were inhibitors of ATPase activity, while smaller groups in this same position, such as the dichlorobenzyl in 115-7c (Fig. 11b), created molecules that actually *promoted* the ability of J proteins to stimulate ATP turnover [136]. Similar findings were observed in chaperone-mediated refolding activity assays [134, 137]. Structural studies finally resolved this dichotomy, showing that the 4 position is oriented toward the incoming J protein, such that large groups

block the PPI, while smaller groups seem to pre-arrange the pocket for binding to the J domain [132].

MAL3-101 has antiproliferative activity in multiple myeloma cells (EC_{50} values estimated to be between 5 and 10 μM), and it was found to be highly synergistic (CI_{50} value 0.01) with the Hsp90 inhibitor, 17-AAG, and the proteasome inhibitor, MG-132 [138]. MAL3-101 was also active in five of seven Merkel cell carcinoma cell lines (EC_{50} values estimated to be between 5 and 30 μM) and in a xenograft model. Interesting, its potency in these cells is correlated with relative Hsp70 expression [139], suggesting that the levels of the chaperone might, in part, predict efficacy.

MAL3-101 and its analogs are accessed through the Biginelli multicomponent reaction [140], which is a synthetic strategy that is amenable to combinatorial synthesis. Indeed, libraries of Hsp70 inhibitors have been generated by this approach [134, 136]. However, potency remains modest at this point, and additional, structure-guided campaigns will likely be required to enhance the affinity of these molecules.

MKT-077

MKT-077 was originally discovered in a screen for antiproliferative activity. It was found to have low micromolar activity against multiple cancer cell lines but little toxicity against either fibroblasts or immortalized epithelial lines [141–143]. MKT-077 exerts its anticancer activity by binding to a novel allosteric site in Hsp70 [84] that is located in a junction between the A and B lobes of the NBD [144] (Fig. 12a). Although this pocket is adjacent to the ATP-binding cleft, MKT-077 analogs do not compete for binding to nucleotide. Rather, binding appears to trap the chaperone in the ADP conformation [83, 144], limiting client release [13, 145–147]. The prolonged interaction between Hsp70 and clients appears to destabilize Akt, Raf1, FoxM1, and Cdk2 in cancer cells [97, 148, 149], possibly by recruiting effectors of the ubiquitin–proteasome system [150].

The first synthetic routes to MKT-077 started with 2-methylthiobenzothiazole, which is methylated on the thiazole nitrogen and then coupled to *N*-ethyl rhodanine and condensed with 1-ethyl-2-methylpyridin-1-ium [151]. However, Takasu and coworkers developed a facile, one-pot, three-step combinatorial synthesis of MKT-077 derivatives by sequential condensation of the methylthioninium, rhodanine, and iminium [152]. Kasmi-Mir et al. refined the synthesis by employing microwave irradiation [153], greatly improving the reaction time and yields (60% in most cases). Taking advantage of these advances, our group assembled MKT-077 analogs and tested their binding to Hsp70 and their antiproliferative activity against breast cancer cells. The benzothiazole only accepted small substitutions, such as halogenations, trifluoromethyl, and methoxy groups [149], presumably due to steric clashes with the sides of the narrow pocket. Conversely, the exposed pyridinium group was amenable to more modifications. For example, analogs with benzyl modifications to the pyridinium were more potent than

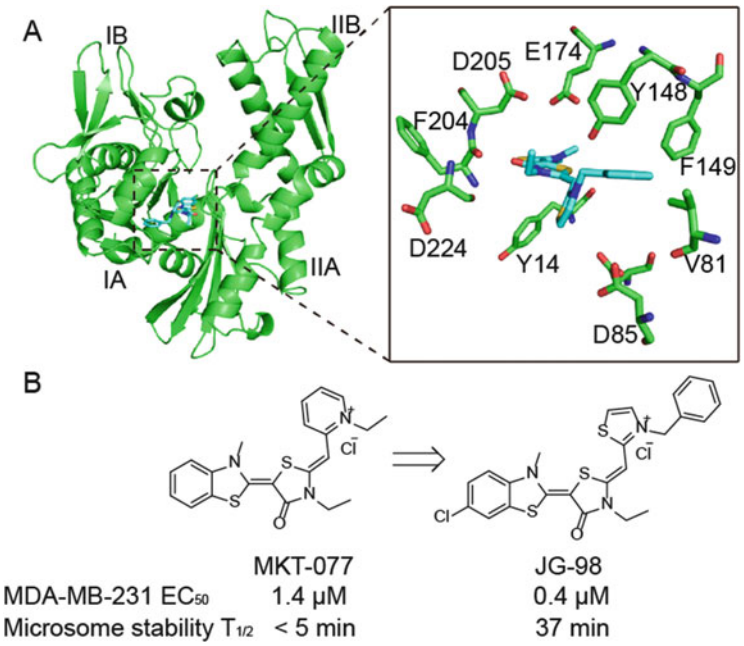

Fig. 12 MKT-077 and its analog JG-98 are allosteric inhibitors of Hsp70 that bind in the NBD. (**a**) Model of JG-98 binding to an allosteric pocket in Hsp70, based on NMR and mutagenesis. JG-98 carbons colored in *cyan* and Hsp70 carbons colored in *green*. (**b**) Modification of metabolically labile positions led to more a potent and stable analog, JG-98

MKT-077 (EC_{50} ~ 1.0 μM), while replacing the pyridinium with a 2-thiazolyl group, such as in JG-98, significantly increased potency (EC_{50} of 0.4 μM in MDA-MB-231 cells and 0.7 μM in MCF7 cells) (Fig. 12b). These changes also enhanced affinity for Hsp70 in vitro by approximately 80-fold ($K_D = 90$ nM), likely by optimizing interactions with a secondary site framed by Y148, V81, P146, and F149.

The MKT-077-binding pocket is deep and significantly more hydrophobic than the nearby ATP-binding site. The benzothiazole of MKT-077 is predicted to reside in the deepest part of this cleft, in a region bounded by F204, D205, and Y14 (Fig. 12a) [144], while the pyridinium is predicted to interact with a nearby region formed by Asp223, Thr224, and His225.

One of the major disadvantages of MKT-077 is its rapid metabolism. For example, MKT-077 is rapidly oxidized by liver microsomes ($t_{1/2}$ ~5 min), and it has a short lifetime in mice [154]. Thus, another goal of recent synthetic efforts has been to improve stability. Metabolite identification studies found that the benzothiazole and pyridinium rings of MKT-077 are the major sites of oxidation by P_{450} enzymes [13]. Consistent with this idea, electron withdrawing groups on the benzothiazole, especially the 3, 4, and 5 positions, improved the lifetimes of the compounds 7-fold in the presence of liver microsomes. Similarly, replacing the

pyridinium with a 2-thiazolyl group limited oxidation and greatly improved stability ($t_{1/2} > 30$ min) [149]. These changes allowed JG-98 to be used in xenograft studies, where it was found to be active against HeLa and MCF7 tumors [97, 155]. The next questions for this series are whether the rhodacyanine can be replaced and whether potency can be further improved.

YK5

The Chiosis group recently reported an exciting new inhibitor of Hsp70 that accesses a distinct binding site [156]. Briefly, they generated a composite Hsp70 model based on multiple reported structures and then examined it for possible cryptic binding sites. From the five most promising sites, they focused on one located between lobes A and B of the NBD. A prominent feature of this cleft is that it contains Cys267, a cysteine that is unique to Hsp72, and not the related cytosolic isoform, Hsc70. Indeed, oxidation of this residue had previously been used to develop early Hsp72-selective inhibitors [157, 158]. The Chiosis group nicely exploited this feature. After modeling suggested that a 2,5′-thiodipyrimidine would be expected to bind in the pocket, an electrophilic acrylamide was installed to favor covalent attachment to Cys267. Optimization of the initial molecule produced YK5, and more recent efforts have further refined the series, producing compounds such as 20a (Fig. 13).

Briefly, at the end of the molecule opposite the acrylamide, the authors found that a variety of moieties could be accepted, including piperazine, morpholine, piperidines, and others. The best was a methylpiperazine, which is likely protonated and might make contacts with His89 (Fig. 13). Additional SAR was obtained around the other substituents on the thiodipyrimidine, including varying the electrophile. Acrylamide was found to be better than other unsaturated amides. On the "ring A" pyrimidine, methoxy (as in YK5) and ethoxy groups (as in compound 20a) were preferred over methyls. Based on the modeling, this molecule was predicted to bind in a pose that positions the dipiperazine for contacts with R261, V59, R264, D234, and E268 of the pocket (Fig. 13a). YK5 bound Hsp72 in lysates [159] and, consistent with the model, mutation of C267S reduced binding. This molecule inhibited ATPase and refolding activity in vitro, consistent with the functional importance of this allosteric site. Because ATP hydrolysis involves large motions of the A and B lobes (see Fig. 1), it seems likely that the molecule would interfere with this motion. In cells, treatment with YK5 destabilized chaperone clients, including Akt and Her2 and activated apoptosis, as measured by PARP cleavage. Finally, YK05 killed SKBr3 cells, with a sub-micromolar EC_{50} value.

Immobilized YK5 was used to ask an important question about Hsp70 function in cancer cells [159]. In SKBr3 cell extracts, YK5 was used to pull down cofactors of the chaperone, revealing Hsp110, Her2, cyclin D, and Raf1 as being Hsp70 bound. However, in MDA-MB-231 cells, CDK1 and PDK1 were identified, while mAR was found in LNCaP cells. Thus, distinct clients appeared to be bound to

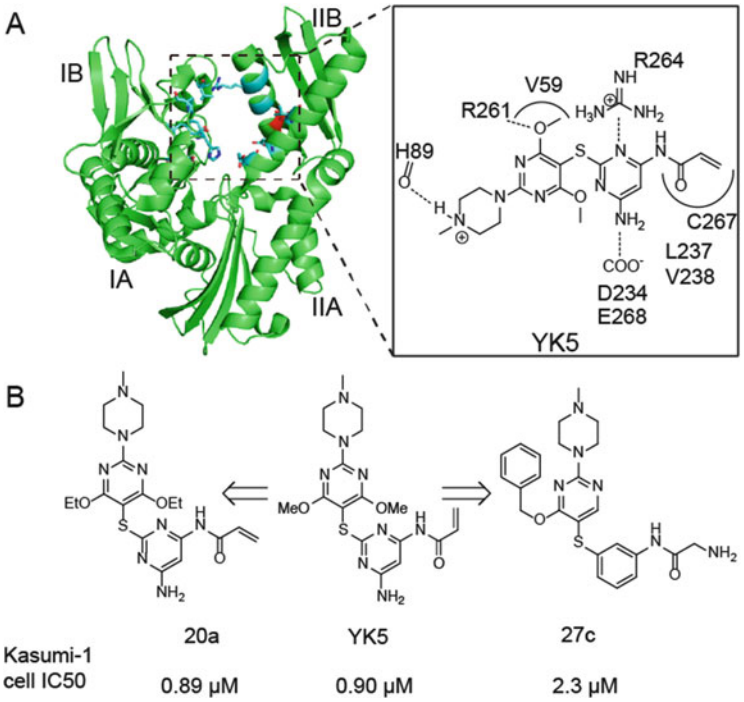

Fig. 13 YK5 and its analogs are allosteric inhibitors of Hsp70 that bind to the NBD. (**a**) Schematic representation of YK5 binding with Hsp70 irreversibly through a covalent bond with C267 (highlighted in *red*). (**b**) Optimization of YK5 led to a slightly more potent, irreversible analog 20a and reversible inhibitor 27c

Hsp70 complexes in different cancer cell types. Treatment with YK5 led to downregulation of these clients in the respective cells, suggesting that they are indeed dependent on Hsp70 for stability.

Recently, the authors converted the covalent YK5 into a series of reversible inhibitors, such as 27c (Fig. 13). Briefly, the acrylamide of YK5 was replaced with an amine to retain interaction with Cys267. At the same time, the pyrimidine adjacent to the acrylamide was replaced with a more electron-rich phenyl group to favor cation-π interactions with Arg 264. To further restore lost affinity, a benzyl group was appended to the other pyrimidine to make contacts with Phe68 and Trp90. The new compounds maintained good antiproliferative activity (EC_{50} of 2.1 μM) and the ability to destabilize clients in cancer cells [160], suggesting that covalent attachment is not required for activity at this allosteric site.

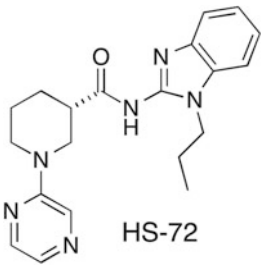

Fig. 14 Chemical structure of allosteric Hsp70 inhibitor HS-72

Other Allosteric Inhibitors

Very recent work has revealed two additional chemical series that target Hsp70. The Haystead group loaded GFP-Hsp70 onto a solid support via binding to immobilized ATP [161]. They then screened a collection of 3,379 purine-containing compounds to identify compounds that could displace the chaperone. Active molecules were defined as those that could release the fluorescent Hsp70 from the resin. Counter-screens against an unrelated protein and additional triage steps focused the follow-up studies on 22 hits. These molecules were rank-ordered based on the ability to activate apoptosis in BT474 cells, and the most active compound was identified as HS-72 (Fig. 14).

HS-72 had antiproliferative activity at mid-micromolar concentrations in multiple cell lines, including BT-474, MCF7, HepG2, HeLa, T47D, and LNCaP, and it destabilized chaperone clients, such as Akt, in these cells. Interestingly, binding studies suggested that HS-72 binds to the stress-inducible Hsp72 and not Hsc70 or BiP. Further, the compound was not competitive with nucleotide binding; rather it selectively interacted with Hsp72 in the ATP-bound form (not the ADP-bound form). This result was somewhat surprising, given the design of the original displacement screen. However, additional mechanistic studies confirmed that HS-72 interacts with Cys306 in Hsp72 or at least requires this residue.

In a unique and powerful approach, the Winssinger group used DNA-immobilized, combinatorial fragment libraries to screen for novel inhibitors of Hsp70 [162]. By combining 125 biologically active fragments with 500 heterocycles on complementary DNA strands, they were able to assemble a microarray containing 62,500 combinations. Incubation of Hsp70 with this array identified fragment pairs with affinity for the chaperone. Based on this screen, they selected the most common fragment motifs and synthesized a 10,000-member-focused collection on PNA templates. After panning this collection and validating the affinity of promising, resynthesized hits using SPR, they settled on ~15 compounds belonging to varied structural classes. These scaffolds provide a rich source of potential leads for further exploration.

3.2.4 Combination Warfare

Most current chemotherapeutic regimens contain some permutation of vinblastine, cisplatin, methotrexate, prednisone, doxorubicin, and any of several other cytotoxic drugs. Cocktails of these active agents are used to maximize therapeutic efficacy while reducing toxicity, side effects, and resistance. In this context, it is worth discussing how Hsp90 and Hsp70 inhibitors, such as the ones reviewed here, might be used in combination with other compounds.

As described earlier, Hsp90 acts as a central hub in cancer cells, directing the fates of hundreds of signaling effectors [2, 26, 27]. Thus, combining Hsp90 inhibitors with conventional chemotherapeutics or radiation therapy creates powerful combinations [163–165]. Hsp90 inhibitors have even been shown to restore sensitivity in cell lines previously resistant to drugs such as tamoxifen, doxorubicin, or trastuzumab [166–168]. Similarly, the idea of combining an Hsp90 inhibitor with a drug targeting Hsp70 has been suggested by many groups [74, 169], and synergy has been experimentally observed in combination with VER-155008 or MAL3-101 [46, 138, 170, 171].

Interestingly, Hsp90 inhibitors that bind different locations (e.g., C-terminal and N-terminal domains) also show synergy in cancer cell viability assays [172]. It seems that interrupting Hsp90 motions in two distinct ways might produce more potent effects. Following this logic, it will be interesting to mix Hsp70 inhibitors with different binding sites. In this way, the Hsp90 and Hsp70 complexes themselves might provide an unusually rich source of novel combinations.

4 Conclusions

Cancer cells must cope with the stress of rapid division and high mutation rates, ultimately becoming “addicted” to chaperones. This same sensitivity is likely why inhibitors of the proteasome and autophagy pathways are effective as anticancer drugs [2, 173, 174], especially in cells that produce high levels of protein. Consistent with this theme, knockdown studies have suggested that Hsp70 might be a particularly good target, either by itself or in combination with other chemotherapeutics [175–177]. However, the suitability of Hsp70 as a drug target remains somewhat of an enigma. Do all of the allosteric binding sites produce a similar effect on Hsp70 clients, as has been seen for many Hsp90 inhibitors? Recent reports suggest that, on the contrary, Hsp70 inhibitors with different binding sites and different mechanisms of action produce distinct effects on cell signaling pathways [178]. Another key question is safety. Hsp70 plays unusually broad roles in normal biology including protein folding, disaggregation, the assembly of multi-protein complexes, subcellular trafficking, protein turnover, antigen presentation, and transcription [179–182]. Current models suggest that these varied activities might arise from combinations of Hsp70 with its diverse sets of co-chaperones. Thus, the

safest "target" for cancer may actually be Hsp70 in a complex with its cancer-related co-chaperones. Until more is known about co-chaperones in cancer, it seems like the best way for the field to hedge its bets is to simultaneously develop multiple inhibitors with different mechanisms of action and binding sites.

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References

1. Powers MV, Workman P (2007) Inhibitors of the heat shock response: biology and pharmacology. FEBS Lett 581:3758–3769
2. Trepel J, Mollapour M, Giaccone G et al (2010) Targeting the dynamic HSP90 complex in cancer. Nat Rev Cancer 10:537–549
3. Jolly C, Morimoto RI (2000) Role of the heat shock response and molecular chaperones in oncogenesis and cell death. J Natl Cancer Inst 92:1564–1572
4. Luo J, Solimini NL, Elledge SJ (2009) Principles of cancer therapy: oncogene and non-oncogene addiction. Cell 136:823–837
5. Ferrarini M, Heltai S, Zocchi MR et al (1992) Unusual expression and localization of heat-shock proteins in human tumor cells. Int J Cancer 51:613–619
6. Gress TM, Muller-Pillasch F, Weber C et al (1994) Differential expression of heat shock proteins in pancreatic carcinoma. Cancer Res 54:547–551
7. Yaglom JA, Gabai VL, Sherman MY (2007) High levels of heat shock protein Hsp72 in cancer cells suppress default senescence pathways. Cancer Res 67:2373–2381
8. Abdel-Magid AF, Carson KG, Harris BD et al (1996) Reductive amination of aldehydes and ketones with sodium triacetoxyborohydride. Studies on direct and indirect reductive amination procedures(1). J Org Chem 61:3849–3862
9. Gabai VL, Budagova KR, Sherman MY (2005) Increased expression of the major heat shock protein Hsp72 in human prostate carcinoma cells is dispensable for their viability but confers resistance to a variety of anticancer agents. Oncogene 24:3328–3338
10. Gabai VL, Yaglom JA, Waldman T et al (2009) Heat shock protein Hsp72 controls oncogene-induced senescence pathways in cancer cells. Mol Cell Biol 29:559–569
11. Yano M, Naito Z, Tanaka S et al (1996) Expression and roles of heat shock proteins in human breast cancer. Jpn J Cancer Res 87:908–915
12. Li J, Buchner J (2013) Structure, function and regulation of the hsp90 machinery. Biomed J 36:106–117
13. Miyata Y, Nakamoto H, Neckers L (2013) The therapeutic target Hsp90 and cancer hallmarks. Curr Pharm Des 19:347–365
14. Pearl LH, Prodromou C (2006) Structure and mechanism of the Hsp90 molecular chaperone machinery. Annu Rev Biochem 75:271–294
15. Krukenberg KA, Street TO, Lavery LA et al (2011) Conformational dynamics of the molecular chaperone Hsp90. Q Rev Biophys 44:229–255
16. Southworth DR, Agard DA (2008) Species-dependent ensembles of conserved conformational states define the Hsp90 chaperone ATPase cycle. Mol Cell 32:631–640
17. Lavery LA, Partridge JR, Ramelot TA et al (2014) Structural asymmetry in the closed state of mitochondrial Hsp90 (TRAP1) supports a two-step ATP hydrolysis mechanism. Mol Cell 53:330–343

18. Kirschke E, Goswami D, Southworth D et al (2014) Glucocorticoid receptor function regulated by coordinated action of the Hsp90 and Hsp70 chaperone cycles. Cell 157:1685–1697
19. Powers MV, Clarke PA, Workman P (2009) Death by chaperone: HSP90, HSP70 or both? Cell Cycle 8:518–526
20. Samant RS, Clarke PA, Workman P (2012) The expanding proteome of the molecular chaperone HSP90. Cell Cycle 11:1301–1308
21. Taipale M, Jarosz DF, Lindquist S (2010) HSP90 at the hub of protein homeostasis: emerging mechanistic insights. Nat Rev Mol Cell Biol 11:515–528
22. Taipale M, Krykbaeva I, Koeva M et al (2012) Quantitative analysis of HSP90-client interactions reveals principles of substrate recognition. Cell 150:987–1001
23. Pratt WB, Morishima Y, Gestwicki JE et al (2014) A model in which heat shock protein 90 targets protein-folding clefts: rationale for a new approach to neuroprotective treatment of protein folding diseases. Exp Biol Med (Maywood) 239:1405–1413
24. Connell P, Ballinger CA, Jiang J et al (2001) The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. Nat Cell Biol 3:93–96
25. Xu W, Mimnaugh EG, Kim JS et al (2002) Hsp90, not Grp94, regulates the intracellular trafficking and stability of nascent ErbB2. Cell Stress Chaperones 7:91–96
26. Pearl LH, Prodromou C, Workman P (2008) The Hsp90 molecular chaperone: an open and shut case for treatment. Biochem J 410:439–453
27. Whitesell L, Lindquist SL (2005) HSP90 and the chaperoning of cancer. Nat Rev Cancer 5:761–772
28. Chaudhury S, Welch TR, Blagg BS (2006) Hsp90 as a target for drug development. ChemMedChem 1:1331–1340
29. Schulte TW, Akinaga S, Soga S et al (1998) Antibiotic radicicol binds to the N-terminal domain of Hsp90 and shares important biologic activities with geldanamycin. Cell Stress Chaperones 3:100–108
30. Schulte TW, Neckers LM (1998) The benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin binds to HSP90 and shares important biologic activities with geldanamycin. Cancer Chemother Pharmacol 42:273–279
31. Whitesell L, Mimnaugh EG, De Costa B et al (1994) Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. Proc Natl Acad Sci U S A 91:8324–8328
32. Donnelly A, Blagg BS (2008) Novobiocin and additional inhibitors of the Hsp90 C-terminal nucleotide-binding pocket. Curr Med Chem 15:2702–2717
33. Eskew JD, Sadikot T, Morales P et al (2011) Development and characterization of a novel C-terminal inhibitor of Hsp90 in androgen dependent and independent prostate cancer cells. BMC Cancer 11:468
34. Patwardhan CA, Fauq A, Peterson LB et al (2013) Gedunin inactivates the co-chaperone p23 protein causing cancer cell death by apoptosis. J Biol Chem 288:7313–7325
35. Pimienta G, Herbert KM, Regan L (2011) A compound that inhibits the HOP-Hsp90 complex formation and has unique killing effects in breast cancer cell lines. Mol Pharm 8:2252–2261
36. Zhang T, Hamza A, Cao X et al (2008) A novel Hsp90 inhibitor to disrupt Hsp90/Cdc37 complex against pancreatic cancer cells. Mol Cancer Ther 7:162–170
37. Ardi VC, Alexander LD, Johnson VA et al (2011) Macrocycles that inhibit the binding between heat shock protein 90 and TPR-containing proteins. ACS Chem Biol 6:1357–1366
38. Zuehlke A, Johnson JL (2010) Hsp90 and co-chaperones twist the functions of diverse client proteins. Biopolymers 93:211–217
39. Li J, Soroka J, Buchner J (2012) The Hsp90 chaperone machinery: conformational dynamics and regulation by co-chaperones. Biochim Biophys Acta 1823:624–635
40. Kamal A, Thao L, Sensintaffar J et al (2003) A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. Nature 425:407–410

41. Rohl A, Rohrberg J, Buchner J (2013) The chaperone Hsp90: changing partners for demanding clients. Trends Biochem Sci 38:253–262
42. Vasko RC, Rodriguez RA, Cunningham CN et al (2010) Mechanistic studies of Sansalvamide A-amide: an allosteric modulator of Hsp90. ACS Med Chem Lett 1:4–8
43. McConnell JR, Alexander LA, McAlpine SR (2014) A heat shock protein 90 inhibitor that modulates the immunophilins and regulates hormone receptors without inducing the heat shock response. Bioorg Med Chem Lett 24:661–666
44. Bertelsen EB, Chang L, Gestwicki JE et al (2009) Solution conformation of wild-type E. coli Hsp70 (DnaK) chaperone complexed with ADP and substrate. Proc Natl Acad Sci U S A 106:8471–8476
45. Bork P, Sander C, Valencia A (1992) An ATPase domain common to prokaryotic cell cycle proteins, sugar kinases, actin, and hsp70 heat shock proteins. Proc Natl Acad Sci U S A 89:7290–7294
46. Massey AJ (2010) ATPases as drug targets: insights from heat shock proteins 70 and 90. J Med Chem 53:7280–7286
47. Wang H, Kurochkin AV, Pang Y et al (1998) NMR solution structure of the 21 kDa chaperone protein DnaK substrate binding domain: a preview of chaperone-protein interaction. Biochemistry 37:7929–7940
48. Smock RG, Rivoire O, Russ WP et al (2010) An interdomain sector mediating allostery in Hsp70 molecular chaperones. Mol Syst Biol 6:414
49. Swain JF, Dinler G, Sivendran R et al (2007) Hsp70 chaperone ligands control domain association via an allosteric mechanism mediated by the interdomain linker. Mol Cell 26:27–39
50. Zuiderweg ER, Bertelsen EB, Rousaki A et al (2013) Allostery in the Hsp70 chaperone proteins. Top Curr Chem 328:99–153
51. Zhang Y, Zuiderweg ER (2004) The 70-kDa heat shock protein chaperone nucleotide-binding domain in solution unveiled as a molecular machine that can reorient its functional subdomains. Proc Natl Acad Sci U S A 101:10272–10277
52. Ahmad A, Bhattacharya A, McDonald RA et al (2011) Heat shock protein 70 kDa chaperone/DnaJ cochaperone complex employs an unusual dynamic interface. Proc Natl Acad Sci U S A 108:18966–18971
53. Mayer MP, Schroder H, Rudiger S et al (2000) Multistep mechanism of substrate binding determines chaperone activity of Hsp70. Nat Struct Biol 7:586–593
54. Vogel M, Bukau B, Mayer MP (2006) Allosteric regulation of Hsp70 chaperones by a proline switch. Mol Cell 21:359–367
55. General IJ, Liu Y, Blackburn ME et al (2014) ATPase subdomain IA is a mediator of interdomain allostery in Hsp70 molecular chaperones. PLoS Comput Biol 10, e1003624
56. Assimon VA, Gillies AT, Rauch JN et al (2013) Hsp70 protein complexes as drug targets. Curr Pharm Des 19:404–417
57. Rauch JN, Gestwicki JE (2014) Binding of human nucleotide exchange factors to heat shock protein 70 (Hsp70) generates functionally distinct complexes in vitro. J Biol Chem 289:1402–1414
58. Sondermann H, Scheufler C, Schneider C et al (2001) Structure of a Bag/Hsc70 complex: convergent functional evolution of Hsp70 nucleotide exchange factors. Science 291:1553–1557
59. Liu FH, Wu SJ, Hu SM et al (1999) Specific interaction of the 70-kDa heat shock cognate protein with the tetratricopeptide repeats. J Biol Chem 274:34425–34432
60. Connarn JN, Assimon VA, Reed RA et al (2014) The molecular chaperone Hsp70 activates protein phosphatase 5 (PP5) by binding the tetratricopeptide repeat (TPR) domain. J Biol Chem 289:2908–2917
61. Smith MC, Scaglione KM, Assimon VA et al (2013) The E3 ubiquitin ligase CHIP and the molecular chaperone Hsc70 form a dynamic, tethered complex. Biochemistry 52:5354–5364
62. Cortajarena AL, Regan L (2006) Ligand binding by TPR domains. Protein Sci 15:1193–1198

63. Chen S, Smith DF (1998) Hop as an adaptor in the heat shock protein 70 (Hsp70) and hsp90 chaperone machinery. J Biol Chem 273:35194–35200
64. Johnson BD, Schumacher RJ, Ross ED et al (1998) Hop modulates Hsp70/Hsp90 interactions in protein folding. J Biol Chem 273:3679–3686
65. Ballinger CA, Connell P, Wu Y et al (1999) Identification of CHIP, a novel tetratricopeptide repeat-containing protein that interacts with heat shock proteins and negatively regulates chaperone functions. Mol Cell Biol 19:4535–4545
66. Hohfeld J, Cyr DM, Patterson C (2001) From the cradle to the grave: molecular chaperones that may choose between folding and degradation. EMBO Rep 2:885–890
67. Muller P, Ruckova E, Halada P et al (2012) C-terminal phosphorylation of Hsp70 and Hsp90 regulates alternate binding to co-chaperones CHIP and HOP to determine cellular protein folding/degradation balances. Oncogene. doi:10.1038/onc.2012.314
68. Rudiger S, Germeroth L, Schneider-Mergener J et al (1997) Substrate specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries. EMBO J 16:1501–1507
69. Zhu X, Zhao X, Burkholder WF et al (1996) Structural analysis of substrate binding by the molecular chaperone DnaK. Science 272:1606–1614
70. Srinivasan SR, Gillies AT, Chang L et al (2012) Molecular chaperones DnaK and DnaJ share predicted binding sites on most proteins in the E. coli proteome. Mol BioSyst 8:2323–2333
71. Koren J 3rd, Jinwal UK, Jin Y et al (2010) Facilitating Akt clearance via manipulation of Hsp70 activity and levels. J Biol Chem 285:2498–2505
72. Meng L, Hunt C, Yaglom JA et al (2011) Heat shock protein Hsp72 plays an essential role in Her2-induced mammary tumorigenesis. Oncogene 30:2836–2845
73. Powers MV, Clarke PA, Workman P (2008) Dual targeting of HSC70 and HSP72 inhibits HSP90 function and induces tumor-specific apoptosis. Cancer Cell 14:250–262
74. Powers MV, Jones K, Barillari C et al (2010) Targeting HSP70: the second potentially druggable heat shock protein and molecular chaperone? Cell Cycle 9:1542–1550
75. Walerych D, Olszewski MB, Gutkowska M et al (2009) Hsp70 molecular chaperones are required to support p53 tumor suppressor activity under stress conditions. Oncogene 28:4284–4294
76. Barouch W, Prasad K, Greene LE et al (1994) ATPase activity associated with the uncoating of clathrin baskets by Hsp70. J Biol Chem 269:28563–28568
77. Jaattela M (1999) Heat shock proteins as cellular lifeguards. Ann Med 31:261–271
78. Sherman MY, Gabai VL (2014) Hsp70 in cancer: back to the future. Oncogene. doi:10.1038/onc.2014.349
79. Jaattela M (1995) Over-expression of hsp70 confers tumorigenicity to mouse fibrosarcoma cells. Int J Cancer 60:689–693
80. Ran Q, Wadhwa R, Kawai R et al (2000) Extramitochondrial localization of mortalin/mthsp70/PBP74/GRP75. Biochem Biophys Res Commun 275:174–179
81. Lu WJ, Lee NP, Kaul SC et al (2011) Mortalin-p53 interaction in cancer cells is stress dependent and constitutes a selective target for cancer therapy. Cell Death Differ 18:1046–1056
82. Knittler MR, Dirks S, Haas IG (1995) Molecular chaperones involved in protein degradation in the endoplasmic reticulum: quantitative interaction of the heat shock cognate protein BiP with partially folded immunoglobulin light chains that are degraded in the endoplasmic reticulum. Proc Natl Acad Sci U S A 92:1764–1768
83. Deocaris CC, Widodo N, Shrestha BG et al (2007) Mortalin sensitizes human cancer cells to MKT-077-induced senescence. Cancer Lett 252:259–269
84. Wadhwa R, Sugihara T, Yoshida A et al (2000) Selective toxicity of MKT-077 to cancer cells is mediated by its binding to the hsp70 family protein mot-2 and reactivation of p53 function. Cancer Res 60:6818–6821
85. Nylandsted J, Brand K, Jaattela M (2000) Heat shock protein 70 is required for the survival of cancer cells. Ann N Y Acad Sci 926:122–125

86. Rohde M, Daugaard M, Jensen MH et al (2005) Members of the heat-shock protein 70 family promote cancer cell growth by distinct mechanisms. Genes Dev 19:570–582
87. Nanbu K, Konishi I, Mandai M et al (1998) Prognostic significance of heat shock proteins HSP70 and HSP90 in endometrial carcinomas. Cancer Detect Prev 22:549–555
88. Ciocca DR, Calderwood SK (2005) Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. Cell Stress Chaperones 10:86–103
89. Jaattela M (1993) Overexpression of major heat shock protein hsp70 inhibits tumor necrosis factor-induced activation of phospholipase A2. J Immunol 151:4286–4294
90. Chalmin F, Ladoire S, Mignot G et al (2010) Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells. J Clin Invest 120:457–471
91. Park HS, Lee JS, Huh SH et al (2001) Hsp72 functions as a natural inhibitory protein of c-Jun N-terminal kinase. EMBO J 20:446–456
92. Saleh A, Srinivasula SM, Balkir L et al (2000) Negative regulation of the Apaf-1 apoptosome by Hsp70. Nat Cell Biol 2:476–483
93. Ravagnan L, Gurbuxani S, Susin SA et al (2001) Heat-shock protein 70 antagonizes apoptosis-inducing factor. Nat Cell Biol 3:839–843
94. Guo F, Sigua C, Bali P et al (2005) Mechanistic role of heat shock protein 70 in Bcr-Abl-mediated resistance to apoptosis in human acute leukemia cells. Blood 105:1246–1255
95. Gotoh T, Terada K, Oyadomari S et al (2004) hsp70-DnaJ chaperone pair prevents nitric oxide- and CHOP-induced apoptosis by inhibiting translocation of Bax to mitochondria. Cell Death Differ 11:390–402
96. Stankiewicz AR, Lachapelle G, Foo CP et al (2005) Hsp70 inhibits heat-induced apoptosis upstream of mitochondria by preventing Bax translocation. J Biol Chem 280:38729–38739
97. Colvin TA, Gabai VL, Gong J et al (2014) Hsp70-Bag3 interactions regulate cancer-related signaling networks. Cancer Res 74:4731–4740
98. Kabakov AE, Gabai VL (1995) Heat shock-induced accumulation of 70-kDa stress protein (HSP70) can protect ATP-depleted tumor cells from necrosis. Exp Cell Res 217:15–21
99. Leu JI, Pimkina J, Frank A et al (2009) A small molecule inhibitor of inducible heat shock protein 70. Mol Cell 36:15–27
100. Evans CG, Chang L, Gestwicki JE (2010) Heat shock protein 70 (hsp70) as an emerging drug target. J Med Chem 53:4585–4602
101. Patury S, Miyata Y, Gestwicki JE (2009) Pharmacological targeting of the Hsp70 chaperone. Curr Top Med Chem 9:1337–1351
102. Brodsky JL, Chiosis G (2006) Hsp70 molecular chaperones: emerging roles in human disease and identification of small molecule modulators. Curr Top Med Chem 6:1215–1225
103. Repalli J, Meruelo D (2015) Screening strategies to identify HSP70 modulators to treat Alzheimer's disease. Drug Des Devel Ther 9:321–331
104. Leu JI, Zhang P, Murphy ME et al (2014) Structural basis for the inhibition of HSP70 and DnaK chaperones by small-molecule targeting of a C-terminal allosteric pocket. ACS Chem Biol 9:2508–2516
105. Balaburski GM, Leu JI, Beeharry N et al (2013) A modified HSP70 inhibitor shows broad activity as an anticancer agent. Mol Cancer Res 11:219–229
106. Leu JI, Pimkina J, Pandey P et al (2011) HSP70 inhibition by the small-molecule 2-phenylethynesulfonamide impairs protein clearance pathways in tumor cells. Mol Cancer Res 9:936–947
107. Kaiser M, Kuhnl A, Reins J et al (2011) Antileukemic activity of the HSP70 inhibitor pifithrin-mu in acute leukemia. Blood Cancer J 1, e28
108. Kirkegaard T, Roth AG, Petersen NH et al (2010) Hsp70 stabilizes lysosomes and reverts Niemann-Pick disease-associated lysosomal pathology. Nature 463:549–553
109. Davis MJ, Gregorka B, Gestwicki JE et al (2012) Inducible renitence limits Listeria monocytogenes escape from vacuoles in macrophages. J Immunol 189:4488–4495

110. Schmitt E, Maingret L, Puig PE et al (2006) Heat shock protein 70 neutralization exerts potent antitumor effects in animal models of colon cancer and melanoma. Cancer Res 66:4191–4197
111. Umezawa H, Kondo S, Iinuma H et al (1981) Structure of an antitumor antibiotic, spergualin. J Antibiot (Tokyo) 34:1622–1624
112. Nishikawa K, Shibasaki C, Takahashi K et al (1986) Antitumor activity of spergualin, a novel antitumor antibiotic. J Antibiot (Tokyo) 39:1461–1466
113. Nemoto K, Abe F, Takita T et al (1987) Suppression of experimental allergic encephalomyelitis in guinea pigs by spergualin and 15-deoxyspergualin. J Antibiot (Tokyo) 40:1193–1194
114. Nemoto K, Hayashi M, Abe F et al (1987) Suppression of humoral immunity in mice by spergualin. Transplant Proc 19:4638–4640
115. Nishizawa R, Takei Y, Yoshida M et al (1988) Synthesis and biological activity of spergualin analogues. I J Antibiot (Tokyo) 41:1629–1643
116. Lebreton L, Annat J, Derrepas P et al (1999) Structure-immunosuppressive activity relationships of new analogues of 15-deoxyspergualin. 1. Structural modifications of the hydroxyglycine moiety. J Med Chem 42:277–290
117. Krieger NR, Emre S (2004) Novel immunosuppressants. Pediatr Transplant 8:594–599
118. Kaufman DB, Gores PF, Kelley S et al (1996) 15-Deoxyspergualin: Immunotherapy in solid organ and cellular transplantation. Transplant Rev 10:160–174
119. Elices MJ (2001) Tresperimus (Laboratoires Fournier). Curr Opin Investig Drugs 2:372–374
120. Nadler SG, Dischino DD, Malacko AR et al (1998) Identification of a binding site on Hsc70 for the immunosuppressant 15-deoxyspergualin. Biochem Biophys Res Commun 253:176–180
121. Nadler SG, Tepper MA, Schacter B et al (1992) Interaction of the immunosuppressant deoxyspergualin with a member of the Hsp70 family of heat shock proteins. Science 258:484–486
122. Umeda Y, Moriguchi M, Ikai K et al (1987) Synthesis and antitumor activity of spergualin analogues. III. Novel method for synthesis of optically active 15-deoxyspergualin and 15-deoxy-11-O-methylspergualin. J Antibiot (Tokyo) 40:1316–1324
123. Havlin KA, Kuhn JG, Koeller J et al (1995) Deoxyspergualin: phase I clinical, immunologic and pharmacokinetic study. Anticancer Drugs 6:229–236
124. Hassan AQ, Kirby CA, Zhou W et al (2015) The novolactone natural product disrupts the allosteric regulation of hsp70. Chem Biol 22:87–97
125. Kragol G, Lovas S, Varadi G et al (2001) The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding. Biochemistry 40:3016–3026
126. Cellitti J, Zhang Z, Wang S et al (2009) Small molecule DnaK modulators targeting the beta-domain. Chem Biol Drug Des 74:349–357
127. Williams DR, Ko SK, Park S et al (2008) An apoptosis-inducing small molecule that binds to heat shock protein 70. Angew Chem Int Ed Engl 47:7466–7469
128. Cho HJ, Gee HY, Baek KH et al (2011) A small molecule that binds to an ATPase domain of Hsc70 promotes membrane trafficking of mutant cystic fibrosis transmembrane conductance regulator. Journal of the American Chemical Society 133:20267–20276
129. Williamson DS, Borgognoni J, Clay A et al (2009) Novel adenosine-derived inhibitors of 70 kDa heat shock protein, discovered through structure-based design. J Med Chem 52:1510–1513
130. Macias AT, Williamson DS, Allen N et al (2011) Adenosine-derived inhibitors of 78 kDa glucose regulated protein (Grp78) ATPase: insights into isoform selectivity. J Med Chem 54:4034–4041
131. Fewell SW, Smith CM, Lyon MA et al (2004) Small molecule modulators of endogenous and co-chaperone-stimulated Hsp70 ATPase activity. J Biol Chem 279:51131–51140

132. Wisen S, Bertelsen EB, Thompson AD et al (2010) Binding of a small molecule at a protein-protein interface regulates the chaperone activity of hsp70-hsp40. ACS chemical biology 5:611–622
133. Jinwal UK, Miyata Y, Koren J 3rd et al (2009) Chemical manipulation of hsp70 ATPase activity regulates tau stability. J Neurosci 29:12079–12088
134. Wisen S, Androsavich J, Evans CG et al (2008) Chemical modulators of heat shock protein 70 (Hsp70) by sequential, microwave-accelerated reactions on solid phase. Bioorg Med Chem Lett 18:60–65
135. Chang L, Bertelsen EB, Wisén S et al (2008) High-throughput screen for small molecules that modulate the ATPase activity of the molecular chaperone DnaK. Anal Biochem 372:167–176
136. Wright CM, Chovatiya RJ, Jameson NE et al (2008) Pyrimidinone-peptoid hybrid molecules with distinct effects on molecular chaperone function and cell proliferation. Bioorg Med Chem 16:3291–3301
137. Wisen S, Gestwicki JE (2008) Identification of small molecules that modify the protein folding activity of heat shock protein 70. Anal Biochem 374:371–377
138. Braunstein MJ, Scott SS, Scott CM et al (2011) Antimyeloma Effects of the Heat Shock Protein 70 Molecular Chaperone Inhibitor MAL3-101. J Oncol 2011:232037
139. Adam C, Baeurle A, Brodsky JL et al (2014) The HSP70 modulator MAL3-101 inhibits Merkel cell carcinoma. PLoS One 9, e92041
140. Huryn DM, Brodsky JL, Brummond KM et al (2011) Chemical methodology as a source of small-molecule checkpoint inhibitors and heat shock protein 70 (Hsp70) modulators. Proc Natl Acad Sci U S A 108:6757–6762
141. Chiba Y, Kubota T, Watanabe M et al (1998) MKT-077, localized lipophilic cation: antitumor activity against human tumor xenografts serially transplanted into nude mice. Anticancer Res 18:1047–1052
142. Chiba Y, Kubota T, Watanabe M et al (1998) Selective antitumor activity of MKT-077, a delocalized lipophilic cation, on normal cells and cancer cells in vitro. J Surg Oncol 69:105–110
143. Koya K, Li Y, Wang H et al (1996) MKT-077, a novel rhodacyanine dye in clinical trials, exhibits anticarcinoma activity in preclinical studies based on selective mitochondrial accumulation. Cancer Res 56:538–543
144. Rousaki A, Miyata Y, Jinwal UK et al (2011) Allosteric drugs: the interaction of antitumor compound MKT-077 with human Hsp70 chaperones. J Mol Biol 411:614–632
145. Abisambra J, Jinwal UK, Miyata Y et al (2013) Allosteric Heat Shock Protein 70 Inhibitors Rapidly Rescue Synaptic Plasticity Deficits by Reducing Aberrant Tau. Biol Psychiatry. doi:10.1016/j.biopsych.2013.02.027
146. Wang AM, Miyata Y, Klinedinst S et al (2013) Activation of Hsp70 reduces neurotoxicity by promoting polyglutamine protein degradation. Nat Chem Biol 9:112–118
147. Miyata Y, Li X, Lee HF et al (2013) Synthesis and initial evaluation of YM-08, a blood-brain barrier permeable derivative of the heat shock protein 70 (Hsp70) inhibitor MKT-077. Which reduces Tau levels. ACS Chem Neurosci. doi:10.1021/cn300210g
148. Koren J 3rd, Miyata Y, Kiray J et al (2012) Rhodacyanine derivative selectively targets cancer cells and overcomes tamoxifen resistance. PLoS One 7, e35566
149. Li X, Srinivasan SR, Connarn J et al (2013) Analogs of the allosteric heat shock protein 70 (Hsp70) inhibitor, MKT-077, as anti-cancer agents. ACS Med Chem Lett 2013:4
150. Miyata Y, Koren J, Kiray J et al (2011) Molecular chaperones and regulation of tau quality control: strategies for drug discovery in tauopathies. Future Med Chem 3:1523–1537
151. Kawakami M, Koya K, Ukai T et al (1998) Structure-activity of novel rhodacyanine dyes as antitumor agents. J Med Chem 41:130–142
152. Takasu K, Terauchi H, Inoue H et al (2003) Parallel synthesis of antimalarial rhodacyanine dyes by the combination of three components in one pot. J Comb Chem 5:211–214
153. Kasmi-Mir S, Djafri A, Hamelin J et al (2007) Synthesis of new rhodacyanines analogous to MKT-077 under microwave irradiation. Synt Commun 37:4017–4034

154. Tatsuta N, Suzuki N, Mochizuki T et al (1999) Pharmacokinetic analysis and antitumor efficacy of MKT-077, a novel antitumor agent. Cancer Chemother Pharmacol 43:295–301
155. Li X, Colvin T, Rauch JN et al (2015) Validation of the Hsp70-Bag3 protein–protein interaction as a potential therapeutic target in cancer. Mol Cancer Ther. doi:10.1158/1535-7163.MCT-14-0650
156. Rodina A, Patel PD, Kang Y et al (2013) Identification of an allosteric pocket on human hsp70 reveals a mode of inhibition of this therapeutically important protein. Chem Biol 20:1469–1480
157. Miyata Y, Rauch JN, Jinwal UK et al (2012) Cysteine reactivity distinguishes redox sensing by the heat-inducible and constitutive forms of heat shock protein 70. Chem Biol 19:1391–1399
158. Wang Y, Gibney PA, West JD et al (2012) The yeast Hsp70 Ssa1 is a sensor for activation of the heat shock response by thiol-reactive compounds. Mol Biol Cell 23:3290–3298
159. Rodina A, Taldone T, Kang Y et al (2014) Affinity purification probes of potential use to investigate the endogenous Hsp70 interactome in cancer. ACS Chem Biol 9:1698–1705
160. Taldone T, Kang Y, Patel HJ et al (2014) Heat shock protein 70 inhibitors. 2. 2,5'-thiodipyrimidines, 5-(phenylthio)pyrimidines, 2-(pyridin-3-ylthio)pyrimidines, and 3-(phenylthio)pyridines as reversible binders to an allosteric site on heat shock protein 70. J Med Chem 57:1208–1224
161. Howe MK, Bodoor K, Carlson DA et al (2014) Identification of an allosteric small-molecule inhibitor selective for the inducible form of heat shock protein 70. Chem Biol 21:1648–1659
162. Daguer JP, Zambaldo C, Ciobanu M et al (2015) DNA display of fragment pairs as a tool for the discovery of novel biologically active small molecules. Chem Rev 6:739–744
163. McNamara AV, Barclay M, Watson AJ et al (2012) Hsp90 inhibitors sensitise human colon cancer cells to topoisomerase I poisons by depletion of key anti-apoptotic and cell cycle checkpoint proteins. Biochem Pharmacol 83:355–367
164. Stingl L, Stuhmer T, Chatterjee M et al (2010) Novel HSP90 inhibitors, NVP-AUY922 and NVP-BEP800, radiosensitise tumour cells through cell-cycle impairment, increased DNA damage and repair protraction. Br J Cancer 102:1578–1591
165. Wainberg ZA, Anghel A, Rogers AM et al (2013) Inhibition of HSP90 with AUY922 induces synergy in HER2-amplified trastuzumab-resistant breast and gastric cancer. Mol Cancer Ther 12:509–519
166. Lu X, Xiao L, Wang L et al (2012) Hsp90 inhibitors and drug resistance in cancer: the potential benefits of combination therapies of Hsp90 inhibitors and other anti-cancer drugs. Biochem Pharmacol 83:995–1004
167. Tatokoro M, Koga F, Yoshida S et al (2012) Potential role of Hsp90 inhibitors in overcoming cisplatin resistance of bladder cancer-initiating cells. Int J Cancer 131:987–996
168. Zhang H, Neely L, Lundgren K et al (2010) BIIB021, a synthetic Hsp90 inhibitor, has broad application against tumors with acquired multidrug resistance. Int J Cancer 126:1226–1234
169. Goloudina AR, Demidov ON, Garrido C (2012) Inhibition of HSP70: a challenging anti-cancer strategy. Cancer Lett 325:117–124
170. Davenport EL, Zeisig A, Aronson LI et al (2010) Targeting heat shock protein 72 enhances Hsp90 inhibitor-induced apoptosis in myeloma. Leukemia 24:1804–1807
171. Wang Y, McAlpine SR (2015) Regulating the cytoprotective response in cancer cells using simultaneous inhibition of Hsp90 and Hsp70. Org Biomol Chem 13:2108–2116
172. Wang Y, McAlpine SR (2015) N-terminal and C-terminal modulation of Hsp90 produce dissimilar phenotypes. Chem Commun (Camb) 51:1410–1413
173. Crawford LJ, Walker B, Irvine AE (2011) Proteasome inhibitors in cancer therapy. J Cell Commun Signal 5:101–110
174. Mathew R, Karantza-Wadsworth V, White E (2007) Role of autophagy in cancer. Nat Rev Cancer 7:961–967

175. Gaspar N, Sharp SY, Pacey S et al (2009) Acquired resistance to 17-allylamino-17-demethoxygeldanamycin (17-AAG, tanespimycin) in glioblastoma cells. Cancer Res 69:1966–1975
176. Kummar S, Gutierrez ME, Gardner ER et al (2010) Phase I trial of 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), a heat shock protein inhibitor, administered twice weekly in patients with advanced malignancies. Eur J Cancer 46:340–347
177. Lancet JE, Gojo I, Burton M et al (2010) Phase I study of the heat shock protein 90 inhibitor alvespimycin (KOS-1022, 17-DMAG) administered intravenously twice weekly to patients with acute myeloid leukemia. Leukemia 24:699–705
178. Budina-Kolomets A, Balaburski GM, Bondar A et al (2014) Comparison of the activity of three different HSP70 inhibitors on apoptosis, cell cycle arrest, autophagy inhibition and HSP90 inhibition. Cancer Biol Ther 15:1–6
179. Bercovich B, Stancovski I, Mayer A et al (1997) Ubiquitin-dependent degradation of certain protein substrates in vitro requires the molecular chaperone Hsc70. J Biol Chem 272:9002–9010
180. Frydman J (2001) Folding of newly translated proteins in vivo: the role of molecular chaperones. Annu Rev Biochem 70:603–647
181. Hartl FU, Bracher A, Hayer-Hartl M (2011) Molecular chaperones in protein folding and proteostasis. Nature 475:324–332
182. Kettern N, Rogon C, Limmer A et al (2011) The Hsc/Hsp70 co-chaperone network controls antigen aggregation and presentation during maturation of professional antigen presenting cells. PLoS One 6, e16398

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Hsp40 Co-chaperones as Drug Targets: Towards the Development of Specific Inhibitors

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Abstract The heat shock protein 40 (Hsp40/DNAJ) family of co-chaperones modulates the activity of the major molecular chaperone heat shock protein 70 (Hsp70) protein group. Hsp40 stimulates the basal ATPase activity of Hsp70 and hence regulates the affinity of Hsp70 for substrate proteins. The number of Hsp40 genes in most organisms is substantially greater than the number of Hsp70 genes. Therefore, different Hsp40 family members may regulate different activities of the same Hsp70. This fact, along with increasing knowledge of the function of Hsp40 in diseases, has led to certain Hsp40 isoforms being considered promising drug targets. Here we review the role of Hsp40 in human disease and recent developments towards the creation of Hsp40-specific inhibitors.

Keywords Cancer, Co-chaperone, DNAJ, Hsp40, Hsp70, Malaria, Neurodegeneration

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Contents

1 Introduction to Hsp40 Co-chaperones

The heat shock protein 40 kDa (Hsp40/DNAJ) family is the largest and most diverse family of Hsps [1–3]. Hsp40 proteins function predominantly as co-chaperones for the Hsp70 (HSPA) chaperones [4–7], although some Hsp40s display independent chaperone activity in that they can bind and prevent the aggregation of substrate proteins [8–13]. A chaperone is defined as a protein that catalyses the folding and conformational change of other proteins but does not constitute part of the final protein product. A co-chaperone would be an accessory protein that regulates the activity of the chaperone, but normally would not be capable of independent chaperone activity. Hsp70 is a major molecular chaperone that regulates the folding, translation, translocation and conformation of nascent and stress-denatured proteins [14, 15]. Hsp70 chaperones are composed of three domains, the 44 kDa N-terminal ATPase or nucleotide-binding domain (NBD), the substrate-binding domain (~18 kDa) and a C-terminal domain of 10 kDa, which terminates in the highly conserved EEVD motif in cytosolic Hsp70s (Fig. 1) [18–20].

The chaperone activity of Hsp70 is cyclical and controlled by the binding and hydrolysis of ATP, which modifies the affinity of the Hsp70 for its protein substrate or client [21]. Hsp70 in an ATP-bound form has a lower affinity for a substrate than ADP-bound Hsp70. Whether Hsp70 is in an ATP- or ADP-bound form depends largely on the ATPase activity of Hsp70. Hsp70 is mostly in the ATP-bound state due to its low basal ATPase activity; however, the ATPase activity can be stimulated by the Hsp40 co-chaperone. Hsp40 captures the non-native conformations of client proteins and delivers them to the Hsp70 chaperone [22]. The subsequent stimulation of the ATPase activity of the Hsp70 by the Hsp40 leads to an increased affinity of the Hsp70 for the client protein, thereby promoting the transfer of the

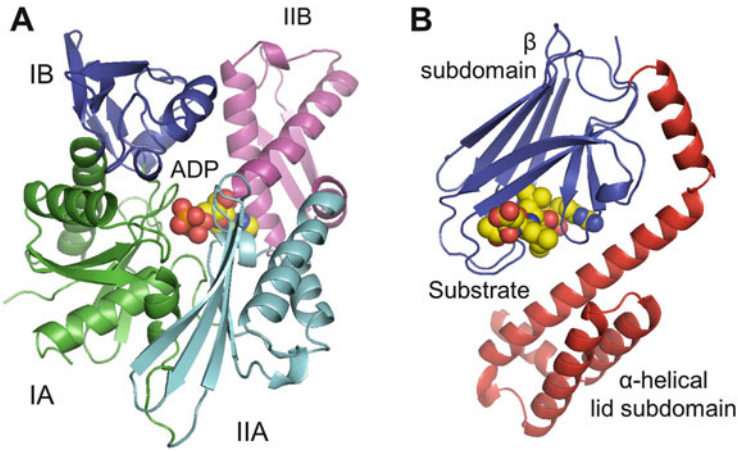

Fig. 1 Structures of key Hsp70 domains. (**a**) The nucleotide-binding domain (NBD) of human Hsc70 (HSPA8) in the ADP-bound form [16]. The four subdomains of the NBD are shown as cartoons and coloured as follows: subdomain IA (residues 1–39, 116–188 and 361–381) is shown in green, subdomain IIA (residues 189–228 and 307–360) is shown in *cyan*, subdomain IB (residues 40–115) is shown in *blue*, subdomain IIB (residues 229–306) is shown in *magenta*. (**b**) The substrate-binding domain (SBD) of *E. coli* DnaK (residues 390–600) bound to a substrate peptide which is shown as spheres [17, 18]. The SBD is divided into two subdomains, the α helical lid subdomain (residues 510–638) shown in *red* and the β subdomain that binds the peptide (residues 390–509) shown in *blue*. The structures were rendered using Pymol and the PDB accession numbers for the Hsc70 NDB and DnaK SBD are 3HSC and 1DKX, respectively

client protein from Hsp40 to Hsp70. The ATP-bound form of the Hsp70 is regenerated by nucleotide exchange which is catalysed by GrpE in prokaryotes and the BAG family of co-chaperones in eukaryotes [23, 24]. Exchange of ADP for ATP on Hsp70 leads to a drop in client affinity and release of the properly folded client protein or leaves the protein awaiting rebinding for successive cycles of chaperoning (Fig. 2).

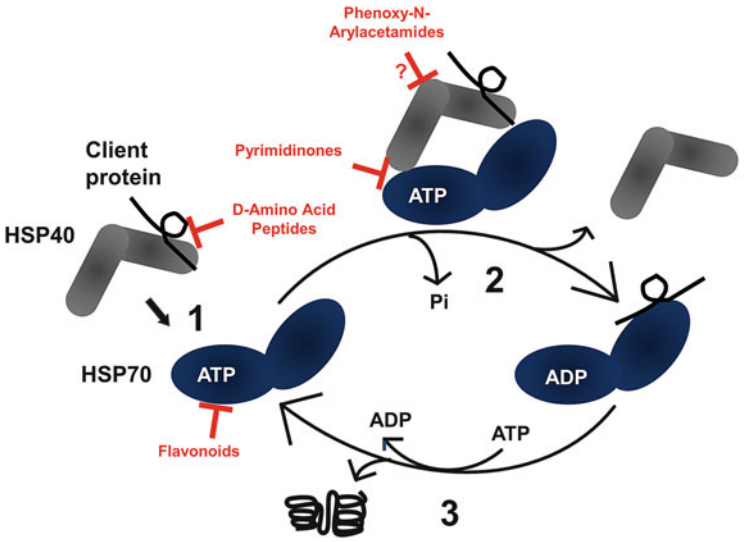

Fig. 2 The Hsp40-Hsp70 chaperone cycle and the site of action of Hsp40 inhibitors. Hsp40 associates with newly synthesised or misfolded protein substrates and delivers them to Hsp70 (*1*). The Hsp40 and substrate stimulate the ATP hydrolysis activity of Hsp70, converting it from a low (ATP-bound) to high (ADP-bound) substrate affinity state (*2*). Hsp70 is restored to its ATP-bound state by nucleotide exchange factors (*3*). The substrate is released to fold into a functional protein or re-enters the Hsp70 cycle. Inhibitors of Hsp40 are indicated in red by name and with a *blunt arrow head* (⊢) to show the site of action. Flavonoids indirectly affect Hsp40 binding to Hsp70 by associating with a subdomain of the N-terminal ATPase domain of Hsp70 and causing an allosteric disruption of Hsp40 binding. D-amino acid peptides inhibit Hsp40 function by competing with substrate binding to Hsp40. Certain pyrimidinones have been shown to bind to the Hsp40-binding site on the underside of the Hsp70 N-terminal ATPase domain thereby directly inhibiting J domain-based Hsp40 interaction with Hsp70. The phenoxy-*N*-arylacetamides bind to Hsp40 and inhibit the interaction of Hsp40 with Hsp70; however, their site of action is not yet known and is thus shown with a *question mark* (?). Hsp70 is shown schematically as navy-filled objects, while Hsp40 is shown as *grey-filled objects*. The unfolded protein substrate is indicated as a *line symbol* with a single coil, while the folded protein is shown as a line completely coiled upon itself

2 Structure and Classification of Hsp40

In humans there are 13 Hsp70s and 47 Hsp40s encoded on the genome [15] and are distributed amongst different subcellular compartments. Hsp70s and Hsp40s have been identified in the mitochondria, endoplasmic reticulum, nucleus and cytoplasm. The number of Hsp40 homologues in each compartment outnumbers the number of Hsp70s [15]. This suggests that the same Hsp70 may interact with different Hsp40s, presumably to fulfil distinct functions. Therefore, various Hsp40s may fine-tune the function of the same Hsp70, depending on the cellular context. Despite the fact that the different Hsp70s and Hsp40s are structurally conserved, they have distinct chaperone activities [11].

The current classification system groups Hsp40 proteins depending on the similarity of the domain architecture to DnaJ, the Hsp40 homologue from *Escherichia coli*. DnaJ contains four distinct domains that define its function, namely, the J domain, a glycine phenylalanine (G/F)-rich region, a cysteine repeat region and a C-terminal region [15, 25, 26]. Type I Hsp40 (DNAJA) shares all four domains with *E. coli* DnaJ. Type II Hsp40 (DNAJB) possesses the J domain, G/F region and C-terminal region, while type III Hsp40 (DNAJC) only contains the J domain in common with *E. coli* DnaJ [15, 26]. In DNAJA and DNAJB, the J domain is located at the N-terminus, while in DNAJC proteins, the J domain can be located anywhere within the protein.

In humans, there are 4 DNAJA, 13 DNAJB and 30 DNAJC genes, although the number of DNAJ proteins is greater due to the fact that some Hsp40s are expressed as different isoforms or splice variants [1, 27]. The type I and II Hsp40 are considered the canonical Hsp70 co-chaperones, in that the function of these Hsp40 proteins is predominantly to facilitate refolding or maturation of de novo synthesised or aggregated client proteins. The type III Hsp40s are the most diverse group and contain a number of members that have multiple functional domains in addition to the J domain. These include protein-protein interaction modules (e.g. tetratricopeptide/TPR domains in DNAJC3 and DNAJC7), protein kinase domains (e.g. DNAJC26) and specialised domains like SANT (Swi3, Ada2, N-CoR and TFIIIB) domains (e.g. DNAJC1 and DNAJC2) and HEPN (higher eukaryotes and prokaryotes nucleotide-binding domain), amongst others. The specific function of many of these domains remains unknown. For a detailed table describing the specific domain architectures of the known Hsp40 proteins in humans and yeast, the reader is referred to the supplementary data accompanying the review by Kampinga and Craig [1]

All Hsp40s contain a J domain, and it is considered the defining characteristic of an Hsp40 protein. The J domain is a highly conserved 70 amino acid motif, forming a three-dimensional structure of four α-helices (numbered sequentially I–IV from the N-terminal to C end of the domain) (Fig. 3). The α-helices are arranged relative to each other in space to form a structure resembling the letter "J" that lends its name to the domain [28, 29]. The helices are connected by loop regions, the most important of which is the loop between helices II and III. This loop region, which varies in size between the different Hsp40 homologues, houses a conserved tripeptide motif, known as the histidine-proline-aspartic acid (HPD) motif [30]. The HPD motif is required for the stimulation of the ATPase activity of the partner Hsp70 chaperone. Mutations of this conserved motif render the Hsp40 incapable of stimulating the ATPase activity of Hsp70 above basal levels and therefore impair co-chaperone activity [31]. While the J domain is an essential part of the Hsp40 proteins, the other domains have important functions as well. The G/F-rich region is largely unstructured and thought to act mainly as a linker region. However, this region has been shown to be important in controlling the specificity of interaction between certain Hsp70s and Hsp40s [32]. The cysteine repeat regions are identified by the presence of four copies of the motif CXXCXGXG, where X is any amino acid. The cysteine repeats form a zinc fingerlike region by the coordination of two

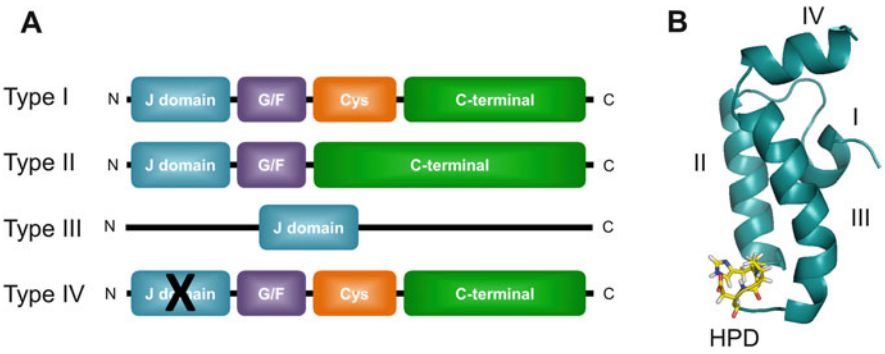

Fig. 3 Domain architecture of Hsp40. (**a**) Schematic representations of the structural domains in type I, II, III and IV Hsp40 proteins. All Hsp40 types contain the signature J domain required for stimulation of the Hsp70 ATPase activity through the conserved tripeptide histidine-proline-aspartic acid (HPD) motif. In type I and II Hsp40, the J domain is usually N-terminal, while in the type III Hsp40, this domain can be found at any position in the protein. The glycine/phenylalanine-rich region (G/F) is found in type I and II Hsp40 and functions as a hinge and regulates the specificity of Hsp70-Hsp40 interactions. The cysteine repeat region (Cys) found only in type I Hsp40 forms a zinc fingerlike structure involved in substrate binding. The C-terminal regions of type I and II Hsp40 are involved in substrate recognition and binding. Type IV Hsp40 is a recently identified subtype in which the conserved HPD motif in the J domain is mutated. (**b**) The J domain of human Hdj2 (DNAJA1) in cartoon format showing the four helices (I–IV) and the location of the highly conserved HPD motif resident in the loop region between helices II and III. The structure was rendered using Pymol, and the PDB accession number for the Hdj1 J domain is 2M6Y

zinc atoms in a tetrahedral arrangement. This region is thought to be involved in the binding of protein substrates and the prevention of aggregation [33, 34]. There is evidence to suggest that the substrate-binding regions are distinct in each Hsp40 and may be specialised for certain types of substrates. Indeed, substrate recognition and binding specificity, as opposed to domain conservation, has been proposed as an alternative approach to classify the Hsp40 members into groups [1]. The remainder of the C-terminus of Hsp40 may contain a number of different domains with distinct functions, including transmembrane domains and a number of protein-protein interaction motifs. Certain eukaryotic Hsp40s contain a CAAX motif at the extreme C-terminus, which is the site of prenylation leading to membrane association that is required for the chaperoning of certain client proteins [35].

3 Hsp40 as Drug Target in Humans

It is only recently that Hsp40 has been considered a possible drug target for the treatment of disease. This is mostly due to the large number of Hsp40 proteins, many of which have not been fully characterised. Indeed, for some Hsp40 genes,

Table 1 Type I Hsp40/DNAJ proteins in humans and links to some human diseases

Genes	General function of Hsp40 class	Protein members		Disease association/ function	Reference
		Name	Alternate name/s		
4	Canonical Hsp70 co-chaperones for client protein binding and delivery to Hsp70 during de novo and stress-related protein folding	DNAJA1	Hsj2/Dj-2/ DJA1, HDJ1	Viral replication, tumour suppressor function in pancreatic cancer, inhibits HIV production in vitro	[36–39]
		DNAJA2	DNAJ/ DNAJ3	Possible link to proarrhythmic cardiac long QT syndrome type 2 via trafficking of hERG	[40]
		DNAJA3	hTid-1/ TID1	Tumour suppressor, low levels prognostic for less favourable outcome in breast cancer; regulation of p53 and Her2	[41–44]
		DNAJA4	Hsj4/DJ4	Possible link to proarrhythmic cardiac long QT syndrome type 2 via trafficking of hERG	[40]

The reader is also referred to the review article by Kampinga and Craig for more information on domain structure and subcellular localisations [1]
hERG the human ether-a-go-go-related gene potassium channel

the presence of a protein product remains to be conclusively demonstrated [1]. There are studies that link many Hsp40 isoforms to human diseases, some of which are summarised in Tables 1, 2 and 3. However, for many of these proteins, we lack the mechanistic insight to determine whether Hsp40s are the causative agents in these disorders or are merely correlated with the disease. Despite this knowledge gap, some Hsp40s have been well described. Those Hsp40s, whose specific function is understood, have demonstrated the potential to be considered drug targets in human diseases, including cancer and neurodegenerative diseases where protein misfolding is a hallmark. In addition, Hsp40s are also involved in a number of infectious diseases, including viral [123] and parasitic diseases [124, 125]. Indeed, many of the Hsp40 proteins of malaria are better characterised than their human counterparts and are considered possible drug targets due to their unique features.

Table 2 Type II Hsp40/DNAJ proteins in humans and their links to some human diseases

Genes	General function of Hsp40 class	Protein members		Disease association/ function	Reference
		Name	Alternate name/s		
13	Specialised co-chaperone, client protein binding and delivery to Hsp70 during de novo and stress-related protein folding, aggregation suppression	DNAJB1	Hdj-1/ Hsp40	Required for HIV replication, inhibits virus production and interacts with Nef, Tat and capsid proteins; promotes cancer cell proliferation through MDM2 and p53	[38]
		DNAJB2[a]	Hsj1	Huntington's disease, neuronal disorders, mutations lead to heredity neuropathies	[45–48]
		DNAJB3	Hsj3/ Msj1/ HCG3	–	–
		DNAJB4	Hlj1/ Hsc40	Tumour suppressor in lung and colon cancer	[49–51]
		DNAJB5	Hsc40/ HSP40-3	–	–
		DNAJB6[a]	MRJ/ HSJ2/ MSJ1	Tumour suppressor and oncoprotein functions, mutations cause hereditary muscular dystrophies, Huntington's disease, suppresses aggregation of polyglutamine-expanded proteins, expression inhibits HIV production in vitro	[13, 27, 38, 52–63]
		DNAJB7	mDj5/Dj-5/HSC3	–	–
		DNAJB8	mDj6	Aggregation prevention of polyglutamine-expanded proteins; tumour initiation in renal cancer stem cells; immunotherapy target	[57, 64, 65]
		DNAJB9	ERdj-4/ Mdg1	Inhibits production of beta-amyloid	[66]

(continued)

Table 2 (continued)

Genes	General function of Hsp40 class	Protein members		Disease association/ function	Reference
		Name	Alternate name/s		
				peptides in Grp78-dependent manner	
		DNAJB11	ERdj-3/ HEDJ	Involved in degradation of glucocerebrosidase variants linked to Gaucher's disease; chaperone for KSHV K1; interacts with cholera toxin, salmonella SlrP	[67–70]
		DNAJB12[a]	mDj10/ DJ10	Chaperone for ER-mediated degradation of CFTR (cystic fibrosis)	[71, 72]
		DNAJB13[b]	TSARG6/ RSPH16A	–	–
		DNAJB14[a]	FLJ14281	–	–

[a]These Hsp40s have multiple splice variants, although not all have been demonstrated experimentally

[b]Although classified as a type II, the DNAJB13 isoform in humans lacks the HPD motif rendering the J domain non-functional. The reader is also referred to the review article by Kampinga and Craig for more information on domain structure and subcellular localisation [1]

CFTR cystic fibrosis transmembrane conductance regulator, *KSHV* Kaposi's sarcoma herpes virsus

3.1 Hsp40s as Drug Targets in Cancer and Neurodegenerative Diseases

There is a growing body of evidence to demonstrate a role for certain Hsp40s in cancer and neurodegenerative diseases in particular (Tables 1, 2 and 3). Hsp40 is interesting in that proteins appear to have both pro- and anticancer activities (reviewed in [2]). For example, DNAJA1, DNAJB4 and DNAJC15 have been shown to have anticancer effects, whereby overexpression of these Hsp40s led to a loss in malignant characteristics or conversely silencing increased malignant characteristics (Tables 1, 2 and 3). In contrast, DNAJC6, DNAJC12 and DNAJC9 are cancer promoting, and overexpression leads to an increase in metastasis and drug resistance [2].

In some instances, different splice variants of the same Hsp40 have been shown to have opposing (i.e. pro- and anticancer) activities. This phenomenon is best described for DNAJB6 (MRJ). DNAJB6 is expressed as two splice variants, DNAJB6a (DNAJB6L) and DNAJB6b (DNAJB6S). The two isoforms are

Table 3 Type III Hsp40/DNAJ proteins in humans and links to human disease

Genes	General function of Hsp40 class	Protein members		Disease association/ function	Reference
		Name	Alternate name/s		
30	Recruitment of Hsp70 for specific client proteins and specialised functions	DNAJC1	ERdj1, Mtj1	In close proximity to SNPs for breast and ovarian cancer risk; susceptibility loci for breast and ovarian cancer	[73, 74]
		DNAJC2	MPP11, zuotin	Overexpressed transcriptional regulator in leukaemia (AML), overexpressed in head and neck squamous cell cancer	[75, 76]
		DNAJC3	PPKRI, P58IPK	Loss of protein leads to diabetes mellitus and multisystemic neurodegeneration; viral infections; breast cancer	[77]
		DNAJC4[a]	HSPF2, MCG18	ND	–
		DNAJC5	CSP	Mutations cause neuronal ceroid lipofuscinoses (family of neurodegenerative diseases); interaction with, degradation and maturation of CFTR; expression inhibits HIV production in vitro	[38, 78–82]
		DNAJC6	Auxilin	Hepatocellular carcinoma; mutants lead to juvenile parkinsonism, deletions associated with obesity, mental retardation and epilepsy	[83–86]
		DNAJC7[a]	TTC2, TPR2, mDJ11	ND	–
		DNAJC8	SPF31	–	–
		DNAJC9	JDD1, HDJC9	Putative candidate gene for schizophrenia, mRNA upregulated in cervical cancer	[87–89]

(continued)

Table 3 (continued)

Genes	General function of Hsp40 class	Protein members		Disease association/ function	Reference
		Name	Alternate name/s		
		DNAJC10[a]	ERdj-5, JD1	Expression sensitised neuroblastoma to apoptosis; knockdown of ERdj5 increased the apoptotic response to fenretinide in neuroectodermal tumour cells; translocation of cholera toxin from ER to cytosol	[90–92]
		DNAJC11	FLJ10737	Mutants associated with motor neuron pathology; candidate tumour suppressor for neuroblastoma	[93, 94]
		DNAJC12[a]	JPD1	Overexpression associated with expression of ER in breast cancer	[95, 96]
		DNAJC13	RME-8, KIAA0678	SNP mutations associated with Parkinson's disease; SNP associated with chronic tic disorder	[97, 98]
		DNAJC14	HDJ3, DRIP78, LIP6	Overexpression modulates flavivirus (e.g. yellow fever virus) infection; overexpression or depletion inhibits flavivirus replication complex formation	[99, 100]
		DNAJC15	DNAJC15, MCJ	Hypermethylation leads to silencing which is associated with malignant paediatric brain tumours and drug resistance in ovarian cancer	[101–103]
		DNAJC16[a]	KIAA0962	ND	
		DNAJC17	FLJ10634	ND	
		DNAJC18	MGC29463	ND	–
		DNAJC19[a]	TIMM14, Tim14	Mutations associated with early-onset dilated cardiomyopathy syndrome (DCMA)	[104–106]

(continued)

Table 3 (continued)

Genes	General function of Hsp40 class	Protein members		Disease association/ function	Reference
		Name	Alternate name/s		
		DNAJC20	HSCB, Hsc20	Putative candidate gene for ataxia susceptibility	[107]
		DNAJC21[a]	DNAJA5, JJJ1	–	–
		DNAJC22	FLJ13236, wus	–	–
		DNAJC23	Sec63L, ERdj2	Mutations cause polycystic liver disease	[108–112]
		DNAJC24	DPH4, JJJ3	Silencing by hypermethylation is associated with immunotoxin resistance	[113]
		DNAJC25	bA16L21.2.1	ND	–
		DNAJC26[a]	GAK	SNPs associated with increased risk of Parkinson's disease; required for osteosarcoma proliferation and survival	[114–118]
		DNAJC27[a]	RBJ, RabJS	–	–
		DNAJC28	Orf28, C21orf55	–	–
		DNAJC29	ARSACS, sacsin	Processing of ataxia-linked proteins, mutations lead to autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS) (neurodegenerative disorder)	[119–121]
		DNAJC30	WBSCR18	Haploinsufficiency associated with Williams–Beuren syndrome (developmental disorder)	[122]

[a]These Hsp40s have multiple splice variants, although not all have been demonstrated experimentally. The reader is also referred to the review article by Kampinga and Craig for more information on domains structure and subcellular localisations [1]
AML acute myeloid leukaemia, *ER* oestrogen receptor, *SNP* single nucleotide polymorphism

identical, except that the longer isoform, DNAJB6a, contains a unique C-terminal extension that contains a functional nuclear localisation sequence (NLS) [15, 27]. Therefore, DNAJB6a is constitutively expressed in the nucleus. DNAJB6b is

expressed predominantly in the cytoplasm but has also been identified in the nucleus under conditions of stress, although the mechanism by which DNAJB6b is transported to the nucleus remains unclear [27, 126]. DNAJB6 appears to regulate cell cycle progression and cytoskeletal dynamics in cancer cells [127, 128]. Whereas DNAJB6b was widely expressed, overexpression of DNAJB6a in breast cancer led to a reduction in malignant characteristics [27]. This role for DNAJB6a as a tumour suppressor was determined to be via the upregulation of the Wnt signalling intermediate DKK1 (Dickkopf WNT signalling pathway inhibitor 1), which led to a reduction in the levels of β-catenin and reversion of epithelial to mesenchymal transition associated with cancer metastasis [52, 129]. This function was dependent on a functional J domain and required an interaction between DNAJB6, HSPA8 and GSK3β (glycogen synthase kinase 3 beta) [53].

Interestingly DNAJB6 is also highly expressed in the nervous system and is required for the maintenance of neural stem cells [130, 131]. Mutations in DNAJB6 have been linked to such diseases as autosomal dominant limb-girdle muscular dystrophy (LGMD), a rare condition characterised by muscle atrophy. In these cases, a novel mutation was identified in the G/F region of DNAJB6, not the J domain [54, 55, 132]. DNAJB6 was also detected in other neurodegenerative conditions, including Huntington's disease models as well as Lewy bodies and Parkinson's astrocytes [56, 130]. DNAJB6 was subsequently shown to be able to prevent the aggregation of polyglutamine-containing proteins, suggesting a protective effect [13]. The aggregation suppression activities of the different isoforms appear substrate specific, with DNAJB6a able to suppress aggregation of proteins in the nucleus but not cytoplasm. In contrast, DNAJB6b could suppress the aggregation of cytosolic polyglutamine-containing proteins [13].

The ability to suppress protein aggregation is also a feature of DNAJB8, another type II Hsp40, which has a very similar domain structure to DNAJB6 [57]. Both DNAJB6 and DNAJB8 can suppress the intracellular aggregation of polyglutamine-containing proteins. The formation of proteasome-resistant intracellular aggregates is the first stage of numerous neurodegenerative diseases, suggesting that activation of DNAJB8 and DNAJB6 may be one approach to prevention or treatment of these types of disorders [12]. DNAJB6 was also able to suppress the aggregation of amyloid in vitro, a protein involved in Alzheimer's disease, in a mechanism that was independent of Hsp70 and could not be recapitulated by expression of another type II Hsp40, DNAJB1, demonstrating that this activity is not a general feature of all Hsp40s, but rather was specific to DNAJB6 [57]. This suggests that DNAJB6 and DNAJB8 may directly capture aggregation-prone proteins and have therapeutic benefits in preventing neurodegeneration.

DNAJB8 has also been linked to the maintenance of renal cancer stem cells (CSCs) [64]. CSCs are a small subpopulation of cancer cells that possess characteristics of stem cells. The CSC subpopulation is required for the initiation and maintenance of the tumour and has been linked with chemoresistance and metastasis [133]. Using the Hoechst side population assay, overexpression of DNAJB8 led to an increase in the proportion of putative CSCs of between 6 and 9% depending on the cell line. Knockdown of DNAJB8 had the opposite effect and

reduced the CSC population to 0%. The serine-rich region of DNAJB8, previously shown to be involved in histone deacetylase (HDAC) binding, was involved in CSC maintenance. The authors subsequently tested the potential of DNAJB8 as an antigen for immunisation, comparing the response to the well-characterised tumour antigen, survivin. Mice were injected with RenCa tumour cells after immunisation with either DNAJB8 or survivin and tumour formation and survival time compared to control mice immunised with PBS or the empty vector backbone. While immunisation with both DNAJB8 and survivin showed a significant reduction in tumour formation compared to the control mice, DNAJB8 produced greater statistically significant antitumour effects compared to survivin [64].

Taken together, these data demonstrate that different Hsp40s and splice variants may have distinct roles in different disorders and provide a proof of concept for Hsp40s as drug targets in a range of conditions. These findings also demonstrate that properties may be shared between Hsp40s that share functional similarities, such as DNAJB6 and DNAJB8. Another Hsp40, DNAJB7, is similar to DNAJB6 and DNAJB8 in that it too contains an HDAC-binding domain in the C-terminus [1]. It is tempting to speculate that DNAJB7 may therefore fulfil similar functions to the structurally related DNAJB6 and DNAJB8.

3.2 Current Approaches and Challenges for Targeting Hsp40 in Humans

The field of Hsp40 inhibitors is in its infancy. There are currently no inhibitors for human Hsp40 in the clinic or in clinical trials and no lead compounds in clinical development. In addition, published reports on specific direct inhibition of Hsp40 are limited. The large number and diversity of Hsp40s mean that it is likely to be difficult to identify a single molecular scaffold that is capable of selectively inhibiting Hsp40s or families. One of the major challenges associated with targeting Hsp40s is the lack of fundamental knowledge on the mechanistic role of these proteins in human diseases. Hsp40s fulfil their function by interacting with other proteins, be it substrate proteins or the Hsp70 chaperone. Therefore, the most likely mechanism of inhibiting Hsp40 is to block the protein-protein interactions of this co-chaperone. The most obvious point of inhibition would be the interaction between the J domain of Hsp40 and the NBD of Hsp70.

3.2.1 Dihydropyrimidines

A study to recognise novel Hsp70 inhibitors identified three compounds containing the dihydropyrimidine scaffold that were able to block the Hsp40-stimulated ATPase activity of Hsp70 but not the basal Hsp70 ATPase activity (Fig. 4) [134]. Two of the dihydropyrimidine molecules, MAL3-39 and MAL3-101, were able to

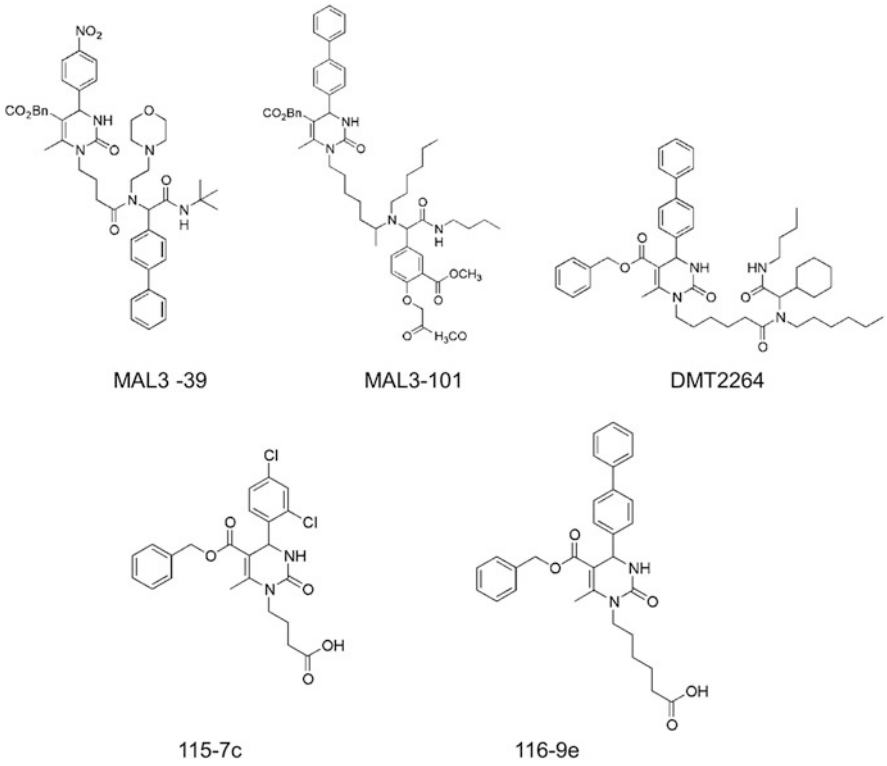

Fig. 4 Structures of selected dihydropyrimidines that inhibit Hsp70-Hsp40 interactions

reduce J domain-dependent activation of yeast Hsp70 and subsequent folding and secretion of a model protein substrate by 60% and 40%, respectively, relative to the uninhibited control [134]. A following study identified a dihydropyrimidine, 115-7c, that is bound to a region on the Hsp70 NBD adjacent to the region recognised by the J domain of Hsp40. This compound was able to either activate or inhibit the Hsp70 chaperone activity via an allosteric mechanism, depending on the nature of specific chemical modifications. The unmodified compound was activating and synergised with Hsp40, while the addition of bulky substitutions produced the compound 116-9e which acted as an inhibitor [135, 136].

3.2.2 Naturally Occurring Inhibitors

Some naturally derived flavonoids, including epicatechin-3-gallate, quercetin and myricetin, have also been shown to disrupt the DnaJ-DnaK (Hsp40-Hsp70 of *E. coli*) system by interfering with the interaction with the Hsp70 NBD (Fig. 5), in addition to their numerous other functions in cells. However, these compounds do not compete with Hsp40 or ATP for binding. Myricetin bound an allosteric site

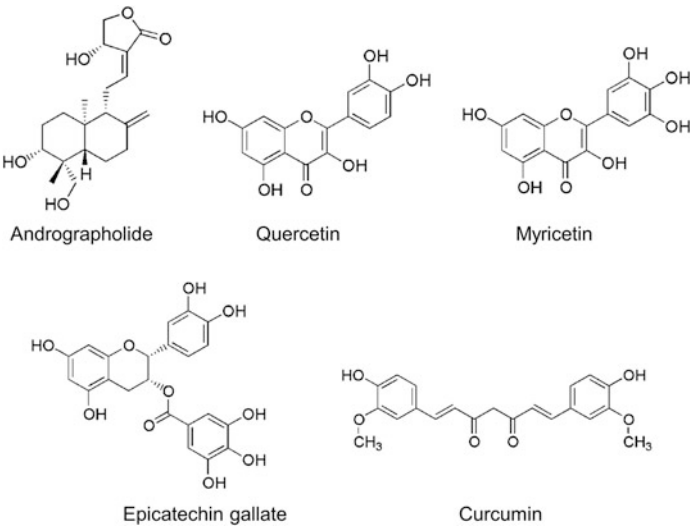

Fig. 5 Structures of some naturally occurring Hsp40 modulators

in the IB and IIB subdomains of Hsp70 (Fig. 1) leading to a change in the conformation of the NBD that inhibited the ability of DnaJ to bind to Hsp70 and to stimulate ATP hydrolysis [137]. Therefore, while these inhibitors do not bind directly to Hsp40, they provide a proof of concept for the disruption of the Hsp70 chaperone through inhibition of Hsp40 binding.

The natural product andrographolide, isolated from a Chinese herb, is another natural compound that influences the function of Hsp40 [138]. This compound was identified in a screen of natural compounds with the ability to activate the promoter of the Hsp40 protein DNAJB4. Andrographolide does not interact with the DNAJB4, but rather increases the transcription of DNAJB4 via AP-1-dependent sites (specifically JunB) in the DNAJB4 promoter. Andrographolide also showed anticancer activity by suppressing the proliferation and invasion of non-small cell lung cancer (NSCLC). DNAJB4 is well characterised as a tumour suppressor [49–51, 139], and therefore the anticancer effects of andrographolide are at least in part due to the increased levels of DNAJB4, as supported by the fact that the anticancer effects of andrographolide were partially reversed by siRNA-mediated silencing of DNAJB4 [138].

Interestingly, andrographolide is not the only compound to activate the DNAJB4 promoter. Curcumin, a component of the spice turmeric, also induces the expression of DNAJB4 by activating the promoter also in an AP-1-dependent manner (through the JNK/JunD pathway) [140]. Curcumin too has anticancer effects, inhibiting lung cancer migration and invasion, which, similar to andrographolide, could be partially reversed upon depletion of DNAJB4 by RNA interference. These two natural compounds are interesting as they demonstrate that stimulation of expression of specific Hsp40 proteins is possible and can be used as an approach to induce anticancer effects. However, it must be remembered that none of these

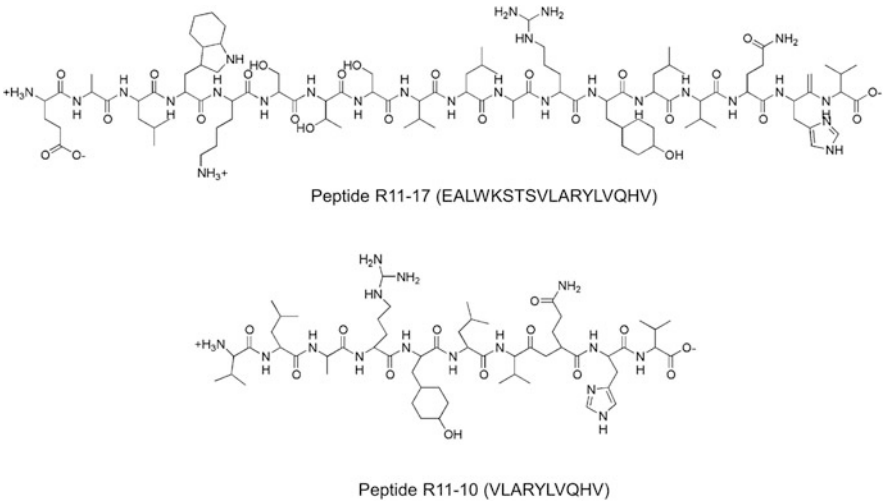

Fig. 6 Structure of D-peptide inhibitors of DnaK-DnaJ interactions [141]

natural compounds are specific for Hsp40 and will have numerous other functions within the cell that may contribute to their overall activity.

3.2.3 D-peptides

In addition to the direct interaction with Hsp70 through its J domain, Hsp40 also interacts with Hsp70 and substrate proteins via its C-terminal region. A previous study has shown proof of concept for the inhibition of Hsp40-Hsp70 interactions via blocking the substrate-binding site of Hsp40 with peptide analogues [141] (Fig. 6). Peptides composed of D-amino acids (R11-17, sequence EALWKSTSV-LARYLVQHV and R11-10, sequence VLARYLVQHV) were shown to compete with natural substrate peptides for binding to DnaJ but not to DnaK. D-peptides bound to DnaJ at low micromolar affinity (R11-17, Kd 1.4 μM and R11-10, Kd 0.8 μM) and inhibited DnaK-mediated chaperone activity. These compounds have been proved useful in delineating the stages of the Hsp70 chaperone cycle but also provide a possible approach for inhibition of Hsp40. The findings demonstrate that it is possible to inhibit chaperone activity by blocking the substrate-binding site of Hsp40 [141]. Therefore, it should be theoretically possible to manipulate Hsp70-mediated folding pathway by controlling the interaction of Hsp40 with substrate peptides [142]. Given that Hsp40 may discriminate between substrates, this could be a mechanism for more selective inhibition of Hsp40 co-chaperone activity. However, this would rely on a detailed understanding of the interaction between the Hsp40 and the specific substrate. In the case of the type I and some type II Hsp40s, it may be relatively easy to inhibit interactions between these co-chaperones and the client protein, given the conserved nature of the C-terminus and substrate-binding motifs [1]. However, in the case of the type III

Hsp40, the nature of the substrate interaction may be more complex. It may involve more than one domain or motif or may occur via a motif that is also found in other non-Hsp40 proteins.

3.2.4 Phenoxy-*N*-arylacetamides

A class of molecules known as the phenoxy-*N*-arylacetamides is the first and only group of compounds shown to bind directly to Hsp40 [143]. This class of compounds was identified in a screen of drug-like compounds to identify novel Hsp90 inhibitors. Using luciferase-refolding assays in rabbit reticulocyte lysate (RRL), the authors identified phenoxy-*N*-arylacetamides as being capable of inhibiting luciferase refolding (not by inhibiting luciferase activity). However, these compounds did not induce Hsp70 upregulation in treated cells. As the induction of the heat shock response is characteristic of N-terminal-binding Hsp90 inhibitors, the authors concluded that Hsp90 was not the target of this compound. As Hsp70-Hsp40 are essential for luciferase refolding in RRL, the effect of the compounds on Hsp70-Hsp40 was tested. They determined that the phenoxy-*N*-arylacetamides inhibited Hsp70-mediated folding of denatured luciferase by binding to Hsp40, not Hsp70. The authors did not identify the binding site of these compounds to Hsp40, although they suggest that the binding is likely to be to an allosteric site on the molecule. The most potent compound was butyl 3-[2-(2,4-dichlorophenoxy)acetamido]benzoate that inhibited Hsp70 refolding of luciferase with an IC_{50} value of 0.13 μM (Fig. 7)

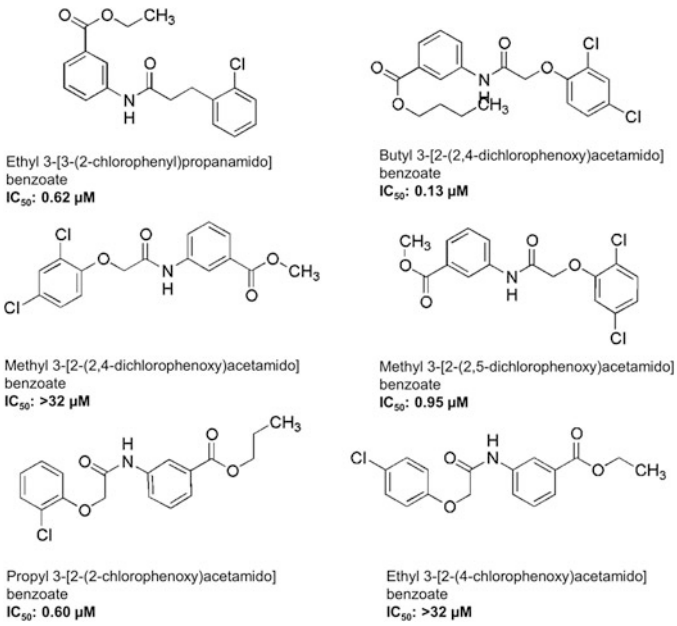

Fig. 7 Structures of phenoxy-*N*-arylacetamide inhibitors of DnaJ

[143]. The compounds did not affect the basal ATPase activity of Hsp70 but did have a moderate effect on the Hsp40-stimulated ATPase activity of Hsp70. The discovery of these molecules holds the potential to identify novel Hsp40 therapeutic inhibitors, although more importantly they are likely to teach us much about the function of Hsp40 itself.

The IC_{50} values indicate the inhibition of refolding of luciferase by Hsp70/DnaJ in the presence of the different compounds [143].

4 Hsp40s as Drug Targets in Malaria

4.1 Structure and Function of Hsp40 in Malaria (Features with Respect to Human Host)

The protozoan parasite *Plasmodium falciparum*, the major cause of human malaria in Africa, is responsible for the most serious form of the disease. Worldwide, malaria infected an estimated 198 million people in 2013 with an approximate death toll of 600,000 individuals [144]. The lack of licensed vaccines and the emergence of parasites resistant to current antimalarial compounds impel us to search for novel antimalarial drugs. Part of the life cycle of *P. falciparum* occurs in humans with the erythrocytic stages being central to its pathology. To thrive within such cell, the parasite has to modify the red blood cell and to succeed it exports a large number of proteins (exportome) into the infected erythrocyte cytosol. Several members of the *P. falciparum* Hsp40 (PfHsp40) family are exported into the host cell cytosol or are predicted to be part of the exportome; they are thought to play a key role in the development and survival of the parasite inside the erythrocyte by facilitating the correct folding, trafficking and assembly of the proteins of the exportome as well as by interacting with human proteins (e.g. cytoskeleton).

The Hsp40 complement of *P. falciparum* is fairly numerous, including at least 49 members [125]. Over the last few years, several reviews have analysed PfHsp40s, and here we will highlight the major features of those PfHsp40 proteins that may represent attractive targets for inhibitor/activator development [124, 125, 145–148]. Besides the canonical type I–III Hsp40s, *P. falciparum* possesses an additional type named type IV [145]. Type IV Hsp40s bear a J domain in which the HPD motif is not conserved (Fig. 3), and hence it is unclear whether they are able to interact with the Hsp70 ATPase domain in a functional manner and therefore act as co-chaperones of Hsp70s. In addition, a number of PfHsp40s exhibit a G/F/S-rich (glycine/phenylalanine/serine-rich) region rather than the common G/F-rich region found in Hsp40s of other organisms [145]. Several PfHsp40s (19) contain a signal sequence at the N-terminus named PEXEL/VTS (*Plasmodium* EXport ELement/ Vacuolar Transport Signal), which targets proteins to the infected erythrocyte cytosol [149–151]. As mentioned earlier, in *falciparum* malaria, the export of

proteins into the infected host cell is a crucial event, and the development and survival of the parasite within the erythrocyte depend on it. As many as ~400 proteins are predicted to constitute the exportome [152], and it is, thus, not surprising that a high number of molecular chaperones are also exported to ensure that the exportome members can reach their destination and fulfil their functions. The likely critical roles of PfHsp40s, in particular of those exported, make the PfHsp40 family an interesting target against which to develop new antimalarial drugs.

Unlike humans and other eukaryotic organisms, *P. falciparum* has merely two type I Hsp40s, PfHsp40/P14_0359/PF3D7_1437900 and Pfj1/PFD0462w/PF3D7_0409400, with only PfHsp40 being cytosolic [153, 154]. Both proteins have been shown to be upregulated by heat shock [154, 155], and accordingly they may be important in the cytoprotection of the parasite during the febrile episodes associated with malaria infection. The in vitro analysis of a possible chaperone/co-chaperone interaction between PfHsp40 and the cytosolic PfHsp70-1/PF08_0054/PF3D7_0818900 indicated that the two proteins could co-operate to suppress protein aggregation and that PfHsp40 is able to stimulate the ATPase activity of PfHsp70-1 [154]. Since both proteins are cytosolic and represent canonical Hsp40 and Hsp70, it is likely that they form a functional partnership in vivo. Similar to its yeast and human homologues Ydj1 and DnaJA1, PfHsp40 contains a putative CAAX box at the C-terminus, which can be farnesylated [35]. In the case of Ydj1, farnesylation is critical for its interaction with certain Hsp90 client proteins [156], and therefore PfHsp40 may also be linked to the PfHsp90-substrate protein network. The other type I Hsp40, Pfj1, contains a putative mitochondrial import signal (RRKVCS) [155] and exhibits an extended C-terminal domain, which is rich in lysine and proline residues. Localisation of Pfj1 to the apicoplast (a plastid-like organelle unique to apicomplexans) has been reported [153], but the data are not conclusive. It was suggested that Pfj1 may be involved in the replication of the apicoplast genome to which Pfj1 would bind via the extended C-terminal region and that it would have a lesser role in protein folding [153]. However, in vitro analysis using model proteins showed that although Pfj1 on its own did not restore the enzymatic activities of denatured glucose-6-phosphate dehydrogenase and α-glucosidase, it was able to stimulate the ability of PfHsp70-1 to do so [157]. The J domain of Pfj1 was also revealed to be functional in a heterologous complementation assay using an *E. coli dnaJ* mutant strain [158]. These in vitro findings should be taken cautiously, since PfHsp70-1 is absent from the apicoplast and mitochondria and thus unlikely to be the in vivo Hsp70 partner of Pfj1. However, the role of this Hsp40 in protein folding should not be dismissed.

The only type II Hsp40s that contain the PEXEL/VTS sequence are PFE0055c/PF3D7_0501100, PFA0660w/PF3D7_0113700 and PFB0090c/PF3D7_0201800. PFE0055c and PFA0660w were both localised within the infected erythrocyte cytosol in novel cholesterol-containing structures named J-dots whose function is not yet understood [159]. PFB0090c was also detected in the infected host cell cytosol in association with two components of the "knobs", KAHRP (knob-associated histidine-rich protein) and PfEMP3 (*Plasmodium falciparum*

erythrocyte membrane protein 3) [160]. Knobs are parasite-induced modifications of the erythrocyte plasma membrane. Through anchorage and display of PfEMP1 (*Plasmodium falciparum* erythrocyte membrane protein 1), knobs enhance the adherence of infected red blood cells to each other and to endothelial cells preventing their clearance by the spleen [161, 162]. PFB0090c has also been reported to co-localise and immunoprecipitate with components of PTEX (*Plasmodium* Translocon of EXported proteins; [163]) through which PEXEL-containing proteins are trafficked into the infected erythrocyte [160, 164, 165]. The three type II Hsp40s appear therefore to be involved in the chaperoning of exported *P. falciparum* proteins that are needed to remodel the host cell in order to sustain the growth and life cycle of the parasite. Furthermore, viable PFA0660w knockout parasite lines could not be obtained suggesting that PFA0660w may be essential to parasite development and survival [166]. The precise role of these three Hsp40s has not been elucidated yet. Nonetheless, the Hsp70 partner of PFE0055c and PFA0660w has been recently proposed to be PfHsp70-x/MAL7P1.228/PF3D7_0831700, which is the only *P. falciparum* Hsp70 present in the infected erythrocyte. PfHsp70-x was found to associate with the J-dots and PfEMP1, and thus together with its putative co-chaperones, it may be playing a central role in parasite virulence [167].

Nearly 40% of the PfHsp40s are presumed to be exported into the infected red blood cell cytosol. Strikingly, half of the PfHsp40s that contain the PEXEL/VTS sequence belongs to the type IV Hsp40s, which, as mentioned earlier, do not contain the conserved HPD motif in their J domain (Fig. 3). The function, mechanism of action and localisation of most of them are still unknown. However, data available for a few members of this class indicate that they, like the other Hsp40 types, may be involved in trafficking and assembly of exported parasite proteins and the general remodelling of the erythrocyte. The remodelling includes not only the formation of knobs but also a change in the physical and biochemical properties of the host cell. Maier and co-workers [166] reported that they were not able to obtain viable parasites in which the genes encoding for the type IV Hsp40s PF11_0034/PF3D7_1102200 or PF11_0509/PF3D7_1149200 were knocked out, indicating that the two proteins may be essential for parasite growth in culture. They also analysed the effect of the disruption of genes encoding for other exported type IV Hsp40s, and they found that the absence of PF10_0381/PF3D7_1039100 caused lack of knobs, and the absence of the ring-infected erythrocyte surface antigen RESA/PFA0110w/PF3D7_0102200 produced a change in the rigidity of the infected erythrocytes [166]. RESA interacts with the erythrocyte cytoskeleton via binding to spectrin assuring its stability during febrile episodes especially in the early stages of parasite development. Changes in the erythrocyte physical properties can affect the cell deformability and the way it circulates in the body [168–171]. Interestingly, RESA and six other exported PfHsp40s (three type III and three type IV) contain a *Plasmodium*-specific PRESAN/PRESAC/PHIST domain (*Plasmodium* RESA N-terminal/*Plasmodium* RESA C-terminal/*Plasmodium* Helical Interspersed SubTelomeric) [125, 172] that may be important for protein-protein interactions within the host cell cytosol, in particular with the components of the

cytoskeleton [173, 174]. Another cytoskeleton-binding domain is the MEC (MESA erythrocyte cytoskeleton-binding) domain, initially discovered in the type IV PfHsp40 MESA/PfEMP2 (Mature parasite-infected Erythrocyte Surface Antigen; PFE0040c/PF3D7_0500800; [175]). The MEC domain has subsequently been found in a number of other exported PfHsp40s (PF10_0378/PF3D7_1038800; PF10_0381; PF11_0034/PF3D7_1102200; PFA0675w/PF2D7_0114000; PFB0925w/PF3D7_0220400; and PFL0055c/PF3D7_1201100). It must be kept in mind that PfHsp40s are structurally quite diverse as they display an array of additional domains (reviewed in [125]). The diversity of the *P. falciparum* chaperone complement can be exploited in the search of new drug targets. Those PfHsp40s members that lack human homologues and possess unique functional domains are particularly attractive candidates. In addition, as the ability of the infected erythrocytes to adhere to the endothelial cells plays an important part in malaria infection, investigating further the PfHsp40s which are known to contribute to the modifications of the red blood cell physical and biochemical characteristics would open new avenues for the development of new therapeutics.

4.2 Current Status on Targeting of Hsp40 as Antimalarial Agents and Future Prospects for Hsp40 Inhibitors as Antimalarials

As we have described in the previous section, the *P. falciparum* genome encodes for a wide variety of Hsp40s. Many of them are unique to the parasite and appear to fulfil crucial if not essential roles. For these reasons, targeting members of the PfHsp40 family and the interaction between them and PfHsp70s seem applicable [124]. However, while a few classes of compounds able to modulate PfHsp70-1 and PfHsp70-x chaperone activities and affect parasite growth in vitro have been identified [176–178], there are no known inhibitors of PfHsp40s. Nevertheless, in a study in which two small molecule modulators of Hsp70s belonging to the pyrimidinone class (MAL3-39 and DMT2264) (Fig. 4) [177] were investigated, the authors found that they had a differential effect on specific Hsp70-Hsp40 partnerships. In particular, the compounds showed inhibitory effects on the Hsj1a-stimulated ATPase activity of a human Hsp70 under steady-state conditions, while no effect was observed on the PfHsp40-stimulated ATPase activity of PfHsp70-1. The latter was inhibited by DMT002264 only under single-turnover conditions [154]. The mechanism of action of the two pyrimidinones on the Hsp40-mediated ATPase activity of Hsp70s is still unclear, but these findings suggest that it is possible to identify small molecule modulators specific to certain Hsp70-Hsp40 pairs and capable of disrupting their functional interaction [154].

One way to identify inhibitors of protein-protein interactions such as chaperone/co-chaperone interfaces can be achieved via high-throughput screening. This is made even easier if the physical interaction of the proteins can be monitored by an

enzymatic reaction as is the case for the Hsp70-Hsp40 partnership measured via ATP hydrolysis. In a recent study, Cesa and colleagues succeeded in screening a library of approximately 3880 compounds for their inhibitory effects on the *E. coli* DnaK-DnaJ and DnaK-GrpE systems by measuring changes in the ATPase activity of DnaK in the presence of a co-chaperone [179]. They were able to isolate molecules with different inhibitory effects on the chaperone/co-chaperone system depending on the presence of DnaJ or GrpE and their stoichiometry with respect to DnaK and therefore specific only to that particular partnership. This type of approach could also be applied to the malaria system since the biochemical properties of certain *P. falciparum* Hsp70-Hsp40 pairs are distinct from the human equivalent making them potential malaria drug targets. The successful disruption of PfHsp70-PfHsp40 interfaces can be hindered by the potential functional redundancy due to the high number of PfHsp40s present in the parasite.

To the best of our knowledge and similar to the human system, there are no known inhibitors of PfHsp40s which directly bind to Hsp40. The phenoxy-*N*-arylacetamide compounds identified by Cassel and co-workers bind directly to DnaJ and may prove useful inhibitors of PfHsp40 [143] (Fig. 7). The Hsp40 binding site has not been identified yet, but it would be interesting to determine whether these molecules can also bind to PfHsp40s. A possible strategy to aid the discovery or design of inhibitors is to consider the *P. falciparum* unique domains mediating protein-protein interaction that in turn are central to the PfHsp40s functions. One promising example is the PRESAN/PRESAC/PHIST domain mentioned in the previous section. Solving the protein structure of such domain in solution may help the rational design of ligands that could block the functionality of that protein region. Other approaches that have been used to target protein-protein interactions include affinity-based techniques like nuclear magnetic resonance-based screening (reviewed in [180]). These approaches could also be utilised for high-throughput screening of compound libraries; however, the difficulty to produce sufficient amount of purified and functional PfHsp40s in vitro represents a considerable obstacle.

Type IV PfHsp40s may represent ideal targets for drug design especially in light of their possible vital involvement in erythrocyte remodelling and because they are not present in humans. Therefore it is a priority to identify the protein-protein interaction network for the type IV PfHsp40s. Mechanistic and structural studies on these proteins will be required to elucidate their mechanism of action and interaction with substrate or partner proteins. This will enable the identification of protein-protein interaction interfaces for inhibitor design.

5 Future Considerations for Inhibition of Hsp40

Many families of molecular chaperones are considered promising drug targets, including Hsp90, Hsp70 and small Hsps. Targeting Hsp40 co-chaperones is a relatively recent pursuit and one that has not yet yielded a substantial number of

inhibitors. One challenging aspect of Hsp40 drug targeting is the fact that contrary to Hsp70s, they do not possess an enzymatic activity which makes the identification of small molecules able to bind them more difficult. The most significant advances have been made in the inhibition of Hsp70-Hsp40 interactions, although a new class of molecules that bind Hsp40, phenoxy-*N*-arylacetamides, have recently been identified [143]. These compounds represent the starting point for the future development of small molecule inhibitors of Hsp40. However, there are still many questions. For example, what is the binding site on Hsp40 for these molecules? Many of the current studies, including this one, have been conducted on *E. coli* DnaJ, which is a canonical type I Hsp40. Will these compounds also be effective against Hsp40 from other species or equally against type II or III Hsp40? What is the effect of different substrates on the inhibition by these compounds? Many studies use an artificial model substrate, luciferase, which may affect the inhibition of the Hsp40 by these compounds. What is the effect of these compounds on cell viability and the stress response? It is therefore likely to be some time before the phenoxy-*N*-arylacetamide-based compounds or other putative Hsp40 inhibitors reach the clinic. Indeed, they are more likely to have most benefit in the short term as tool compounds to understand Hsp40 function and to act as scaffolds to guide development of future small molecule inhibitors.

An alternative approach to the development of inhibitors of Hsp40 may be to control the expression of the proteins themselves [2]. From the published studies, it is clear that human Hsp40 may act either as tumour suppressors or as oncoproteins [3]. Some Hsp40s also function as suppressors of protein aggregation [57]. Therefore, by regulating the expression of well-characterised Hsp40s, it may be possible to treat the disease using a gene therapy or biological approach. Knockdown of selected proteins or even isoforms using allele- or isoform-specific RNA interference may also be a feasible approach. However, this will require a detailed understanding of the functions, interactions and expression patterns of the individual isoforms. While some Hsp40s are poorly described at best, there are many human Hsp40s for which we do not have a function. Therefore, there needs to be a concerted effort to investigate the fundamental roles of these Hsp40 co-chaperones to complement the growing efforts to identify novel inhibitors.

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References

1. Kampinga HH, Craig EA (2010) The HSP70 chaperone machinery: J proteins as drivers of functional specificity. Nat Rev Mol Cell Biol 11:579–592
2. Sterrenberg JN, Blatch GL, Edkins AL (2011) Human DNAJ in cancer and stem cells. Cancer Lett 312:129–142
3. Mitra A, Shevde LA, Samant RS (2009) Multi-faceted role of HSP40 in cancer. Clin Exp Metastasis 26:559–567
4. Cyr DM, Lu X, Douglas MG (1992) Regulation of Hsp70 function by a eukaryotic DnaJ homolog. J Biol Chem 267:20927–20931
5. Suh WC, Burkholder WF, Lu CZ et al (1998) Interaction of the Hsp70 molecular chaperone, DnaK, with its cochaperone DnaJ. Proc Natl Acad Sci U S A 95:15223–15228
6. Laufen T, Mayer MP, Beisel C et al (1999) Mechanism of regulation of hsp70 chaperones by DnaJ cochaperones. Proc Natl Acad Sci U S A 96:5452–5457
7. Terada K, Mori M (2000) Human DnaJ homologs dj2 and dj3, and bag-1 are positive cochaperones of hsc70. J Biol Chem 275:24728–24734
8. Langer T, Lu C, Echols H et al (1992) Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding. Nature 356:683–689
9. Lu Z, Cyr DM (1998) Protein folding activity of Hsp70 is modified differentially by the hsp40 co-chaperones Sis1 and Ydj1. J Biol Chem 273:27824–27830
10. Fan CY, Ren HY, Lee P et al (2005) The type I Hsp40 zinc finger-like region is required for Hsp70 to capture non-native polypeptides from Ydj1. J Biol Chem 280:695–702
11. Hageman J, van Waarde MA, Zylicz A et al (2011) The diverse members of the mammalian HSP70 machine show distinct chaperone-like activities. Biochem J 435:127–142
12. Mansson C, Kakkar V, Monsellier E et al (2013) DNAJB6 is a peptide-binding chaperone which can suppress amyloid fibrillation of polyglutamine peptides at substoichiometric molar ratios. Cell Stress Chaperones. doi:10.1007/s12192-013-0448-5
13. Hageman J, Rujano MA, van Waarde MA et al (2010) A DNAJB chaperone subfamily with HDAC-dependent activities suppresses toxic protein aggregation. Mol Cell 37:355–369
14. Vos MJ, Hageman J, Carra S et al (2008) Structural and functional diversities between members of the human HSPB, HSPH, HSPA, and DNAJ chaperone families. Biochemistry 47:7001–7011
15. Hageman J, Kampinga HH (2009) Computational analysis of the human HSPH/HSPA/DNAJ family and cloning of a human HSPH/HSPA/DNAJ expression library. Cell Stress Chaperones 14:1–21
16. Bhattacharya A, Kurochkin AV, Yip GN et al (2009) Allostery in Hsp70 chaperones is transduced by subdomain rotations. J Mol Biol 388:475–490
17. Swain JF, Schulz EG, Gierasch LM (2006) Direct comparison of a stable isolated Hsp70 substrate-binding domain in the empty and substrate-bound states. J Biol Chem 281:1605–1611
18. Bertelsen EB, Chang L, Gestwicki JE et al (2009) Solution conformation of wild-type *E. coli* Hsp70 (DnaK) chaperone complexed with ADP and substrate. Proc Natl Acad Sci U S A 106:8471–8476
19. Zhu X, Zhao X, Burkholder WF et al (1996) Structural analysis of substrate binding by the molecular chaperone DnaK. Science 272:1606–1614
20. Mayer MP, Bukau B (2005) Hsp70 chaperones: cellular functions and molecular mechanism. Cell Mol Life Sci 62:670–684
21. Szabo A, Langer T, Schroder H et al (1994) The ATP hydrolysis-dependent reaction cycle of the Escherichia coli Hsp70 system DnaK, DnaJ, and GrpE. Proc Natl Acad Sci U S A 91:10345–10349
22. Summers DW, Douglas PM, Ramos CH et al (2009) Polypeptide transfer from Hsp40 to Hsp70 molecular chaperones. Trends Biochem Sci 34:230–233

23. Kabani M, Beckerich JM, Brodsky JL (2002) Nucleotide exchange factor for the yeast Hsp70 molecular chaperone Ssa1p. Mol Cell Biol 22:4677–4689
24. Choglay AA, Chapple JP, Blatch GL et al (2001) Identification and characterization of a human mitochondrial homologue of the bacterial co-chaperone GrpE. Gene 267:125–134
25. Kampinga HH, Hageman J, Vos MJ et al (2009) Guidelines for the nomenclature of the human heat shock proteins. Cell Stress Chaperones 14:105–111
26. Cheetham ME, Caplan AJ (1998) Structure, function and evolution of DnaJ: conservation and adaptation of chaperone function. Cell Stress Chaperones 3:28–36
27. Mitra A, Fillmore RA, Metge BJ et al (2008) Large isoform of MRJ (DNAJB6) reduces malignant activity of breast cancer. Breast Cancer Res 10:R22
28. Qian YQ, Patel D, Hartl FU et al (1996) Nuclear magnetic resonance solution structure of the human Hsp40 (HDJ-1) J-domain. J Mol Biol 260:224–235
29. Pellecchia M, Szyperski T, Wall D et al (1996) NMR structure of the J-domain and the Gly/Phe-rich region of the *Escherichia coli* DnaJ chaperone. J Mol Biol 260:236–250
30. Tsai J, Douglas MG (1996) A conserved HPD sequence of the J-domain is necessary for YDJ1 stimulation of Hsp70 ATPase activity at a site distinct from substrate binding. J Biol Chem 271:9347–9354
31. Hennessy F, Boshoff A, Blatch GL (2005) Rational mutagenesis of a 40 kDa heat shock protein from Agrobacterium tumefaciens identifies amino acid residues critical to its in vivo function. Int J Biochem Cell Biol 37:177–191
32. Yan W, Craig EA (1999) The glycine-phenylalanine-rich region determines the specificity of the yeast Hsp40 Sis1. Mol Cell Biol 19:7751–7758
33. Banecki B, Liberek K, Wall D et al (1996) Structure-function analysis of the zinc finger region of the DnaJ molecular chaperone. J Biol Chem 271:14840–14848
34. Szabo A, Korszun R, Hartl FU et al (1996) A zinc finger-like domain of the molecular chaperone DnaJ is involved in binding to denatured protein substrates. EMBO J 15:408–417
35. Farh L, Mitchell DA, Deschenes RJ (1995) Farnesylation and proteolysis are sequential, but distinct steps in the CaaX box modification pathway. Arch Biochem Biophys 318:113–121
36. Cao M, Wei C, Zhao L et al (2014) DnaJA1/Hsp40 is co-opted by influenza A virus to enhance its viral RNA polymerase activity. J Virol 88:14078–14089
37. Stark JL, Mehla K, Chaika N et al (2014) Structure and function of human DnaJ homologue subfamily a member 1 (DNAJA1) and its relationship to pancreatic cancer. Biochemistry 53:1360–1372
38. Urano E, Morikawa Y, Komano J (2013) Novel role of HSP40/DNAJ in the regulation of HIV-1 replication. J Acquir Immune Defic Syndr 64:154–162
39. Tang D, Khaleque MA, Jones EL et al (2005) Expression of heat shock proteins and heat shock protein messenger ribonucleic acid in human prostate carcinoma in vitro and in tumors in vivo. Cell Stress Chaperones 10:46–58
40. Walker VE, Wong MJ, Atanasiu R et al (2010) Hsp40 chaperones promote degradation of the HERG potassium channel. J Biol Chem 285:3319–3329
41. Jan CI, Yu CC, Hung MC et al (2011) Tid1, CHIP and ErbB2 interactions and their prognostic implications for breast cancer patients. J Pathol 225:424–437
42. Cheng H, Cenciarelli C, Nelkin G et al (2005) Molecular mechanism of hTid-1, the human homolog of Drosophila tumor suppressor l(2)Tid, in the regulation of NF-kappaB activity and suppression of tumor growth. Mol Cell Biol 25:44–59
43. Copeland E, Balgobin S, Lee CM et al (2011) hTID-1 defines a novel regulator of c-Met Receptor signaling in renal cell carcinomas. Oncogene 30:2252–2263
44. Kurzik-Dumke U, Horner M, Czaja J et al (2008) Progression of colorectal cancers correlates with overexpression and loss of polarization of expression of the htid-1 tumor suppressor. Int J Mol Med 21:19–31
45. Blumen SC, Astord S, Robin V et al (2012) A rare recessive distal hereditary motor neuropathy with HSJ1 chaperone mutation. Ann Neurol 71:509–519

46. Borrell-Pages M, Canals JM, Cordelieres FP et al (2006) Cystamine and cysteamine increase brain levels of BDNF in Huntington disease via HSJ1b and transglutaminase. J Clin Invest 116:1410–1424
47. Gao XC, Zhou CJ, Zhou ZR et al (2011) Co-chaperone HSJ1a dually regulates the proteasomal degradation of ataxin-3. PLoS One 6, e19763
48. Gess B, Auer-Grumbach M, Schirmacher A et al (2014) HSJ1-related hereditary neuropathies: novel mutations and extended clinical spectrum. Neurology 83:1726–1732
49. Chang TP, Yu SL, Lin SY et al (2010) Tumor suppressor HLJ1 binds and functionally alters nucleophosmin via activating enhancer binding protein 2alpha complex formation. Cancer Res 70:1656–1667
50. Liu Y, Zhou J, Zhang C et al (2014) HLJ1 is a novel biomarker for colorectal carcinoma progression and overall patient survival. Int J Clin Exp Pathol 7:969–977
51. Tsai MF, Wang CC, Chang GC et al (2006) A new tumor suppressor DnaJ-like heat shock protein, HLJ1, and survival of patients with non-small-cell lung carcinoma. J Natl Cancer Inst 98:825–838
52. Menezes ME, Mitra A, Shevde LA et al (2012) DNAJB6 governs a novel regulatory loop determining Wnt/beta-catenin signalling activity. Biochem J 444:573–580
53. Mitra A, Menezes ME, Pannell LK et al (2012) DNAJB6 chaperones PP2A mediated dephosphorylation of GSK3beta to downregulate beta-catenin transcription target, osteopontin. Oncogene 31:4472–4483
54. Harms MB, Sommerville RB, Allred P et al (2012) Exome sequencing reveals DNAJB6 mutations in dominantly-inherited myopathy. Ann Neurol 71:407–416
55. Sandell SM, Mahjneh I, Palmio J et al (2013) 'Pathognomonic' muscle imaging findings in DNAJB6 mutated LGMD1D. Eur J Neurol. doi:10.1111/ene.12239
56. Durrenberger PF, Filiou MD, Moran LB et al (2009) DnaJB6 is present in the core of Lewy bodies and is highly up-regulated in parkinsonian astrocytes. J Neurosci Res 87:238–245
57. Gillis J, Schipper-Krom S, Juenemann K et al (2013) The DNAJB6 and DNAJB8 protein chaperones prevent intracellular aggregation of polyglutamine peptides. J Biol Chem 288:17225–17237
58. Couthouis J, Raphael AR, Siskind C et al (2014) Exome sequencing identifies a DNAJB6 mutation in a family with dominantly-inherited limb-girdle muscular dystrophy. Neuromuscul Disord 24:431–435
59. Mansson C, Arosio P, Hussein R et al (2014) Interaction of the molecular chaperone DNAJB6 with growing amyloid-beta 42 (Abeta42) aggregates leads to sub-stoichiometric inhibition of amyloid formation. J Biol Chem 289:31066–31076
60. Mansson C, Kakkar V, Monsellier E et al (2014) DNAJB6 is a peptide-binding chaperone which can suppress amyloid fibrillation of polyglutamine peptides at substoichiometric molar ratios. Cell Stress Chaperones 19:227–239
61. Mitra A, Menezes ME, Shevde LA et al (2010) DNAJB6 induces degradation of {beta}-catenin and causes partial reversal of mesenchymal phenotype. J Biol Chem 285:24686–24694
62. Sarparanta J, Jonson PH, Golzio C et al (2012) Mutations affecting the cytoplasmic functions of the co-chaperone DNAJB6 cause limb-girdle muscular dystrophy. Nat Genet 44(450-455): S451–S452
63. Zhang TT, Jiang YY, Shang L et al (2014) Overexpression of DNAJB6 promotes colorectal cancer cell invasion through an IQGAP1/ERK-dependent signaling pathway. Mol Carcinog. doi:10.1002/mc.22194
64. Nishizawa S, Hirohashi Y, Torigoe T et al (2012) HSP DNAJB8 controls tumor-initiating ability in renal cancer stem-like cells. Cancer Res 72:2844–2854
65. Morita R, Nishizawa S, Torigoe T et al (2014) Heat shock protein DNAJB8 is a novel target for immunotherapy of colon cancer-initiating cells. Cancer Sci 105:389–395
66. Hoshino T, Nakaya T, Araki W et al (2007) Endoplasmic reticulum chaperones inhibit the production of amyloid-beta peptides. Biochem J 402:581–589

67. Bernal-Bayard J, Cardenal-Munoz E, Ramos-Morales F (2010) The Salmonella type III secretion effector, salmonella leucine-rich repeat protein (SlrP), targets the human chaperone ERdj3. J Biol Chem 285:16360–16368
68. Massey S, Burress H, Taylor M et al (2011) Structural and functional interactions between the cholera toxin A1 subunit and ERdj3/HEDJ, a chaperone of the endoplasmic reticulum. Infect Immun 79:4739–4747
69. Tan YL, Genereux JC, Pankow S et al (2014) ERdj3 is an endoplasmic reticulum degradation factor for mutant glucocerebrosidase variants linked to Gaucher's disease. Chem Biol 21:967–976
70. Wen KW, Damania B (2010) Hsp90 and Hsp40/Erdj3 are required for the expression and anti-apoptotic function of KSHV K1. Oncogene 29:3532–3544
71. Grove DE, Fan CY, Ren HY et al (2011) The endoplasmic reticulum-associated Hsp40 DNAJB12 and Hsc70 cooperate to facilitate RMA1 E3-dependent degradation of nascent CFTRDeltaF508. Mol Biol Cell 22:301–314
72. Yamamoto YH, Kimura T, Momohara S et al (2010) A novel ER J-protein DNAJB12 accelerates ER-associated degradation of membrane proteins including CFTR. Cell Struct Funct 35:107–116
73. Michailidou K, Hall P, Gonzalez-Neira A et al (2013) Large-scale genotyping identifies 41 new loci associated with breast cancer risk. Nat Genet 45(353-361):361e351–361e352
74. Pharoah PD, Tsai YY, Ramus SJ et al (2013) GWAS meta-analysis and replication identifies three new susceptibility loci for ovarian cancer. Nat Genet 45(362-370):370e361–370e362
75. Greiner J, Ringhoffer M, Taniguchi M et al (2004) mRNA expression of leukemia-associated antigens in patients with acute myeloid leukemia for the development of specific immunotherapies. Int J Cancer 108:704–711
76. Resto VA, Caballero OL, Buta MR et al (2000) A putative oncogenic role for MPP11 in head and neck squamous cell cancer. Cancer Res 60:5529–5535
77. Synofzik M, Haack TB, Kopajtich R et al (2014) Absence of BiP co-chaperone DNAJC3 causes diabetes mellitus and multisystemic neurodegeneration. Am J Hum Genet 95:689–697
78. Benitez BA, Alvarado D, Cai Y et al (2011) Exome-sequencing confirms DNAJC5 mutations as cause of adult neuronal ceroid-lipofuscinosis. PLoS One 6, e26741
79. Cadieux-Dion M, Andermann E, Lachance-Touchette P et al (2013) Recurrent mutations in DNAJC5 cause autosomal dominant Kufs disease. Clin Genet 83:571–575
80. Greaves J, Lemonidis K, Gorleku OA et al (2012) Palmitoylation-induced aggregation of cysteine-string protein mutants that cause neuronal ceroid lipofuscinosis. J Biol Chem 287:37330–37339
81. Noskova L, Stranecky V, Hartmannova H et al (2011) Mutations in DNAJC5, encoding cysteine-string protein alpha, cause autosomal-dominant adult-onset neuronal ceroid lipofuscinosis. Am J Hum Genet 89:241–252
82. Velinov M, Dolzhanskaya N, Gonzalez M et al (2012) Mutations in the gene DNAJC5 cause autosomal dominant Kufs disease in a proportion of cases: study of the Parry family and 8 other families. PLoS One 7, e29729
83. Edvardson S, Cinnamon Y, Ta-Shma A et al (2012) A deleterious mutation in DNAJC6 encoding the neuronal-specific clathrin-uncoating co-chaperone auxilin, is associated with juvenile Parkinsonism. PLoS One 7, e36458
84. Koroglu C, Baysal L, Cetinkaya M et al (2013) DNAJC6 is responsible for juvenile parkinsonism with phenotypic variability. Parkinsonism Relat Disord 19:320–324
85. Vauthier V, Jaillard S, Journel H et al (2012) Homozygous deletion of an 80 kb region comprising part of DNAJC6 and LEPR genes on chromosome 1P31.3 is associated with early onset obesity, mental retardation and epilepsy. Mol Genet Metab 106:345–350
86. Yang T, Li XN, Li XG et al (2014) DNAJC6 promotes hepatocellular carcinoma progression through induction of epithelial-mesenchymal transition. Biochem Biophys Res Commun 455:298–304

87. Liu CM, Fann CS, Chen CY et al (2011) ANXA7, PPP3CB, DNAJC9, and ZMYND17 genes at chromosome 10q22 associated with the subgroup of schizophrenia with deficits in attention and executive function. Biol Psychiatry 70:51–58
88. Pan Z, Chen S, Pan X et al (2010) Differential gene expression identified in Uigur women cervical squamous cell carcinoma by suppression subtractive hybridization. Neoplasma 57:123–128
89. Lyng H, Brovig RS, Svendsrud DH et al (2006) Gene expressions and copy numbers associated with metastatic phenotypes of uterine cervical cancer. BMC Genomics 7:268
90. Corazzari M, Lovat PE, Armstrong JL et al (2007) Targeting homeostatic mechanisms of endoplasmic reticulum stress to increase susceptibility of cancer cells to fenretinide-induced apoptosis: the role of stress proteins ERdj5 and ERp57. Br J Cancer 96:1062–1071
91. Thomas CG, Spyrou G (2009) ERdj5 sensitizes neuroblastoma cells to endoplasmic reticulum stress-induced apoptosis. J Biol Chem 284:6282–6290
92. Williams JM, Inoue T, Banks L et al (2013) The ERdj5-Sel1L complex facilitates cholera toxin retrotranslocation. Mol Biol Cell 24:785–795
93. Ioakeimidis F, Ott C, Kozjak-Pavlovic V et al (2014) A splicing mutation in the novel mitochondrial protein DNAJC11 causes motor neuron pathology associated with cristae disorganization, and lymphoid abnormalities in mice. PLoS One 9, e104237
94. Katoh M, Katoh M (2003) Identification and characterization of FLJ10737 and CAMTA1 genes on the commonly deleted region of neuroblastoma at human chromosome 1p36.31-p36.23. Int J Oncol 23:1219–1224
95. De Bessa SA, Salaorni S, Patrao DF et al (2006) JDP1 (DNAJC12/Hsp40) expression in breast cancer and its association with estrogen receptor status. Int J Mol Med 17:363–367
96. He HL, Lee YE, Chen HP et al (2015) Overexpression of DNAJC12 predicts poor response to neoadjuvant concurrent chemoradiotherapy in patients with rectal cancer. Exp Mol Pathol 98:338–345
97. Sundaram SK, Huq AM, Sun Z et al (2011) Exome sequencing of a pedigree with Tourette syndrome or chronic tic disorder. Ann Neurol 69:901–904
98. Vilarino-Guell C, Rajput A, Milnerwood AJ et al (2014) DNAJC13 mutations in Parkinson disease. Hum Mol Genet 23:1794–1801
99. Yi Z, Sperzel L, Nurnberger C et al (2011) Identification and characterization of the host protein DNAJC14 as a broadly active flavivirus replication modulator. PLoS Pathog 7, e1001255
100. Yi Z, Yuan Z, Rice CM et al (2012) Flavivirus replication complex assembly revealed by DNAJC14 functional mapping. J Virol 86:11815–11832
101. Lindsey JC, Lusher ME, Strathdee G et al (2006) Epigenetic inactivation of MCJ (DNAJD1) in malignant paediatric brain tumours. Int J Cancer 118:346–352
102. Shridhar V, Bible KC, Staub J et al (2001) Loss of expression of a new member of the DNAJ protein family confers resistance to chemotherapeutic agents used in the treatment of ovarian cancer. Cancer Res 61:4258–4265
103. Strathdee G, Vass JK, Oien KA et al (2005) Demethylation of the MCJ gene in stage III/IV epithelial ovarian cancer and response to chemotherapy. Gynecol Oncol 97:898–903
104. Davey KM, Parboosingh JS, McLeod DR et al (2006) Mutation of DNAJC19, a human homologue of yeast inner mitochondrial membrane co-chaperones, causes DCMA syndrome, a novel autosomal recessive Barth syndrome-like condition. J Med Genet 43:385–393
105. Ojala T, Polinati P, Manninen T et al (2012) New mutation of mitochondrial DNAJC19 causing dilated and noncompaction cardiomyopathy, anemia, ataxia, and male genital anomalies. Pediatr Res 72:432–437
106. Sparkes R, Patton D, Bernier F (2007) Cardiac features of a novel autosomal recessive dilated cardiomyopathic syndrome due to defective importation of mitochondrial protein. Cardiol Young 17:215–217

107. Sun G, Gargus JJ, Ta DT et al (2003) Identification of a novel candidate gene in the iron-sulfur pathway implicated in ataxia-susceptibility: human gene encoding HscB, a J-type co-chaperone. J Hum Genet 48:415–419
108. Davila S, Furu L, Gharavi AG et al (2004) Mutations in SEC63 cause autosomal dominant polycystic liver disease. Nat Genet 36:575–577
109. Janssen MJ, Salomon J, Te Morsche RH et al (2012) Loss of heterozygosity is present in SEC63 germline carriers with polycystic liver disease. PLoS One 7, e50324
110. Waanders E, Croes HJ, Maass CN et al (2008) Cysts of PRKCSH mutated polycystic liver disease patients lack hepatocystin but express Sec63p. Histochem Cell Biol 129:301–310
111. Waanders E, te Morsche RH, de Man RA et al (2006) Extensive mutational analysis of PRKCSH and SEC63 broadens the spectrum of polycystic liver disease. Hum Mutat 27:830
112. Waanders E, Venselaar H, te Morsche RH et al (2010) Secondary and tertiary structure modeling reveals effects of novel mutations in polycystic liver disease genes PRKCSH and SEC63. Clin Genet 78:47–56
113. Wei H, Xiang L, Wayne AS et al (2012) Immunotoxin resistance via reversible methylation of the DPH4 promoter is a unique survival strategy. Proc Natl Acad Sci U S A 109:6898–6903
114. Chen YP, Song W, Huang R et al (2013) GAK rs1564282 and DGKQ rs11248060 increase the risk for Parkinson's disease in a Chinese population. J Clin Neurosci 20:880–883
115. Li NN, Chang XL, Mao XY et al (2012) GWAS-linked GAK locus in Parkinson's disease in Han Chinese and meta-analysis. Hum Genet 131:1089–1093
116. Lin CH, Chen ML, Tai YC et al (2013) Reaffirmation of GAK, but not HLA-DRA, as a Parkinson's disease susceptibility gene in a Taiwanese population. Am J Med Genet B Neuropsychiatr Genet 162B:841–846
117. Pankratz N, Wilk JB, Latourelle JC et al (2009) Genomewide association study for susceptibility genes contributing to familial Parkinson disease. Hum Genet 124:593–605
118. Susa M, Choy E, Liu X et al (2010) Cyclin G-associated kinase is necessary for osteosarcoma cell proliferation and receptor trafficking. Mol Cancer Ther 9:3342–3350
119. Baets J, Deconinck T, Smets K et al (2010) Mutations in SACS cause atypical and late-onset forms of ARSACS. Neurology 75:1181–1188
120. Miyatake S, Miyake N, Doi H et al (2012) A novel SACS mutation in an atypical case with autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS). Intern Med 51:2221–2226
121. Parfitt DA, Michael GJ, Vermeulen EG et al (2009) The ataxia protein sacsin is a functional co-chaperone that protects against polyglutamine-expanded ataxin-1. Hum Mol Genet 18:1556–1565
122. Merla G, Ucla C, Guipponi M et al (2002) Identification of additional transcripts in the Williams-Beuren syndrome critical region. Hum Genet 110:429–438
123. Knox C, Luke GA, Blatch GL et al (2011) Heat shock protein 40 (Hsp40) plays a key role in the virus life cycle. Virus Res 160:15–24
124. Pesce ER, Cockburn IL, Goble JL et al (2010) Malaria heat shock proteins: drug targets that chaperone other drug targets. Infect Disord Drug Targets 10:147–157
125. Njunge JM, Ludewig MH, Boshoff A et al (2013) Hsp70s and J proteins of Plasmodium parasites infecting rodents and primates: structure, function, clinical relevance, and drug targets. Curr Pharm Des 19:387–403
126. Andrews JF, Sykora LJ, Letostak TB et al (2012) Cellular stress stimulates nuclear localization signal (NLS) independent nuclear transport of MRJ. Exp Cell Res 318:1086–1093
127. Izawa I, Nishizawa M, Ohtakara K et al (2000) Identification of Mrj, a DnaJ/Hsp40 family protein, as a keratin 8/18 filament regulatory protein. J Biol Chem 275:34521–34527
128. Dey S, Banerjee P, Saha P (2009) Cell cycle specific expression and nucleolar localization of human J-domain containing co-chaperone Mrj. Mol Cell Biochem 322:137–142
129. Mitra A, Menezes ME, Shevde LA et al (2010) DNAJB6 induces degradation of beta-catenin and causes partial reversal of mesenchymal phenotype. J Biol Chem 285:24686–24694

130. Chuang JZ, Zhou H, Zhu M et al (2002) Characterization of a brain-enriched chaperone, MRJ, that inhibits Huntingtin aggregation and toxicity independently. J Biol Chem 277:19831–19838
131. Watson ED, Mattar P, Schuurmans C et al (2009) Neural stem cell self-renewal requires the Mrj co-chaperone. Dev Dyn 238:2564–2574
132. Sato T, Hayashi YK, Oya Y et al (2013) DNAJB6 myopathy in an Asian cohort and cytoplasmic/nuclear inclusions. Neuromuscul Disord 23:269–276
133. Lawson JC, Blatch GL, Edkins AL (2009) Cancer stem cells in breast cancer and metastasis. Breast Cancer Res Treat 118:241–254
134. Fewell SW, Smith CM, Lyon MA et al (2004) Small molecule modulators of endogenous and co-chaperone-stimulated Hsp70 ATPase activity. J Biol Chem 279:51131–51140
135. Chang L, Bertelsen EB, Wisen S et al (2008) High-throughput screen for small molecules that modulate the ATPase activity of the molecular chaperone DnaK. Anal Biochem 372:167–176
136. Wisen S, Bertelsen EB, Thompson AD et al (2010) Binding of a small molecule at a protein-protein interface regulates the chaperone activity of hsp70-hsp40. ACS Chem Biol 5:611–622
137. Chang L, Miyata Y, Ung PM et al (2011) Chemical screens against a reconstituted multiprotein complex: myricetin blocks DnaJ regulation of DnaK through an allosteric mechanism. Chem Biol 18:210–221
138. Lai YH, Yu SL, Chen HY et al (2013) The HLJ1-targeting drug screening identified Chinese herb andrographolide that can suppress tumour growth and invasion in non-small-cell lung cancer. Carcinogenesis 34:1069–1080
139. Wang CC, Tsai MF, Hong TM et al (2005) The transcriptional factor YY1 upregulates the novel invasion suppressor HLJ1 expression and inhibits cancer cell invasion. Oncogene 24:4081–4093
140. Chen HW, Lee JY, Huang JY et al (2008) Curcumin inhibits lung cancer cell invasion and metastasis through the tumor suppressor HLJ1. Cancer Res 68:7428–7438
141. Bischofberger P, Han W, Feifel B et al (2003) D-Peptides as inhibitors of the DnaK/DnaJ/GrpE chaperone system. J Biol Chem 278:19044–19047
142. Siegenthaler RK, Christen P (2006) Tuning of DnaK chaperone action by nonnative protein sensor DnaJ and thermosensor GrpE. J Biol Chem 281:34448–34456
143. Cassel JA, Ilyin S, McDonnell ME et al (2012) Novel inhibitors of heat shock protein Hsp70-mediated luciferase refolding that bind to DnaJ. Bioorg Med Chem 20:3609–3614
144. World Health Organisation (2014) World Malaria Report 2014. World Health Organisation, Geneva
145. Botha M, Pesce ER, Blatch GL (2007) The Hsp40 proteins of *Plasmodium falciparum* and other apicomplexa: regulating chaperone power in the parasite and the host. Int J Biochem Cell Biol 39:1781–1803
146. Acharya P, Kumar R, Tatu U (2007) Chaperoning a cellular upheaval in malaria: heat shock proteins in *Plasmodium falciparum*. Mol Biochem Parasitol 153:85–94
147. Rug M, Maier AG (2011) The heat shock protein 40 family of the malaria parasite *Plasmodium falciparum*. IUBMB Life 63:1081–1086
148. Shonhai A, Maier AG, Przyborski JM et al (2011) Intracellular protozoan parasites of humans: the role of molecular chaperones in development and pathogenesis. Protein Pept Lett 18:143–157
149. Hiller NL, Bhattacharjee S, van Ooij C et al (2004) A host-targeting signal in virulence proteins reveals a secretome in malarial infection. Science 306:1934–1937
150. Marti M, Good RT, Rug M et al (2004) Targeting malaria virulence and remodeling proteins to the host erythrocyte. Science 306:1930–1933
151. Hiss JA, Przyborski JM, Schwarte F et al (2008) The Plasmodium export element revisited. PLoS One 3, e1560
152. Boddey JA, Carvalho TG, Hodder AN et al (2013) Role of plasmepsin V in export of diverse protein families from the *Plasmodium falciparum* exportome. Traffic 14:532–550

153. Kumar A, Tanveer A, Biswas S et al (2010) Nuclear-encoded DnaJ homolog of *Plasmodium falciparum* interacts with replication ori of the apicoplast genome. Mol Microbiol. doi:10.1111/j.1365-2958.2010.07033.x
154. Botha M, Chiang AN, Needham PG et al (2011) *Plasmodium falciparum* encodes a single cytosolic type I Hsp40 that functionally interacts with Hsp70 and is upregulated by heat shock. Cell Stress Chaperones 16:389–401
155. Watanabe J (1997) Cloning and characterization of heat shock protein DnaJ homologues from *Plasmodium falciparum* and comparison with ring infected erythrocyte surface antigen. Mol Biochem Parasitol 88:253–258
156. Flom GA, Lemieszek M, Fortunato EA et al (2008) Farnesylation of Ydj1 is required for in vivo interaction with Hsp90 client proteins. Mol Biol Cell 19:5249–5258
157. Misra G, Ramachandran R (2009) Hsp70-1 from *Plasmodium falciparum*: protein stability, domain analysis and chaperone activity. Biophys Chem 142:55–64
158. Nicoll WS, Botha M, McNamara C et al (2007) Cytosolic and ER J-domains of mammalian and parasitic origin can functionally interact with DnaK. Int J Biochem Cell Biol 39:736–751
159. Kulzer S, Rug M, Brinkmann K et al (2010) Parasite-encoded Hsp40 proteins define novel mobile structures in the cytosol of the *P. falciparum*-infected erythrocyte. Cell Microbiol 12:1398–1420
160. Acharya P, Chaubey S, Grover M et al (2012) An exported heat shock protein 40 associates with pathogenesis-related knobs in *Plasmodium falciparum* infected erythrocytes. PLoS One 7, e44605
161. Crabb BS, Cooke BM, Reeder JC et al (1997) Targeted gene disruption shows that knobs enable malaria-infected red cells to cytoadhere under physiological shear stress. Cell 89:287–296
162. Miller LH, Baruch DI, Marsh K et al (2002) The pathogenic basis of malaria. Nature 415:673–679
163. de Koning-Ward TF, Gilson PR, Boddey JA et al (2009) A newly discovered protein export machine in malaria parasites. Nature 459:945–949
164. Bullen HE, Charnaud SC, Kalanon M et al (2012) Biosynthesis, localization, and macromolecular arrangement of the *Plasmodium falciparum* translocon of exported proteins (PTEX). J Biol Chem 287:7871–7884
165. Riglar DT, Rogers KL, Hanssen E et al (2013) Spatial association with PTEX complexes defines regions for effector export into *Plasmodium falciparum*-infected erythrocytes. Nat Commun 4:1415
166. Maier AG, Rug M, O'Neill MT et al (2008) Exported proteins required for virulence and rigidity of *Plasmodium falciparum*-infected human erythrocytes. Cell 134:48–61
167. Kulzer S, Charnaud S, Dagan T et al (2012) *Plasmodium falciparum*-encoded exported hsp70/hsp40 chaperone/co-chaperone complexes within the host erythrocyte. Cell Microbiol 14:1784–1795
168. Da Silva E, Foley M, Dluzewski AR et al (1994) The *Plasmodium falciparum* protein RESA interacts with the erythrocyte cytoskeleton and modifies erythrocyte thermal stability. Mol Biochem Parasitol 66:59–69
169. Silva MD, Cooke BM, Guillotte M et al (2005) A role for the *Plasmodium falciparum* RESA protein in resistance against heat shock demonstrated using gene disruption. Mol Microbiol 56:990–1003
170. Pei X, Guo X, Coppel R et al (2007) The ring-infected erythrocyte surface antigen (RESA) of *Plasmodium falciparum* stabilizes spectrin tetramers and suppresses further invasion. Blood 110:1036–1042
171. Diez-Silva M, Park Y, Huang S et al (2012) Pf155/RESA protein influences the dynamic microcirculatory behavior of ring-stage *Plasmodium falciparum* infected red blood cells. Sci Rep 2:614
172. Sargeant TJ, Marti M, Caler E et al (2006) Lineage-specific expansion of proteins exported to erythrocytes in malaria parasites. Genome Biol 7:R12

173. Mayer C, Slater L, Erat MC et al (2012) Structural analysis of the *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) intracellular domain reveals a conserved interaction epitope. J Biol Chem 287:7182–7189
174. Parish LA, Mai DW, Jones ML et al (2013) A member of the *Plasmodium falciparum* PHIST family binds to the erythrocyte cytoskeleton component band 4.1. Malar J 12:160
175. Kilili GK, LaCount DJ (2011) An erythrocyte cytoskeleton-binding motif in exported *Plasmodium falciparum* proteins. Eukaryot Cell 10:1439–1447
176. Ramya TN, Surolia N, Surolia A (2006) 15-Deoxyspergualin modulates *Plasmodium falciparum* heat shock protein function. Biochem Biophys Res Commun 348:585–592
177. Chiang AN, Valderramos JC, Balachandran R et al (2009) Select pyrimidinones inhibit the propagation of the malarial parasite, *Plasmodium falciparum*. Bioorg Med Chem 17:1527–1533
178. Cockburn IL, Pesce ER, Pryzborski JM et al (2011) Screening for small molecule modulators of Hsp70 chaperone activity using protein aggregation suppression assays: inhibition of the plasmodial chaperone PfHsp70-1. Biol Chem 392:431–438
179. Cesa LC, Patury S, Komiyama T et al (2013) Inhibitors of Difficult Protein-Protein Interactions Identified by High-Throughput Screening of Multiprotein Complexes. ACS Chem Biol. doi:10.1021/cb400356m
180. Makley LN, Gestwicki JE (2013) Expanding the number of 'druggable' targets: non-enzymes and protein–protein interactions. Chem Biol Drug Des 81:22–32

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HSP47: The New Heat Shock Protein Therapeutic Target

George Sharbeen, Shelli McAlpine, and Phoebe Phillips

Abstract Heat shock proteins (HSPs) are part of a highly conserved genetic survival system originally discovered by Ferruccio Ritossa in 1962 (Ritossa, Riv Ist Sieroter Ital 37:79–108, 1962). Members of this family function as molecular chaperones that stabilize protein folding (De Maio, Shock 11:1–12, 1999). HSP47 is a molecular chaperone that is essential for collagen biosynthesis (Ishida et al., Mol Biol Cell 17:2346–2355, 2006; Matsuoka et al., Mol Biol Cell 15:4467–4475, 2004; Nagai et al., J Cell Biol 150:1499–1506, 2000). This chaperone is a potential therapeutic target, as it has been observed to be upregulated in collagen-producing cells in several fibrotic conditions (Taguchi et al., Acta Histochem Cytochem 44:35–41, 2011). The recent resolution of a HSP47 crystal structure has provided new insights into the chaperone's mechanism of action (Widmer et al., Proc Natl Acad Sci U S A 109:13243–13247, 2012) with implications for future drug design. This review will summarize our current understanding of the biochemistry of HSP47-collagen interactions and the potential of HSP47 as a therapeutic target in fibrotic conditions. It will also discuss the current pharmacological inhibitors and the identification of new HSP47 small-molecule inhibitors.

Keywords Cancer, Collagen, Fibrosis, Hsp47, Serpin

Contents

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1 Introduction

1.1 Heat Shock Proteins as Chaperones

All prokaryotic and eukaryotic cells are intrinsically driven to survive and have evolved mechanisms to serve this basic need. The cellular response to heat, originally observed by Ferruccio Ritossa as chromosome "puffing" in *Drosophila* [1], is the most highly conserved survival mechanism, involving expression of a series of proteins called heat shock proteins (HSPs). Since the initial discovery of stress-induced protein expression in *Drosophila* [2], the HSP family of proteins has continued to grow, encompassing a variety of members with different subcellular localizations, expression patterns, and functions [3]. The nomenclature for proteins in this family follows their size, for example, HSP47 is ~47 kDa, HSP70 is ~70 kDa, and HSP90 is ~90 kDa. HSPs primarily function as molecular chaperones that stabilize protein folding either constitutively or during cellular stress [3]. Stabilization is achieved (1) by binding and holding unfolded/unstable polypeptide sequences [4–6] and (2) by forming large protein complexes that facilitate ATP-dependent protein folding [7–9]. HSPs have also been identified as attractive therapeutic targets as they are differentially upregulated in several human diseases including cardiovascular disease, neurodegenerative disease, and cancer [10–15].

1.2 HSP47: A Collagen-Specific Chaperone

HSP47 (also known as colligin, SERPINH1, gp46, and J6 protein) was originally identified as collagen-binding protein in mouse embryo parietal endoderm cells [16], mouse teratocarcinoma stem cells [17], rat skeletal myoblasts [18, 19], and chick embryo fibroblasts [20, 21]. This protein also belongs to the serine protease inhibitor (serpin) family despite a lack of serine protease inhibitory activity

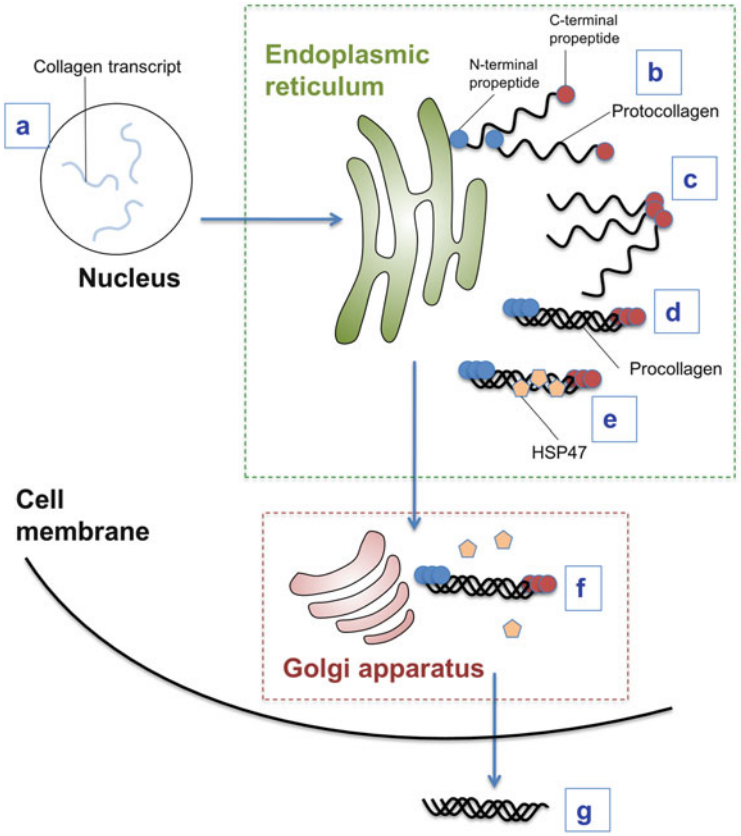

Fig. 1 Collagen biosynthesis. (*a*) Collagen genes are first transcribed in the nucleus then (*b*) translated into monomeric peptide chains (protocollagen) in the endoplasmic reticulum. (*c*–*d*) Collagen peptide chains are assembled into triple-helical trimers (procollagen) by P4H, P3H, lysyl hydroxylase, and protein disulfide isomerase. (*e*) HSP47 binds and stabilizes procollagen under the neutral pH in the endoplasmic reticulum. (*f*) The procollagen is delivered to the *cis*-Golgi, where the slightly acidic pH of the organelle causes release of HSP47. (*g*) The procollagen is delivered out of the cell where propeptides are cleaved to produce collagen

[22]. Unlike other serpin family members, HSP47 localizes to the endoplasmic reticulum [21, 23] and has only one known client protein, collagen [24]. However, it should be noted that recent work by Bianchi et al. [25] has implicated HSP47 as a binding partner of amyloid precursor protein. While this interaction was not specifically shown to be a chaperone function of HSP47, it suggests HSP47 may have undiscovered client proteins and supplemental roles.

HSP47 plays a critical role in collagen biosynthesis by aiding in the assembly of triple helices in procollagen (Fig. 1). Nagai et al. [26] showed the reliance of collagen production on HSP47 in a mouse model. The team observed embryonic lethality in *hsp47*$^{-/-}$ mice that was associated with a loss of correctly folded triple-helical type I collagen [26]. Two subsequent studies confirmed this observation.

Matsuoka and colleagues [27] observed a significant drop in type IV collagen secretion in *hsp47*$^{-/-}$ mouse embryonic stem cells. The type IV collagen that was secreted was more sensitive to protease digestion, suggesting incorrect triple-helix formation [27]. Ishida and colleagues showed that *hsp47*$^{-/-}$ embryonic mouse cells developed insoluble intracellular aggregates of type I collagen due to slower secretion [28]. The type I collagen that was secreted was thin and abnormally branched and contained an unprocessed N-terminal pro-peptide [28].

Dafforn et al. [29] were the first to show HSP47's molecular chaperone function. They demonstrated the ability of HSP47 to bind and fold a peptide that mimics the structure of partially folded procollagen. This action resulted in higher-order assemblies of the folded monomeric peptide into collagen-like polypeptide chains. Ono et al. [30] have since provided direct in vitro and in vivo evidence for the interaction between HSP47 and procollagen triple helices. Collagen is a major component of the extracellular matrix [31]. Fibrosis can arise when collagen-secreting cells are phenotypically altered to overproduce collagen and other extracellular matrix proteins [32, 33]. HSP47 upregulation has been observed in several fibrotic conditions making it a potential therapeutic target [34].

1.3 Structure of HSP47

Serpins have a highly conserved structural fold that consists of a core of three β-sheets surrounded by nine α-helices [35] (Fig. 2). Serpin conformational changes are controlled by four conserved structural regions: hinge, breach, gate, and shutter domains [36] (Fig. 2).

Collagens similarly share highly conserved structures, consisting of three polyproline α-chains that consist of characteristic Gly-X–Y repeats (where X and Y are any amino acids; X is often proline and Y is often hydroxyproline) and trimerize into a right-handed supercoil [37] (Fig. 3). Synthetic collagen peptides used to probe the function of HSP47 mimic this sequence and fold.

Until recently, the lack of a crystal structure has limited the design of small-molecule inhibitors that can functionally target HSP47. In 2012, Widmer et al. [38] obtained crystal structures of canine HSP47 in both its free form and bound to a synthetic trimeric collagen peptide containing Gly-X-Arg repeats. While the structure was of canine HSP47, it shares 97% homology with the human HSP47 amino acid sequence [39] (Fig. 4), making it a *trans*-species representative of HSP47.

The overall structure of HSP47 was consistent with the serpin structural fold of three β-sheets and nine α-helices and was not found to undergo significant conformational change upon binding to collagen (Fig. 5). One to two HSP47 proteins engaged each binding site on the collagen triple helix. In situations where HSP47 bound either the leading or lagging α-helix or both, binding to the middle α-helix was sterically hindered [38]. While contacts were primarily made between a HSP47 monomer and its target collagen strand, additional contacts were also observed between the same HSP47 monomer and the other two strands of the trimeric

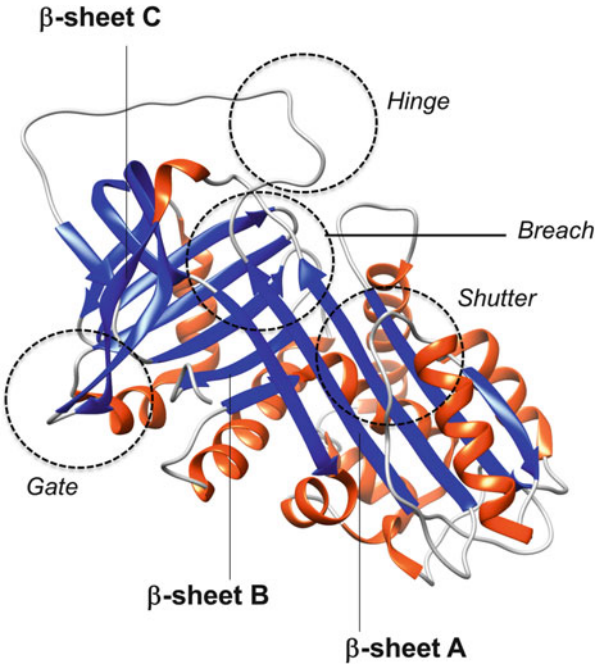

Fig. 2 Structure of a serpin family member. The crystal structure of human α1-proteinase inhibitor in its native conformation is shown. α-Helices and β-strands are depicted in ribbon form. α-Helices are colored in *red* and β-strands are colored *blue*. The three highly conserved β-sheets (β-sheet A–C) are labeled. Regions critical for conformational changes (breach, shutter, gate, and hinge regions) are *circled* and labeled in *italics*. The structure was constructed using the UCSF Chimera package and PDB file 1OO8 [103]

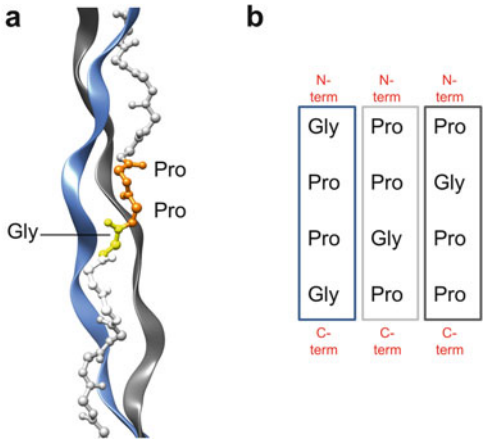

Fig. 3 The structure of collagen. (**a**) The crystal structure of a triple-helical collagen-mimetic peptide. Individual collagen peptides are colored *blue*, *gray*, or *white* and are depicted in ribbon form or in ball-and-stick form. The repeating Gly-Pro–Pro unit is highlighted and labeled on the ball-and-stick chain. (**b**) Schematic diagram showing the staggered alignment of the three collagen chains as a result of supercoiling. The structure was constructed using the UCSF Chimera package and PDB file 1CAG [104]

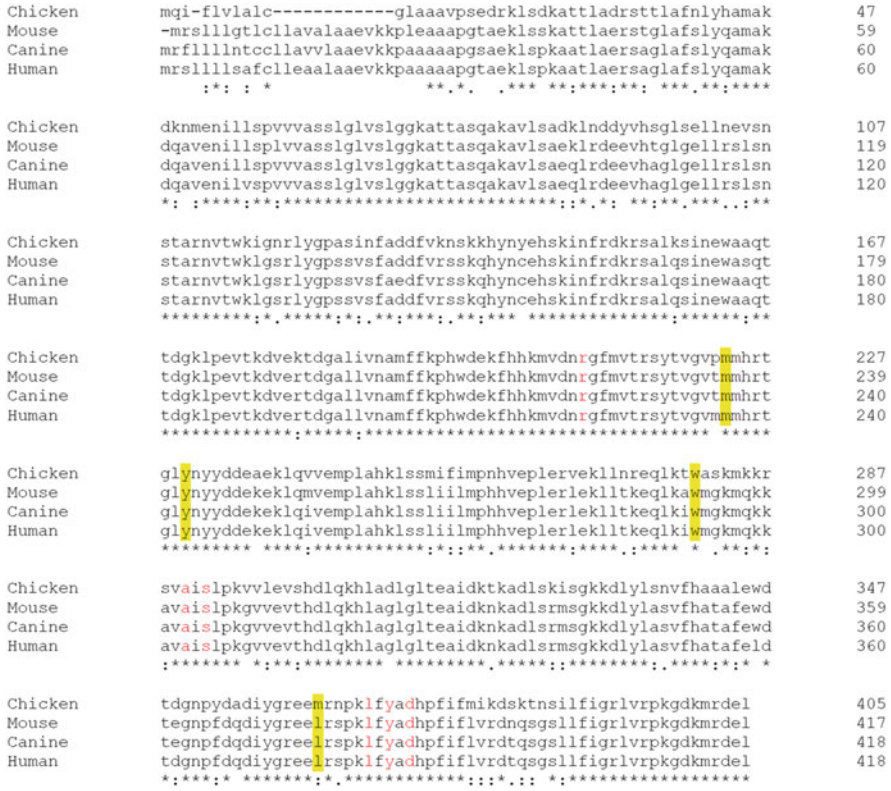

Fig. 4 Protein sequence alignment of human, canine, mouse and chicken HSP47. Sequences from the following accessions were aligned in CLUSTAL Omega: NP_001226 (human), XP_542305 (canine), NP_990622 (chicken), and NP_033955 (mouse). Key residues identified in canine HSP47 are colored *red* [38], and those identified in chicken HSP47 are highlighted in *yellow* [40]. *Asterisks* indicate fully conserved residues. A *colon* indicates conservation between residues of strongly similar properties. *Periods* indicate conservation between residues of weakly similar properties

collagen peptide [38]. The chaperone docked to the Gly-X-Arg sequence on its target strand via a salt bridge between Arg8 of the collagen sequence and Asp385 of HSP47 (Fig. 6). In addition, the authors detected hydrogen bonding between Arg222 of HSP47 and Pro5/Gly6 of the collagen peptide (Fig. 6). In nature, threonine is favored over proline in the Pro5 position [41]. The structure explained this preference, as HSP47 Ser305 and Ala303 are better able to form contacts with a threonine at this position (Fig. 6). Hydrophobic interactions were also detected, particularly involving Leu381 and Tyr383 of the chaperone (Fig. 6). Mutagenesis of Asp385, Leu381, and Tyr383 dramatically reduced HSP47 affinity for collagen, confirming the structural predictions [38].

In the same year, Yagi-Utsumi et al. [40] published their work on characterizing the active site of chicken HSP47 in solution. Their initial multidimensional nuclear magnetic resonance (NMR) approach failed due to HSP47's large size and its

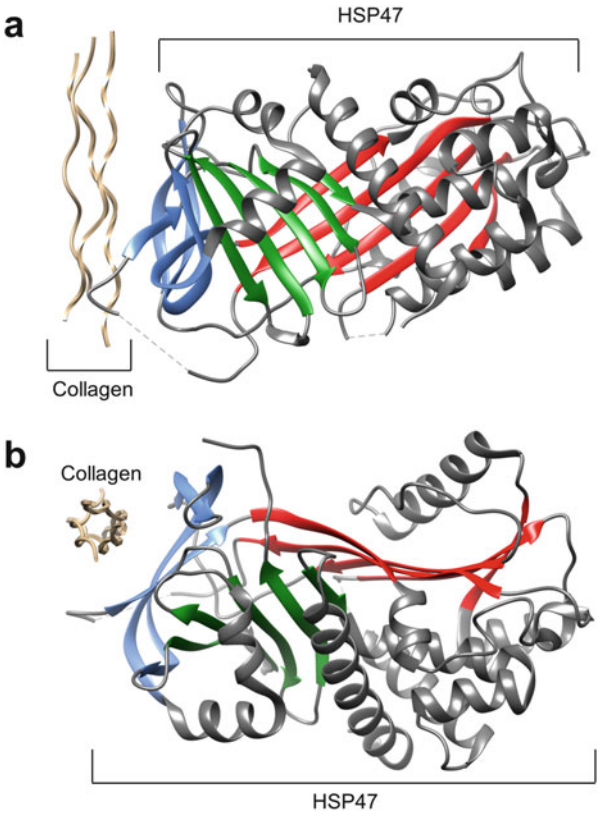

Fig. 5 Crystal structure of HSP47 bound to a triple-helical collagen-mimetic peptide. The overall fold of the protein is shown in ribbon form, from (**a**) a side view and (**b**) a top view. The three highly conserved β-sheets that characterize the serpin family are colored (*red* = β-sheet A, *green* = β-sheet B, *blue* = β-sheet C). *Dashed lines* in the *gray* HSP47 structure represent poorly structured loops that could not be resolved. The structure was constructed using the UCSF Chimera package and PDB file 4AU2 [38]

tendency to aggregate. The authors instead opted for an amino acid selective-labeling approach combined with site-directed mutagenesis and NMR. In this approach, the peaks of highly conserved histidine and tryptophan residues were used as NMR spectroscopic references or probes to test the effect of site-directed mutations on HSP47 collagen binding. Residues that were flagged as important were functionally tested with a collagen-binding assay. The authors determined that HSP47 binds collagen using its B/C β-barrel domain and a nearby loop and involves the residues Met223, Tyr230, Trp280, and Met363. These do not match the key residues identified in the canine HSP47 structure, and most are not at the collagen-binding interface of HSP47 when mapped to canine HSP47. While there is some difference between chicken and canine HSP47 sequences (79% homology, Fig. 4), all the key residues and surrounding sequences identified in the canine HSP47 are present in the chicken sequence. The residues identified in the chicken sequence do appear to sit around the edges of β-sheet C (which contains the collagen-binding interface) in canine HSP47, so it is possible that mutation of these residues may have interfered with the flexibility or structural integrity of the region. Ultimately, in the absence of a 3D structure of chicken HSP47, we can only speculate as to the cause of this difference. What is important to note is that canine HSP47 shares

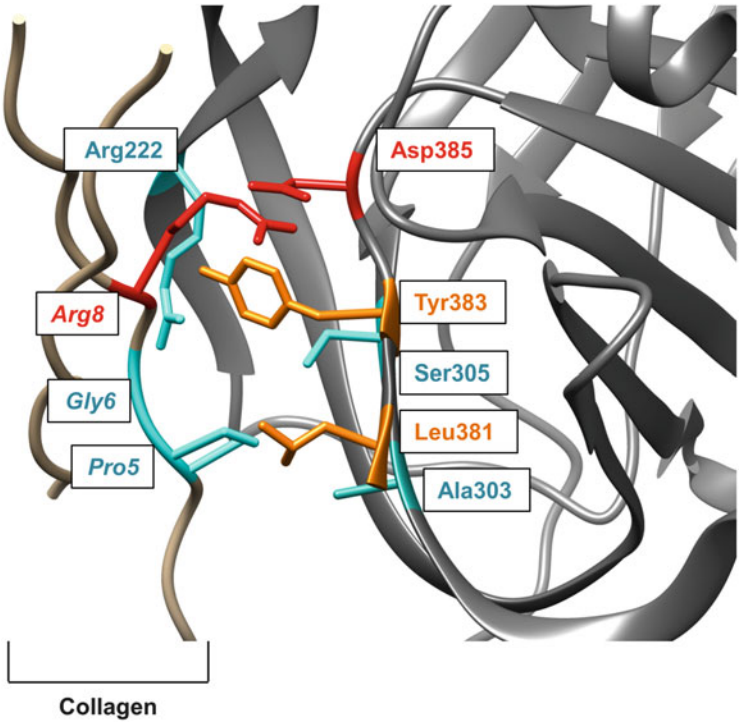

Fig. 6 Key residues involved in HSP47/collagen interactions. The side chains of key residues are shown in *stick form* and *colored* according to predicted interactions (*red* = salt bridge, *blue* = hydrogen bonding/water-based interactions, *orange* = hydrophobic interactions). The name and position of key residues are also shown. Labels for residues in the collagen chain are *italicized*. The middle collagen strand and residues 376–380 off the HSP47 structure have been hidden for clarity. The structure was constructed using the UCSF Chimera package and PDB file 4AU2 [38]

much higher homology with human HSP47 (97% homology, Fig. 4), making it highly relevant to the study of human HSP47.

The identification of key binding-site residues in β-sheet C of HSP47 (Figs. 2 and 5) represents a significant advance for therapeutic development by facilitating structure-based drug design. Based on the high degree of homology between canine and human HSP47 (Fig. 4), competitive HSP47 inhibitors would aim to target the human equivalents of residues Arg222, Ala303, Ser305, Leu381, Tyr383, and Asp385.

2 Molecular Mechanisms That Govern HSP47 Binding to Collagen

2.1 pH Sensitivity

HSP47 has been shown to dissociate from procollagen upon transport of the client protein out of the ER and into the *cis*-Golgi [42]. The disengaged HSP47 is targeted back to the ER via an RDEL ER-retention sequence [23]. Release of collagen from HSP47 has been attributed to a change in pH between the neutral ER and the slightly acidic *cis*-Golgi. The impact of acidic pH on HSP47's affinity for collagen has been demonstrated in vitro and correlated with *trans*-conformational changes in the chaperone [43]. Recently, Abdul-Wahab and colleagues [44] characterized the molecular switch that drives collagen release by mouse HSP47. The team investigated the role of six evolutionarily conserved HSP47 histidine residues in binding to collagen, using a combination of homology-based 3D modeling, site-directed histidine to alanine mutations, and collagen-binding/stabilization assays. The six HSP47 histidine residues were located in the "breach," "gate," and "shutter" domains of the protein. Only mutations of histidines in the breach cluster, specifically His191, His197, and His198 (His208, 214, and 215 on the full mouse protein sequence), were found to affect collagen binding and release. Collagen affinity chromatography showed that the His191 HSP47 mutant released collagen at a slightly higher pH than WT HSP47 and other HSP47 mutants, while His197/8 mutants lost their ability to release their client protein in a pH-dependent manner. Complementary to this work, the authors [44] mutated highly conserved HSP47 tryptophan residues and used circular dichroism spectroscopy to measure secondary structure changes in response to a pH titration. Mutation of Trp192, which resides in the breach region, markedly reduced the conformational change of HSP47 relative to the WT protein, suggesting the breach region experiences a significant conformational change as part of the pH switch. This work revealed that the molecular mechanism for pH-mediated collagen release from HSP47 resides in the breach region. Importantly, the study suggests that histidines in this domain might represent drug targets as interference in pH-dependent conformational changes of the breach region could either impede initial collagen binding or prevent release of collagen from HSP47, thereby preventing collagen secretion.

2.2 Collagen Primary Sequence: Gly-X-Arg

The primary collagen sequence is critical to HSP47 binding. The triple-helical arrangement of collagen spatially restricts every third residue in the primary collagen sequence to a small, inert glycine [37]. As mentioned in Sect. 1, collagen α-helices consist of Gly-X–Y triplets, where X and Y are any amino acid [37] (Fig. 3b). Several studies have investigated the interaction of HSP47 with synthetic

triple-helical collagen-mimetic peptides based on this sequence. In 2002, Tasab et al. [45] showed that HSP47 binding to collagen required a minimum of one Gly-X-Arg sequence in the triple helix. In the same year, Koide et al. [46] demonstrated that replacing the proline with arginine in the Y position of a Gly-Pro–Pro collagen peptide enhanced its binding to HSP47. The same group also corroborated the requirement of a single Gly-X-Arg sequence for HSP47 binding, in a subsequent study [47].

2.3 Collagen Tertiary Structure: Triple-Helical Fold

Effective HSP47 binding to collagen also requires a correctly folded triple-helical collagen structure. In 2000, Tasab and colleagues [48] used a cell-based assay to show that only triple-helical collagen peptides could be co-immunoprecipitated with HSP47. This observation was reiterated in 2006 by Koide and colleagues [47]. The team measured the affinity of human and mouse HSP47 for conformation – restricted triple-helical and monomeric collagen peptides in vitro and observed preferential HSP47 binding to correctly folded triple-helical collagen [47]. Ono et al. [30] more recently demonstrated this requirement both in vitro and in live cells. The team developed an elegant in vitro fluorescence resonance energy transfer (FRET) assay to measure the interaction of chicken and human HSP47 with a monomeric or trimeric collagen peptide. They observed a dose-dependent increase in FRET signal with increasing concentrations of the trimeric peptide but no increase with the monomeric form. A similar approach was used in vivo. However, instead of utilizing FRET, the authors divided the fluorophore mKG into two protein fragments, the C-terminal fragment attached to the monomeric or trimeric collagen peptide and the N-terminal fragment attached to HSP47. Binding of collagen by HSP47 then allowed the two fragments to sterically interact, forming a functional fluorescent protein. The in vivo results reproduced in vitro observations and showed that HSP47 only binds the trimeric collagen peptide. HSP47's preference for properly folded collagen is explained by Widmer et al.'s [38] resolution of the collagen-bound HSP47 crystal structure. The structure showed that HSP47/collagen complex formation buried approximately 1,000 $Å^2$ of solvent-accessible surface on HSP47. These contacts were only possible in the correct triple-helical configuration of collagen. Thus, HSP47 has higher affinity for correctly folded collagen as this form maximizes interactions between the chaperone and client protein.

3 Therapeutic Potential of Targeting HSP47

Fibrosis refers to the excessive deposition of extracellular matrix (ECM) proteins, particularly collagens, in response to tissue damage and inflammation [33]. It occurs when the balance of ECM degradation and production, required for maintenance of normal organ structure, is skewed in favor of ECM production [33]. Excessive collagen deposition is observed in a variety of diseases including liver cirrhosis, pulmonary fibrosis, and pancreatic cancer, to name just a few [32, 49, 50]. The perpetrators of fibrosis are ECM-producing myofibroblasts responding to chronic tissue damage [51]. During wound healing, growth factors secreted by immune cells and platelets, such as transforming growth factor-β (TGFβ), interleukin-1β (IL-1β), and platelet-derived growth factor (PDGF), activate fibroblasts to produce ECM proteins including collagen [51]. Chronic activation of fibroblasts results in excessive deposition of ECM proteins and thus fibrosis [51]. There are currently no effective therapeutic treatments for fibrosis, necessitating the need for new targets and approaches.

HSP47 is suggested to be an ideal therapeutic target for fibrosis, as it is specifically expressed in collagen-producing cells [52] and its upregulation has been observed in various fibrotic diseases including liver cirrhosis and idiopathic pulmonary fibrosis [53–60]. Furthermore, in animal models of fibrosis, inhibition of HSP47 has been shown to suppress collagen secretion [54, 61, 62]. Conversely, retroviral expression of HSP47 in human vascular smooth muscle cells increased procollagen expression, suggesting HSP47 also directly influences collagen expression [63].

Chronic liver damage ultimately leads to liver cirrhosis and is characterized by excessive collagen deposition that distorts the normal liver tissue architecture. The result is impairment of hepatic blood flow leading to an accumulation of blood in the hepatic portal vein, a condition known as portal hypertension [64]. Hepatic cells lining the sinusoids normally maintain close contact with blood from the hepatic portal vein and play a critical role in regulating systemic glucose and lipid supplies [64]. Cirrhosis forces blood to bypass these cells and return to the circulation via abnormal routes [64]. Hepatic stellate cells (HSCs) are key pro-fibrogenic, collagen-secreting cells in the liver that are activated by cytokines released in response to tissue damage [50]. Brown et al. [53] measured HSP47 in human samples of the normal and fibrotic liver, finding that HSP47 was specifically expressed in HSCs and was elevated in tissue samples obtained from chronic liver disease patients. Earlier, Masuda and colleagues [55] had measured the change in HSP47 expression in a carbon tetrachloride (CCl_4)-inducible rat model of liver fibrosis. They observed a similar increase in HSP47 expression concomitant with induction of liver fibrosis in animals with repeated CCl_4 treatments, whereas animals receiving only a single dose of CCl_4 failed to produce the same increase. The results indicated that HSP47 upregulation was linked to the development of fibrosis and not a direct response to the CCl_4 [55].

Inhibition of collagen synthesis appears to be a promising treatment approach for liver cirrhosis. Issa and colleagues [65] observed that regression of CCl_4-induced liver fibrosis in rats was associated with downregulation of collagen I and tissue inhibitor of metalloproteinase (TIMP) and upregulation of collagen-degrading matrix metalloproteinases (MMPs). This reversibility has also been observed in human patients [66]. Alternatively, some studies have attempted to therapeutically treat liver fibrosis by increasing expression of MMPs, for example, Parsons et al. [67] showed that inhibition of TIMP-1 could attenuate liver fibrosis in CCL_4-treated rats. However, translation of these findings to clinical outcomes has been hindered by the lack of specificity of these approaches.

In 2008, Sato and colleagues [62] took a significant step toward developing a targeted, anti-fibrotic treatment for liver cirrhosis. Their approach utilized vitamin A-coupled liposomes that carried siRNA against HSP47. Activated HSCs absorb vitamin A [68], thereby allowing the particles to specifically target these pro-fibrogenic liver cells. Their theory was that specifically silencing HSP47 in HSCs would halt and/or regress fibrosis in the liver. The team used three different rat models of liver cirrhosis: (1) treatment with dimethylnitrosamine (DMN), which induces a lethal hepatic fibrosis; (2) treatment with CCL4, which is a milder and non-lethal liver fibrosis model; or (3) bile duct ligation which is a chronic model of liver cirrhosis. Cirrhotic animals were treated with repeated intravenous injections of HSP47 siRNA-loaded vitamin A liposomes while cirrhosis was still being induced. In all three models, reversal of liver fibrosis was observed after five administrations of HSP47 siRNA, as evidenced by a reduction in the fibrotic area and collagen in the liver. Additionally, survival of DMN-treated rats was significantly improved by HSP47 siRNA treatments in a dose-dependent manner, with the highest dose giving a 100% survival rate after 10 weeks of DMN treatment compared to control groups which exhibited 100% lethality by the eighth week [62].

More recently, Park and colleagues [69] successfully suppressed collagen production in vitro in the human HSC line LX-2. They achieved this by treating LX-2 cells with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), an anticancer agent that largely does not affect normal cells [70, 71]. Both HSP47 transcription and protein activity are under the control of the stress-response transcription factor heat shock factor-1 (HSF1) [72]. The authors demonstrated that TRAIL treatment of LX-2 cells led to dephosphorylation of HSF1, thus inactivating it and consequently reducing expression of HSP47. The result was reduction in soluble collagen secretion that was enhanced with increasing doses of TRAIL and by the addition of HSP47 siRNA. Conversely, overexpression of HSP47 was able to overcome TRAIL-mediated suppression of collagen production, directly implicating HSP47 in HSC collagen production. While the translation of this study to clinical applications is limited by its use of immortalized HSCs, the work nicely demonstrates the effectiveness of HSP47 as a therapeutic target to inhibit collagen secretion in fibrotic liver [69].

Pulmonary fibrosis is a consequence of many lung diseases, whereby excessive collagen deposits thicken the interstitial lung tissue that separates blood vessels

from air spaces in the lungs [49]. This impairs the diffusion of gases across the membrane, thereby hindering effective oxygen delivery into the body and carbon dioxide out of the body and ultimately leading to alveolar collapse [49]. HSP47 is elevated in the fibrotic regions of lung tissue from idiopathic pulmonary fibrosis (IPF) patients [73, 74]. A study of IPF patient lung biopsies by Amenomori and colleagues [75] revealed increased HSP47 expression in activated lung fibroblasts that correlated with poorer patient survival. More recently, Kakugawa et al. [76] showed elevated HSP47 expression in tissue samples and serum from patients with acutely exacerbated IPF.

Bleomycin-induced pulmonary fibrosis animal models have been used extensively to study lung fibrosis [77]. In 2003, Ishi et al. [78] characterized HSP47 expression in mice after bleomycin delivery. They observed upregulation of HSP47, primarily in cells positively staining for α-smoooth muscle actin (α-SMA, a myofibroblast marker), surfactant protein-A (SP-A, a marker of type II pneumocytes), or F4/80 (a macrophage marker). The upregulation significantly correlated with increased lung collagen content [78]. A follow-up study by the same group [79] similarly characterized HSP47 expression in mice after 5 days of daily intravenous bleomycin administration. Lung tissue sections were probed for HSP47 mRNA via in situ hybridization and adjacent tissue sections probed for α-SMA, SP-A, and F4/80 cell markers by immunohistochemistry. Consistent with the 2003 study and with clinical data, HSP47 mRNA was not detected in control lung tissue but was significantly elevated in bleomycin-treated murine lungs. Immunohistochemistry confirmed that HSP47-expressing cells fell into three major groups of cells, α-SMA-positive lung myofibroblasts in the interstitial space, SP-A-positive type II pneumocytes in the alveolar walls, and F4/80-positive macrophages [79].

In 2004, Kakugawa and colleagues [80] investigated the mechanism of the anti-fibrotic drug pirfenidone in a similar bleomycin-induced mouse model of IPF. After 14 days of bleomycin administration, mice had received daily oral doses of pirfenidone till the completion of the model. Pirfenidone treatment was found to inhibit collagen deposition, as determined by measurement of hydroxyproline content in homogenized lung tissue samples. Immunohistochemistry was used to measure HSP47 expression in the treated murine lungs, as well as the myofibroblast marker α-SMA. The group observed that in response to pirfenidone treatment, the number of α-SMA-positive cells as well as the proportion of α-SMA-positive cells expressing HSP47 was substantially reduced in bleomycin-treated murine lungs, suggesting its anti-fibrotic activity was linked to HSP47 downregulation [80]. However, whether pirfenidone directly inhibits HSP47 or its collagen-binding capacity has not been tested (discussed in Sect. 4.2).

Fibrosis is a characteristic feature of pancreatitis (inflammation of the pancreas and risk factor for pancreatic cancer) and pancreatic cancer and is a consequence of pancreatic stellate cell (PSC; key fibrogenic cells in the pancreas) activation [32, 81, 82]. Additionally, activated PSCs have been shown to directly stimulate pancreatic cancer cells into a more aggressive and chemoresistant phenotype [32]. The extensive fibrosis in pancreatic cancer impairs normal pancreatic endocrine and exocrine function by deforming the tissue architecture but also creates a hypoxic

tumor microenvironment by hindering blood flow through the tumor vasculature [32, 83]. Hypoxia drives the transformation of benign pancreatic cancer cells into a more aggressive, metastatic, and chemoresistant phenotype [84, 85]. In addition, pancreatic fibrosis inhibits effective delivery of chemotherapeutics by sequestering drugs away from pancreatic cancer cells [32, 86–88]. Pancreatic cancer is notoriously hard to treat due to its acquired chemoresistance and metastatic spread [84, 85]. Inhibition of fibrosis in pancreatic cancer is therefore critical in improving disease outcome. The efficacy of anti-fibrotic approaches for pancreatic cancer has been demonstrated in several animal model studies [86–88].

There is growing evidence that HSP47 is a potential therapeutic target for ablating fibrosis in pancreatic cancer. Iacobuzio-Donahue and colleagues [89] carried out a global gene expression screen of normal pancreatic tissue, resected pancreatic cancer tissue, and pancreatic cancer cell lines. HSP47 exhibited a 6.41-fold increase in expression in pancreatic cancer samples relative to normal pancreas. The authors validated HSP47 gene expression by immunohistochemistry, observing abundant HSP47 staining in desmoplastic regions of pancreatic cancer sections. In the same year, the group published a larger immunohistochemical study of patient pancreatic cancer samples for some of the genes they had identified, including HSP47 [90]. HSP47 expression was only observed in 12% of cases in nonneoplastic pancreatic stroma and was limited to scattered fibroblasts. In contrast, the chaperone was extensively expressed in intratumoral and peritumoral stroma/fibrotic areas of all pancreatic cancer samples. Further linking HSP47 expression to pancreatic cancer development was the observation that it was progressively upregulated from noninvasive pancreatic intraepithelial neoplasias (PanINs; precursor lesions of pancreatic cancer) to highly fibrotic pancreatic cancer.

In 2012, Ishiwatari and colleagues [61] demonstrated the potency of HSP47 as a therapeutic target to ablate pancreatic fibrosis in a model of pancreatitis. Like HSCs, PSCs exhibit a vitamin A-storing phenotype [91]. As in their 2008 study on HSP47 in liver fibrosis [62], the group exploited this phenotype by using vitamin A-coupled liposomes to deliver HSP47 siRNA to PSCs. Pancreatic fibrosis was induced in rats by dibutyltin dichloride treatment or cerulean treatment before administration of the liposomes. HSP47 silencing was found to resolve pancreatic fibrosis, as determined by the improved histology, reduced hydroxyproline (collagen) content, and reduced α-SMA-positive cells (activated PSCs). The authors probed pancreatic tissue sections from treated rats for apoptosis via terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. They found it was increased and co-localized with α-SMA positive in HSP47-silenced pancreas samples. In vitro assays confirmed that HSP47 silencing in in situ-activated rat and human PSCs not only reduced collagen secretion in a dose-dependent manner but significantly increased apoptosis [61]. The results suggest that HSP47 silencing could ablate pancreatic fibrosis by a dual function, both inhibiting soluble collagen production and eliminating the activated PSCs that produce it. This work has significant potential implications for pancreatic cancer, as inhibition of HSP47 may inhibit pancreatic cancer-associated fibrosis, which in turn reduces the

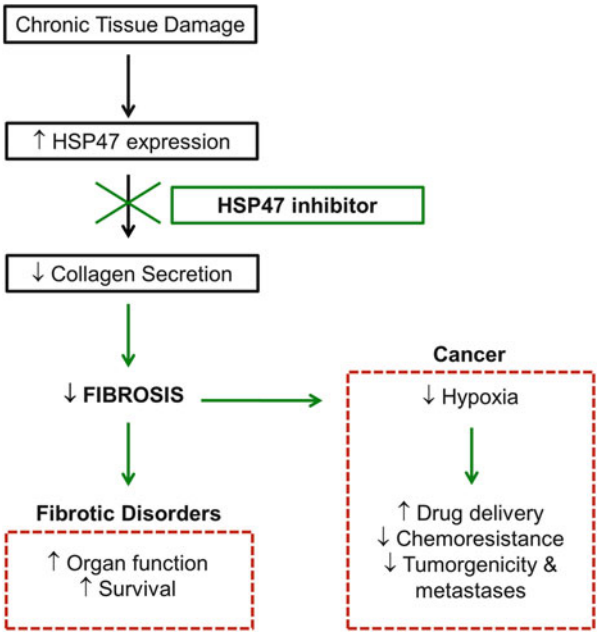

Fig. 7 Therapeutic targeting of HSP47 can inhibit fibrosis and increase patient survival. Chronic tissue damage/inflammation leads to elevated expression of the collagen chaperone HSP47 and increased collagen secretion, resulting in fibrosis [92]. Fibrosis deforms the normal organ architecture, inhibiting organ function and creating a hypoxic microenvironment. Hypoxia drives epithelial to mesenchymal transition (EMT) in cancer cells, which is associated with more aggressive disease and chemoresistance [84, 85]. In addition, fibrosis forms a physical barrier to chemotherapeutics [32, 86–88]. HSP47 inhibitors can halt fibrosis by blocking HSP47 collagen binding, thus impairing correct processing and secretion of collagen. In fibrotic conditions such as liver cirrhosis and pulmonary fibrosis, the primary downstream effect of HSP47 inhibition would be restoration of organ function by resolution of fibrosis, thus increasing patient survival. In cancer, inhibition/resolution of fibrosis by HSP47 inhibition removes the physical barrier to chemotherapeutics and reduces hypoxia, thereby increasing drug delivery to cancer cells, reducing EMT and chemoresistance. HSP47 inhibition also has the potential to eliminate pro-fibrogenic cancer-associated fibroblasts (CA fibroblasts), impairing stimulatory signaling from CA fibroblasts to cancer cells, thus reducing tumor growth and spread [32, 82]

tumorigenicity of pancreatic cancer cells by reducing hypoxia. Moreover, decreasing fibrosis has the potential to increase drug delivery to tumors and decrease tumor progression and metastases. Figure 7 illustrates how HSP47 inhibition may influence cancer progression and clinical outcome in other fibrotic diseases.

4 Pharmacological Inhibitors for HSP47

4.1 Screening Systems to Identify Small-Molecule Inhibitors for HSP47

Given that HSP47 has been implicated in the pathogenesis of several fibrotic disorders (outlined in Sect. 3), it is a research priority to identify potential pharmacological inhibitors that could be used as anti-fibrotic agents. In 2005, Thomson et al. [93] described the development of a high-throughput assay to screen for potential compounds that inhibit HSP47 collagen chaperone activity. The method utilized the ability of mature triple-helical collagen to spontaneously associate to form collagen fibrils, which can be monitored via measuring turbidity by spectrophotometry. Therefore, in the presence of functional HSP47, the collagen fibril formation is reduced and a strong inhibitor of HSP47 restores collagen fibril formation. This assay took advantage of the fact that HSP47 inhibits collagen fibril formation in vitro [94], which supports HSP47's role in preventing the premature association of collagen triple helices in the ER [95]. Using this novel approach, the authors screened 2,080 compounds, identifying four inhibitors of HSP47 with IC50 values ranging from 6.3 to 26.6 μM. The advantage of using this approach to identify inhibitors for HSP47 is that they are likely to have reduced side effects and increased specificity because collagen is the only known substrate for HSP47. As pointed out by the authors, the main limitation of this approach is that it is difficult to isolate active HSP47 [94, 96].

Based on the same principle of fibril formation, Okano-Kosugi et al. [97] developed a high-throughput 384-well platform for screening inhibitors of HSP47. The study emphasized the flexibility of the approach, extending it to other collagen-binding proteins. This was possible because the method relies on the increased turbidity caused by fibril formation and not on any specific changes in the collagen-binding proteins. Thus various combinations of fibril-forming collagens and collagen-binding proteins could be tested. The authors used this platform to compare the inhibitory ability of the four compounds identified as inhibitors in the Thomson et al. study [93] on purified HSP47. Unlike the previous findings, they did not observe any evidence of HSP47 competitive inhibition, despite validating the system with a HSP47 peptide inhibitor. However, the two studies are not directly comparable due to significant methodology variations including: solubility of inhibitors, temperature, and incubation time. Firstly, the Thomson et al. [93] study solubilized the inhibitors in 100% DMSO, whereas Okanu-Kosugi et al. [97] used only 2% DMSO. In addition, incubation of the inhibitor with HSP47 was carried out at 34°C by Thomson and colleagues [93] and for the full length of the assay. In contrast, Okanu-Kosugi et al. [97] carried out a pre-assay incubation step for 10 min and at 4°C. The shorter incubation time and lower temperature may have prevented efficient binding of the inhibitors to HSP47 and may have additionally caused precipitation of the inhibitors.

A limitation in the field is that, although the above two studies have identified assays suitable for high-throughput screening of potential HSP47 inhibitors, there is no subsequent biological validation of the inhibitors. Future studies should combine our knowledge of key regions (as identified by recent structure-function studies, Sect. 1) which are essential for HSP47's interaction with collagen and high-throughput screening systems described above to design and test effective HSP47 inhibitors. Careful attention must also be paid to temperature, compound kinetics, and solubility in these assays. Most importantly, all positive assay results must be validated in a biological cell system, by testing the ability of these inhibitors to effectively suppress collagen secretion from a collagen-producing cell, thus proving their therapeutic potential.

4.2 Pirfenidone

Pirfenidone is an anti-fibrotic and anti-inflammatory agent that was originally designed as a treatment for idiopathic pulmonary fibrosis [80, 98]. The drug has been shown to suppress HSP47 and collagen I expression in TGF-β-stimulated human lung fibroblasts in vitro [98] as well as lung myofibroblasts and type II pneumocytes in a bleomycin-induced mouse model of pulmonary fibrosis [80]. More recently, Hisatomi and colleagues [99] extended these findings to alveolar epithelial cells. The group stimulated the human alveolar epithelial cell line, A549, with TGF-β in vitro, inducing an epithelial to mesenchymal transition and enhancing collagen type I and HSP47 expression. Pirfenidone treatment of these cells was able to reduce expression of both HSP47 and collagen, consistent with findings in lung fibroblasts and type I pneumocytes [99]. Pirfenidone was approved for treatment of idiopathic pulmonary fibrosis in Europe in 2011, supported by several positive clinical studies [100]. Despite these promising results, pirfenidone is still limited by its lack of specificity for HSP47 and its poorly understood mechanism of action.

4.3 Terutroban

Terutroban is an antiplatelet drug that binds the thromboxane/prostaglandin endoperoxide receptor and prevents the development of aorta hyperplasia [101]. In 2011, Gelosa and colleagues [102] investigated the impact of terutroban in a spontaneous, stroke-prone rat model of hypertension (SHRSP). When placed on a high-sodium diet, SHRSP rats developed thicker aortic walls compared to counterparts on a standard diet. The aortic walls of these rats exhibited increased collagen deposition, as determine by Sirius red staining, and increased fibronectin. Coadministration of terutroban with a high-sodium diet prevented these effects. Immunohistochemistry of aorta tissue sections showed extensive HSP47 staining in

high-sodium SHRSP rats relative to standard diet controls, and a striking suppression of HSP47 expression in terutroban-treated counterparts, to levels lower than that of both high-sodium and standard diet SHRSP rats. In addition, quantitative PCR for TGF-β revealed that terutroban also suppresses expression of this pro-fibrotic growth factor [102]. Like pirfenidone, terutroban is limited in its application as a HSP47 inhibitor, as it was not originally designed as a HSP47 inhibitor and is likely to only be indirectly affecting HSP47 expression.

5 Concluding Statements

HSP47 is a key therapeutic target in a variety of fibrotic conditions, due its collagen chaperone function and its disease-associated upregulation (Fig. 7). While existing pharmacotherapeutics indirectly inhibit HSP47, the effects are nonspecific and the mechanisms poorly understood. Recent resolution of HSP47's crystal structure and advances in our understanding of its molecular biology provide key information for the development of more targeted, structure-based therapeutics that can potentially improve the outcome of several fibrotic conditions. Importantly inhibition of HSP47 can already be achieved using a highly specific siRNA nanotechnology approach that has broad therapeutic applicability.

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References

1. Ritossa P (1962) Problems of prophylactic vaccinations of infants. Riv Ist Sieroter Ital 37:79–108
2. Tissieres A, Mitchell HK, Tracy UM (1974) Protein synthesis in salivary glands of Drosophila melanogaster: relation to chromosome puffs. J Mol Biol 84:389–398
3. De Maio A (1999) Heat shock proteins: facts, thoughts, and dreams. Shock 11:1–12
4. Mayer MP, Bukau B (2005) Hsp70 chaperones: cellular functions and molecular mechanism. Cell Mol Life Sci 62:670–684
5. Pratt WB, Toft DO (2003) Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. Exp Biol Med (Maywood) 228:111–133
6. Wegele H, Muller L, Buchner J (2004) Hsp70 and Hsp90–a relay team for protein folding. Rev Physiol Biochem Pharmacol 151:1–44
7. Arrigo AP (2005) Heat shock proteins as molecular chaperones. Med Sci (Paris) 21:619–625
8. Spiess C, Meyer AS, Reissmann S et al (2004) Mechanism of the eukaryotic chaperonin: protein folding in the chamber of secrets. Trends Cell Biol 14:598–604
9. Young JC, Agashe VR, Siegers K et al (2004) Pathways of chaperone-mediated protein folding in the cytosol. Nat Rev Mol Cell Biol 5:781–791
10. Adachi H, Katsuno M, Waza M et al (2009) Heat shock proteins in neurodegenerative diseases: pathogenic roles and therapeutic implications. Int J Hyperthermia 25:647–654

11. Aghdassi A, Phillips P, Dudeja V et al (2007) Heat shock protein 70 increases tumorigenicity and inhibits apoptosis in pancreatic adenocarcinoma. Cancer Res 67:616–625
12. Khalil AA, Kabapy NF, Deraz SF et al (2011) Heat shock proteins in oncology: diagnostic biomarkers or therapeutic targets? Biochim Biophys Acta 1816:89–104
13. Lu X, Kakkar V (2010) The role of heat shock protein (HSP) in atherosclerosis: pathophysiology and clinical opportunities. Curr Med Chem 17:957–973
14. Madrigal-Matute J, Martin-Ventura JL, Blanco-Colio LM et al (2011) Heat-shock proteins in cardiovascular disease. Adv Clin Chem 54:1–43
15. Martins AS, Davies FE, Workman P (2012) Inhibiting the molecular evolution of cancer through HSP90. Oncotarget 3:1054–1056
16. Kurkinen M, Taylor A, Garrels JI et al (1984) Cell surface-associated proteins which bind native type IV collagen or gelatin. J Biol Chem 259:5915–5922
17. Wang SY, Gudas LJ (1990) A retinoic acid-inducible mRNA from F9 teratocarcinoma cells encodes a novel protease inhibitor homologue. J Biol Chem 265:15818–15822
18. Cates GA, Brickenden AM, Sanwal BD (1984) Possible involvement of a cell surface glycoprotein in the differentiation of skeletal myoblasts. J Biol Chem 259:2646–2650
19. Cates GA, Kaur H, Sanwal BD (1984) Inhibition of fusion of skeletal myoblasts by tunicamycin and its reversal by N-acetylglucosamine. Can J Biochem Cell Biol 62:28–35
20. Nagata K, Saga S, Yamada KM (1986) A major collagen-binding protein of chick embryo fibroblasts is a novel heat shock protein. J Cell Biol 103:223–229
21. Saga S, Nagata K, Chen WT et al (1987) pH-dependent function, purification, and intracellular location of a major collagen-binding glycoprotein. J Cell Biol 105:517–527
22. Ragg H (2007) The role of serpins in the surveillance of the secretory pathway. Cell Mol Life Sci 64:2763–2770
23. Satoh M, Hirayoshi K, Yokota S et al (1996) Intracellular interaction of collagen-specific stress protein HSP47 with newly synthesized procollagen. J Cell Biol 133:469–483
24. Ishida Y, Nagata K (2011) Hsp47 as a collagen-specific molecular chaperone. Methods Enzymol 499:167–182
25. Bianchi FT, Camera P, Ala U et al (2011) The collagen chaperone HSP47 is a new interactor of APP that affects the levels of extracellular beta-amyloid peptides. PLoS One 6, e22370
26. Nagai N, Hosokawa M, Itohara S et al (2000) Embryonic lethality of molecular chaperone hsp47 knockout mice is associated with defects in collagen biosynthesis. J Cell Biol 150:1499–1506
27. Matsuoka Y, Kubota H, Adachi E et al (2004) Insufficient folding of type IV collagen and formation of abnormal basement membrane-like structure in embryoid bodies derived from Hsp47-null embryonic stem cells. Mol Biol Cell 15:4467–4475
28. Ishida Y, Kubota H, Yamamoto A et al (2006) Type I collagen in Hsp47-null cells is aggregated in endoplasmic reticulum and deficient in N-propeptide processing and fibrillogenesis. Mol Biol Cell 17:2346–2355
29. Dafforn TR, Della M, Miller AD (2001) The molecular interactions of heat shock protein 47 (Hsp47) and their implications for collagen biosynthesis. J Biol Chem 276:49310–49319
30. Ono T, Miyazaki T, Ishida Y et al (2012) Direct in vitro and in vivo evidence for interaction between Hsp47 protein and collagen triple helix. J Biol Chem 287:6810–6818
31. Gordon MK, Hahn RA (2010) Collagens. Cell Tissue Res 339:247–257
32. Phillips P (2012) Pancreatic stellate cells and fibrosis. In: Grippo PJ, Munshi HG (eds) Pancreatic cancer and tumor microenvironment. Trivandrum, Transworld Research Network
33. Wynn TA, Ramalingam TR (2012) Mechanisms of fibrosis: therapeutic translation for fibrotic disease. Nat Med 18:1028–1040
34. Taguchi T, Nazneen A, Al-Shihri AA et al (2011) Heat shock protein 47: a novel biomarker of phenotypically altered collagen-producing cells. Acta Histochem Cytochem 44:35–41
35. Schreuder HA, de Boer B, Dijkema R et al (1994) The intact and cleaved human antithrombin III complex as a model for serpin-proteinase interactions. Nat Struct Biol 1:48–54

36. Irving JA, Pike RN, Lesk AM et al (2000) Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function. Genome Res 10:1845–1864
37. Shoulders MD, Raines RT (2009) Collagen structure and stability. Annu Rev Biochem 78:929–958
38. Widmer C, Gebauer JM, Brunstein E et al (2012) Molecular basis for the action of the collagen-specific chaperone Hsp47/SERPINH1 and its structure-specific client recognition. Proc Natl Acad Sci U S A 109:13243–13247
39. Drogemuller C, Becker D, Brunner A et al (2009) A missense mutation in the SERPINH1 gene in Dachshunds with osteogenesis imperfecta. PLoS Genet 5, e1000579
40. Yagi-Utsumi M, Yoshikawa S, Yamaguchi Y et al (2012) NMR and mutational identification of the collagen-binding site of the chaperone Hsp47. PLoS One 7, e45930
41. Nishikawa Y, Takahara Y, Asada S et al (2010) A structure-activity relationship study elucidating the mechanism of sequence-specific collagen recognition by the chaperone HSP47. Bioorg Med Chem 18:3767–3775
42. Smith T, Ferreira LR, Hebert C et al (1995) Hsp47 and cyclophilin B traverse the endoplasmic reticulum with procollagen into pre-Golgi intermediate vesicles. A role for Hsp47 and cyclophilin B in the export of procollagen from the endoplasmic reticulum. J Biol Chem 270:18323–18328
43. El-Thaher SH, Drake AF, Yokota S et al (1996) The pH-dependent, ATP-independent interaction of collagen specific serpin/stress protein HSP47. Protein Peptide Letters 3:1–8
44. Abdul-Wahab MF, Homma T, Wright M et al (2013) The pH sensitivity of murine heat shock protein 47 (HSP47) binding to collagen is affected by mutations in the breach histidine cluster. J Biol Chem 288:4452–4461
45. Tasab M, Jenkinson L, Bulleid NJ (2002) Sequence-specific recognition of collagen triple helices by the collagen-specific molecular chaperone HSP47. J Biol Chem 277:35007–35012
46. Koide T, Takahara Y, Asada S et al (2002) Xaa-Arg-Gly triplets in the collagen triple helix are dominant binding sites for the molecular chaperone HSP47. J Biol Chem 277:6178–6182
47. Koide T, Asada S, Takahara Y et al (2006) Specific recognition of the collagen triple helix by chaperone HSP47: minimal structural requirement and spatial molecular orientation. J Biol Chem 281:3432–3438
48. Tasab M, Batten MR, Bulleid NJ (2000) Hsp47: a molecular chaperone that interacts with and stabilizes correctly-folded procollagen. EMBO J 19:2204–2211
49. Crouch E (1990) Pathobiology of pulmonary fibrosis. Am J Physiol 259:L159–L184
50. Yin C, Evason KJ, Asahina K et al (2013) Hepatic stellate cells in liver development, regeneration, and cancer. J Clin Invest 123:1902–1910
51. Kalluri R, Zeisberg M (2006) Fibroblasts in cancer. Nat Rev Cancer 6:392–401
52. Nagata K (2003) Therapeutic strategy for fibrotic diseases by regulating the expression of collagen-specific molecular chaperone HSP47. Nihon Yakurigaku Zasshi 121:4–14
53. Brown KE, Broadhurst KA, Mathahs MM et al (2005) Expression of HSP47, a collagen-specific chaperone, in normal and diseased human liver. Lab Invest 85:789–797
54. Hagiwara S, Iwasaka H, Matsumoto S et al (2007) Introduction of antisense oligonucleotides to heat shock protein 47 prevents pulmonary fibrosis in lipopolysaccharide-induced pneumopathy of the rat. Eur J Pharmacol 564:174–180
55. Masuda H, Fukumoto M, Hirayoshi K et al (1994) Coexpression of the collagen-binding stress protein HSP47 gene and the alpha 1(I) and alpha 1(III) collagen genes in carbon tetrachloride-induced rat liver fibrosis. J Clin Invest 94:2481–2488
56. Murakami S, Toda Y, Seki T et al (2001) Heat shock protein (HSP) 47 and collagen are upregulated during neointimal formation in the balloon-injured rat carotid artery. Atherosclerosis 157:361–368
57. Naitoh M, Hosokawa N, Kubota H et al (2001) Upregulation of HSP47 and collagen type III in the dermal fibrotic disease, keloid. Biochem Biophys Res Commun 280:1316–1322

58. Razzaque MS, Kumatori A, Harada T et al (1998) Coexpression of collagens and collagen-binding heat shock protein 47 in human diabetic nephropathy and IgA nephropathy. Nephron 80:434–443
59. Razzaque MS, Nazneen A, Taguchi T (1998) Immunolocalization of collagen and collagen-binding heat shock protein 47 in fibrotic lung diseases. Mod Pathol 11:1183–1188
60. Rocnik E, Chow LH, Pickering JG (2000) Heat shock protein 47 is expressed in fibrous regions of human atheroma and Is regulated by growth factors and oxidized low-density lipoprotein. Circulation 101:1229–1233
61. Ishiwatari H, Sato Y, Murase K et al (2012) Treatment of pancreatic fibrosis with siRNA against a collagen-specific chaperone in vitamin A-coupled liposomes. Gut. doi:10.1136/gutjnl-2011-301746
62. Sato Y, Murase K, Kato J et al (2008) Resolution of liver cirrhosis using vitamin A-coupled liposomes to deliver siRNA against a collagen-specific chaperone. Nat Biotechnol 26:431–442
63. Rocnik EF, van der Veer E, Cao H et al (2002) Functional linkage between the endoplasmic reticulum protein Hsp47 and procollagen expression in human vascular smooth muscle cells. J Biol Chem 277:38571–38578
64. Ishak KG, Zimmerman HJ, Ray MB (1991) Alcoholic liver disease: pathologic, pathogenetic and clinical aspects. Alcohol Clin Exp Res 15:45–66
65. Issa R, Zhou X, Constandinou CM et al (2004) Spontaneous recovery from micronodular cirrhosis: evidence for incomplete resolution associated with matrix cross-linking. Gastroenterology 126:1795–1808
66. Arthur MJ (2002) Reversibility of liver fibrosis and cirrhosis following treatment for hepatitis C. Gastroenterology 122:1525–1528
67. Parsons CJ, Bradford BU, Pan CQ et al (2004) Antifibrotic effects of a tissue inhibitor of metalloproteinase-1 antibody on established liver fibrosis in rats. Hepatology 40:1106–1115
68. Blomhoff R, Wake K (1991) Perisinusoidal stellate cells of the liver: important roles in retinol metabolism and fibrosis. FASEB J 5:271–277
69. Park SJ, Sohn HY, Park SI (2013) TRAIL regulates collagen production through HSF1-dependent Hsp47 expression in activated hepatic stellate cells. Cell Signal 25:1635–1643
70. Johnstone RW, Frew AJ, Smyth MJ (2008) The TRAIL apoptotic pathway in cancer onset, progression and therapy. Nat Rev Cancer 8:782–798
71. Wiley SR, Schooley K, Smolak PJ et al (1995) Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 3:673–682
72. Christians ES, Yan LJ, Benjamin IJ (2002) Heat shock factor 1 and heat shock proteins: critical partners in protection against acute cell injury. Crit Care Med 30:S43–S50
73. Iwashita T, Kadota J, Naito S et al (2000) Involvement of collagen-binding heat shock protein 47 and procollagen type I synthesis in idiopathic pulmonary fibrosis: contribution of type II pneumocytes to fibrosis. Hum Pathol 31:1498–1505
74. Kakugawa T, Mukae H, Hayashi T et al (2005) Expression of HSP47 in usual interstitial pneumonia and nonspecific interstitial pneumonia. Respir Res 6:57
75. Amenomori M, Mukae H, Sakamoto N et al (2010) HSP47 in lung fibroblasts is a predictor of survival in fibrotic nonspecific interstitial pneumonia. Respir Med 104:895–901
76. Kakugawa T, Yokota SI, Ishimatsu Y et al (2013) Serum heat shock protein 47 levels are elevated in acute exacerbation of idiopathic pulmonary fibrosis. Cell Stress Chaperones. doi:10.1007/s12192-013-0411-5
77. Moeller A, Ask K, Warburton D et al (2008) The bleomycin animal model: a useful tool to investigate treatment options for idiopathic pulmonary fibrosis? Int J Biochem Cell Biol 40:362–382
78. Ishii H, Mukae H, Kakugawa T et al (2003) Increased expression of collagen-binding heat shock protein 47 in murine bleomycin-induced pneumopathy. Am J Physiol Lung Cell Mol Physiol 285:L957–L963

79. Kakugawa T, Mukae H, Hishikawa Y et al (2010) Localization of HSP47 mRNA in murine bleomycin-induced pulmonary fibrosis. Virchows Arch 456:309–315
80. Kakugawa T, Mukae H, Hayashi T et al (2004) Pirfenidone attenuates expression of HSP47 in murine bleomycin-induced pulmonary fibrosis. Eur Respir J 24:57–65
81. Vonlaufen A, Joshi S, Qu C et al (2008) Pancreatic stellate cells: partners in crime with pancreatic cancer cells. Cancer Res 68:2085–2093
82. Vonlaufen A, Phillips PA, Xu Z et al (2008) Pancreatic stellate cells and pancreatic cancer cells: an unholy alliance. Cancer Res 68:7707–7710
83. Bristow RG, Hill RP (2008) Hypoxia and metabolism. Hypoxia, DNA repair and genetic instability. Nat Rev Cancer 8:180–192
84. Wang Z, Li Y, Ahmad A et al (2011) Pancreatic cancer: understanding and overcoming chemoresistance. Nat Rev Gastroenterol Hepatol 8:27–33
85. Zalatnai A, Molnar J (2007) Review. Molecular background of chemoresistance in pancreatic cancer. In Vivo 21:339–347
86. Jacobetz MA, Chan DS, Neesse A et al (2013) Hyaluronan impairs vascular function and drug delivery in a mouse model of pancreatic cancer. Gut 62:112–120
87. Olive KP, Jacobetz MA, Davidson CJ et al (2009) Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. Science 324:1457–1461
88. Provenzano PP, Cuevas C, Chang AE et al (2012) Enzymatic targeting of the stroma ablates physical barriers to treatment of pancreatic ductal adenocarcinoma. Cancer Cell 21:418–429
89. Iacobuzio-Donahue CA, Maitra A, Shen-Ong GL et al (2002) Discovery of novel tumor markers of pancreatic cancer using global gene expression technology. Am J Pathol 160:1239–1249
90. Maitra A, Iacobuzio-Donahue C, Rahman A et al (2002) Immunohistochemical validation of a novel epithelial and a novel stromal marker of pancreatic ductal adenocarcinoma identified by global expression microarrays: sea urchin fascin homolog and heat shock protein 47. Am J Clin Pathol 118:52–59
91. McCarroll JA, Phillips PA, Santucci N et al (2006) Vitamin A inhibits pancreatic stellate cell activation: implications for treatment of pancreatic fibrosis. Gut 55:79–89
92. Taguchi T, Razzaque MS (2007) The collagen-specific molecular chaperone HSP47: is there a role in fibrosis? Trends Mol Med 13:45–53
93. Thomson CA, Atkinson HM, Ananthanarayanan VS (2005) Identification of small molecule chemical inhibitors of the collagen-specific chaperone Hsp47. J Med Chem 48:1680–1684
94. Lasky JA, Ortiz LA (2001) Antifibrotic therapy for the treatment of pulmonary fibrosis. Am J Med Sci 322:213–221
95. Nagata K (1998) Expression and function of heat shock protein 47: a collagen-specific molecular chaperone in the endoplasmic reticulum. Matrix Biol 16:379–386
96. Thomson CA, Ananthanarayanan VS (2001) A method for expression and purification of soluble, active Hsp47, a collagen-specific molecular chaperone. Protein Expr Purif 23:8–13
97. Okano-Kosugi H, Matsushita O, Asada S et al (2009) Development of a high-throughput screening system for the compounds that inhibit collagen-protein interactions. Anal Biochem 394:125–131
98. Nakayama S, Mukae H, Sakamoto N et al (2008) Pirfenidone inhibits the expression of HSP47 in TGF-beta1-stimulated human lung fibroblasts. Life Sci 82:210–217
99. Hisatomi K, Mukae H, Sakamoto N et al (2012) Pirfenidone inhibits TGF-beta1-induced over-expression of collagen type I and heat shock protein 47 in A549 cells. BMC Pulm Med 12:24
100. Cottin V (2013) The role of pirfenidone in the treatment of idiopathic pulmonary fibrosis. Respir Res 14(Suppl 1):S5
101. Chamorro A (2009) TP receptor antagonism: a new concept in atherothrombosis and stroke prevention. Cerebrovasc Dis 27(Suppl 3):20–27

102. Gelosa P, Sevin G, Pignieri A et al (2011) Terutroban, a thromboxane/prostaglandin endoperoxide receptor antagonist, prevents hypertensive vascular hypertrophy and fibrosis. Am J Physiol Heart Circ Physiol 300:H762–H768
103. Dementiev A, Simonovic M, Volz K, Gettins PG (2003) Canonical inhibitor-like interactions explain reactivity of alpha1-proteinase inhibitor Pittsburgh and antithrombin with proteinases. J Biol Chem 278:37881–37887
104. Bella J, Eaton M, Brodsky B, Berman HM (1994) Crystal and molecular structure of a collagen-like peptide at 1.9 A resolution. Science 266:75–81

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Heat Shock Protein 27: Structure, Function, Cellular Role and Inhibitors

Rashid Mehmood and Shelli R. McAlpine

Abstract Hsp27 is an important heat shock protein found in all organisms from prokaryotes to mammals. It is a structurally conserved ATP-independent protein, present in both normal and abnormal tissues. Its expression and induction occur under stressed conditions. The self-association to form oligomers and subsequent equilibrium between oligomer and dimer play critical roles in regulating Hsp27's function. Site-specific phosphorylation of Hsp27 regulates this equilibrium, controlling the activity of Hsp27. Hsp27's molecular chaperone activity includes interacting with a large number of proteins and regulating their folding states. Significant levels of Hsp27 expression have been observed in many diseases, including cancer, neuronal diseases and cardiac diseases. Herein we describe the current understanding of Hsp27's structure, function, cellular role and inhibitors.

Keywords ATP independent, Cancer, Heat shock proteins, Hsp27, Natural products, Small molecules

Contents

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1 Introduction

Heat shock protein 27 kDa (Hsp27) has a molecular weight of ~27,000 g/mol (27 kDa) and belongs to a family of small heat shock proteins (Hsp) that are produced in virtually all organisms from prokaryotes to mammals [1]. The expression and induction of Hsp27 occur amongst many cell types and tissues under both normal and stress conditions [2]. Under normal conditions, Hsp27 regulates protein-folding events. It exists as polydispersed assemblies [3, 4]. These assemblies are regulated by phosphorylation events, where three sequential phosphorylation events drive the inactive oligomers to the active dimer form [5]. The molecular chaperone exists in the inactive state as large oligomers, with sizes up to 800 kDa (29-mers), and an average size of 14-mers [5]. Unlike most of the heat shock proteins that regulate protein folding and aggregation, Hsp27 is not dependent on ATP [6].

Hsp27 expression is upregulated in cellular differentiation and development [7] as well as in cells under environmentally stressed conditions such as heat and oxidative surroundings. Hsp27's expression is also increased during inflammation events [8]. High levels of Hsp27 expression have been observed in numerous diseases including cardiovascular and neuronal disorders and cancer, which is logical since these diseases produce a stressed environment in the cell [9–13]. The gene encoding for Hsp27 (heat shock protein beta-1 or *HSPB1*) contains a regulatory DNA motif known as the heat shock element (HSE). This HSE motif, which is located upstream of the *HSPB1* gene, is used by heat shock factor-1 (HSF-1) to induce transcription of Hsp27. Specifically under stressed conditions, HSF-1 binds to the HSE motif, whereupon recruitment of other transcription factors leads to the transcription of Hsp27 [14–16].

2 Structure and Function

There are 205 amino acid residues in the full-length Hsp27 protein [17]. The structure of Hsp27 is divided into three parts as shown in Fig. 1.

The N-terminal domain contains two regions, including a hydrophobic domain of amino acids 16–19, which is highly conserved across species. These four amino acids are referred to as the WDPF domain, which denotes the sequence Trp–Asp–Pro–Phe (Fig. 1). The remainder of the N-terminal sequence (from residues 1–15

Fig. 1 Full-length structural model of Hsp27. Full-length Hsp27 (205 amino acids) is divided into three portions; (**a**) N-terminus covers amino acid residues 1–39 and contains the WDPF (Trp–Asp–Pro–Phe) domain, (**b**) the α-crystallin domain which is from residues 87–167 and (**c**) the C-terminus, which consists of residues 179–205, which contain the IXI/V motif (specifically the residues between 179 and 182)

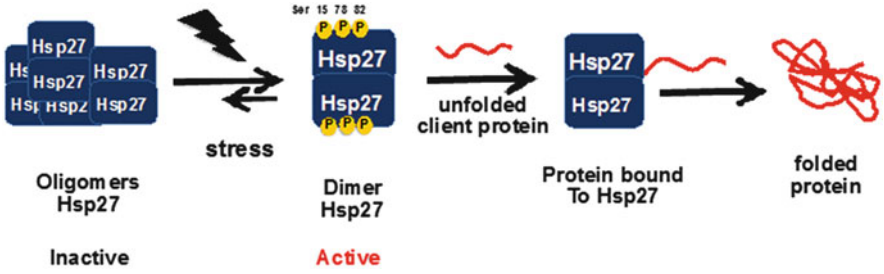


Fig. 2 Phosphorylation of three serines (15, 78 and 82) drives Hsp27 from oligomers to dimer formation. Hsp27 exists as an inactive oligomeric state, which upon serine phosphorylation under stress forms the active dimer. This dimer picks up the unfolded client proteins and facilitates protein folding

and 19–38) varies widely across species. This region is highly flexible and does not have ordered secondary structure. Although most of the N-terminus is not highly conserved, it contains many of the regulatory post-translational modification sites 17 [17].These sites, once modified, control Hsp27 structure, function and even expression.

Recent reports show that phosphorylation of Hsp27 at serine residue 15 in the N-terminus, and serines 78 and 82 in the ACD, promotes oligomer disassociation of Hsp27 and the formation of the active dimer (Fig. 2) [5, 18]. Initial observations showed that an increase in chaperone activity correlated to a decrease in the oligomer size [19–22]. Extensive studies now show that the active chaperone species is the dimer form of Hsp27, which picks up the unfolded proteins that are regulated by Hsp27, and refolds or de-aggregates these proteins (Fig. 2) [5].

The ACD is generally defined as residues 87–167 (Fig. 1), and it is highly conserved across all species of Hsp27. This region is a structured β-sheet with several contact points that facilitate formation of the Hsp27 oligomers. These folds are responsible for the oligomerisation and stabilisation of the inactive Hsp27 protein [23]. The C-terminal domain of Hsp27, defined as residues 179–205, is a highly conserved region across species but is structurally very flexible [24]. The C-terminus also contains a short, flexible and motile extension that protrudes out of the domain. The flexible tail region of C-terminal domain has no defined secondary structure but is negatively charged (Fig. 1).

3 Hsp27 and Its Role in the Cell

One of Hsp27's primary roles is to facilitate folding proteins by transferring unfolded or aggregated proteins to Hsp90 (Fig. 3). Stressed conditions or high levels of unfolded protein accumulation drive Hsp27 from the oligomer to the active dimer form (Fig. 2). The Hsp27 dimer binds to the unfolded protein and transfers it to Hsp40 [11]. Hsp40 then forms a complex with Hsp70 via heat shock interacting protein (HIP). Then this complex of Hsp40 and Hsp70 binds to Hsp90 via heat shock organising protein (HOP). The client protein is transferred to Hsp90 from the complex, whereupon Hsp90 binds ATP. A dephosphorylation event of ATP provides the energy required for Hsp90 to fold the protein, and the folded client is released along with ADP [25, 26]

3.1 Apoptosis

Apoptosis is a process in which cells commit suicide by activating an intracellular death programme [27]. Disruption of apoptosis can promote tumour initiation, progression and drug resistance. Hsp27 can inhibit apoptosis by several mechanisms and as such has attracted attention for its pro-survival role in the cell. As a chaperone, Hsp27 folds mis-folded or unfolded polypeptides, de-aggregates proteins or promotes proteolytic degradation (Fig. 2) [19].

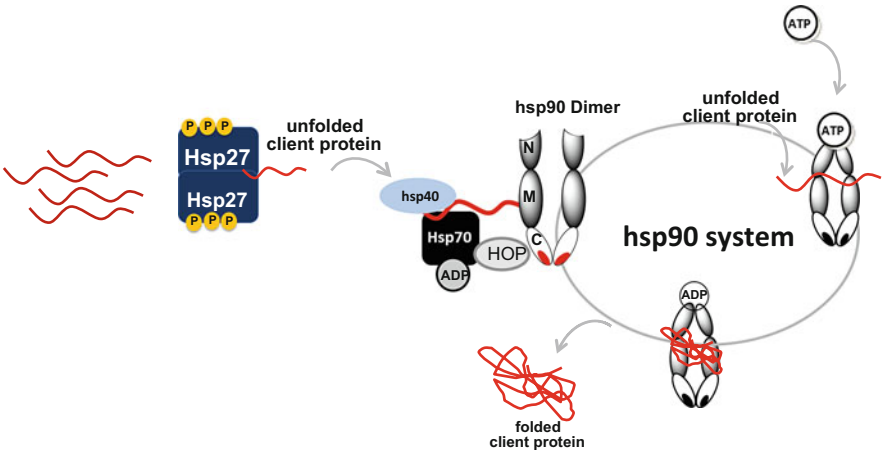


Fig. 3 Integration of Hsp27 into the Hsp70–Hsp90 folding cycle. Dimeric phosphorylated form of Hsp27 picks up the unfolded client protein and transfers it to Hsp40. Hsp40 forms a complex with Hsp70, which then binds to Hsp90 via HOP. Unfolded client protein is transferred to Hsp90 via this complex, where it is released as a folded client protein

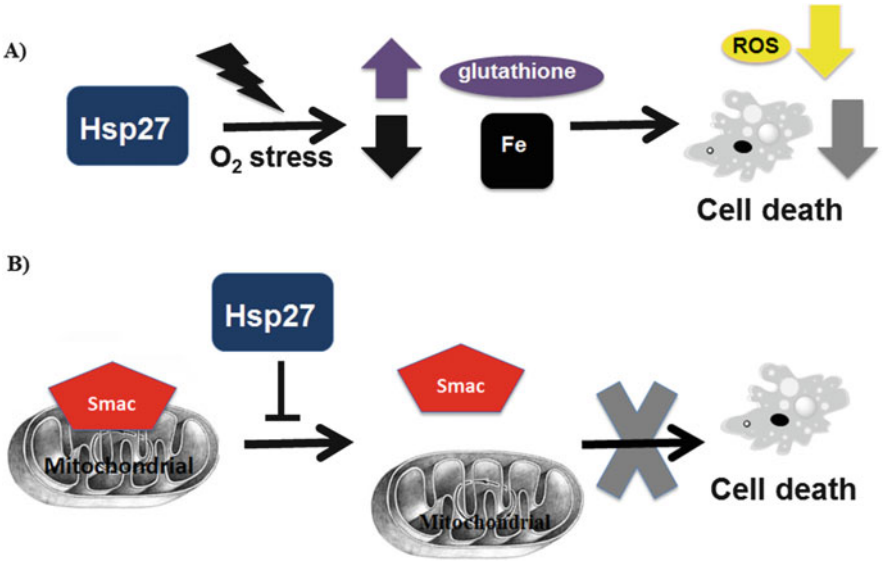


Fig. 4 The role of Hsp27 in facilitating cell survival. (**a**) Under stress conditions, Hsp27 increases glutathione and reduces iron concentrations. This leads to a reduction in reactive oxygen species and reduces cell death. (**b**) Hsp27 inhibits the release of Smac, a pro-apoptotic protein, from the mitochondria, which reduces apoptosis

One anti-apoptotic (or pro-survival) mechanism regulated by Hsp27 involves signal transduction and regulation of F-actin [28]. Hsp27's cytoprotective roles involve binding to and stabilising actin polymers. Stabilising F-actin prevents disruption of the cell's cytoskeleton. Overexpression of Hsp27 under stressed conditions causes an increase in F-actin levels, [29] where F-actin is responsible for cellular mobility and contraction during cell division [30]. Thus, stabilising actin allows Hsp27 to facilitate cell mobility and division, thereby leading to a reduction in apoptosis.

Hsp27 also facilitates cell survival via other mechanisms. During oxidative stress, Hsp27 functions as an antioxidant, lowering the levels of reactive oxygen species (ROS) by raising levels of intracellular glutathione and lowering the levels of intracellular iron (Fig. 4a) [31]. Hsp27 has also been shown to inhibit the mitochondrial release of Smac (second mitochondria-derived activator of caspases), where Smac promotes apoptosis via activation of caspases (Fig. 4b) [32]. Thus, inhibition of Smac release inhibits apoptosis. Furthermore, there is evidence that Hsp27 exerts its anti-apoptotic effect on cytochrome c release by preventing caspase activation [33].

3.2 Cancer

Given the pro-survival role played by Hsp27, the presence of Hsp27 is reported as a risk factor for the malignant progression in benign proliferating breast lesions. Indeed, its expression in lesions is now used as an indicator that the benign proliferating cells are likely to become cancerous [34]. Furthermore, when patients are treated with the drug Herceptin, which targets the Her2 receptor with the goal of inducing degradation of this protein, Hsp27 actively induces resistance by increasing the stability of Her2. Hsp27's active protection of the oncogenic protein increases the life of cancer cells [35], and by enhancing its stability and survival, Hsp27 reduces the impact of cancer therapy.

Hsp27 is highly expressed in prostate cancer and is associated with aggressive tumour behaviour, metastasis and poor prognosis [36]. Hsp27 drives a process referred to as epithelial to mesenchymal transition (EMT). EMT is a process whereby cancerous epithelial cells convert to a mesenchymal phenotype with defined morphology, protein expression and gene signatures, thus producing fully functioning tumour cells [37].

In lung cancer, Hsp27 is also associated with poor patient prognosis [38]. Lung cancer stem cells showed elevated Hsp27 protein levels when they were treated by numerous traditional chemotherapies. Treating lung cancer stem cells with a combination of traditional chemotherapy produced no results; however, combined treatment with quercetin (Fig. 4), an inhibitor to downregulate the expression of Hsp27, effectively decreased the survival of lung cancer stem cells. Evidence supporting these observations of Hsp27's impact on lung cancer was seen when patients were treated with the drug interferon gamma (IFN-γ).

IFN-γ has an anti-proliferation and immuno-modulatory effect, and is used to treat patients with several types of cancer. Investigation of its mechanism shows that it downregulates Hsp27 expression and enhances tumour cell death in vitro and tumour suppression in vivo [39].

3.3 Cardiac Diseases

A relatively high level of endogenous Hsp27 expression is observed in developing human cardiac muscle tissues, suggesting it plays a role in facilitating development and survival of cardiac muscles. Indeed, Hsp27 plays a key role in many cardiac diseases including atherosclerosis, cardiomyopathy and atrial fibrillation [40]. Hsp27 is also reported to be associated with coronary artery disease, abdominal aortic aneurysm and peripheral artery disease [41]. As discussed earlier in cancer (Fig. 4a), high levels of Hsp27 play an important role in the decreasing cardiomyopathy by increasing glutathione peroxidase activity and decreasing levels of reactive oxygen species [42].

3.4 Neuronal Diseases

The protein-folding role of Hsp27 is critical in several neuronal diseases including Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis (ALS) [43–45]. All of these neuronal diseases are a function of accumulated unfolded or aggregated protein. Hsp27's role in regulating proteins associated with these diseases is now well established, and there are recent advances that connect the chaperone-active Hsp27 to a reduction in these diseases [43–45]. Alzheimer's disease is associated with the protein Tau, which aggregates when hyper-phosphorylated to produce plaques that are associated with decreased neuronal function and Alzheimer's disease. Hsp27 binds to Tau, inhibiting its hyper-phosphorylation, which then blocks the formation of aggregated Tau or plaques. Inhibiting plaque formation leads to a decrease in Alzheimer's disease [46]. Huntington's disease is a genetic, neurodegenerative disease caused by an extended poly-glutamine region in the Huntington protein. The disease causes involuntary muscle movement, cognitive decline and psychiatric disturbances. Hsp27 plays a protective role as a chaperone in Huntington's disease by preventing neuronal dysfunction through protection of proteins involved in these pathways, which inhibits nerve damage and death [47].

4 Hsp27 Inhibitors

There are four classes of inhibitors that successfully regulate Hsp27 protein levels: Antisense oligonucleotides, small interfering RNA (siRNAs), small molecules and peptide aptamers. However, except peptide aptamers none of these inhibitors directly target the Hsp27 protein. Rather they regulate the protein levels by preventing its translation from mRNA into protein.

4.1 Antisense Oligonucleotides

Oligonucleotides are unmodified or chemically modified single-stranded DNA molecules. Antisense oligonucleotides are nucleic acid sequences that bind to mRNA in order to inhibit protein synthesis. Recently, an antisense oligonucleotide, 2′-*O*-methoxyethyl phosphorothioate (OGX-427), inhibited Hsp27 expression and enhanced drug efficacy when used in combination with traditional chemotherapies in cancer xenograft models [48].

4.2 Small Interfering RNA (siRNAs)

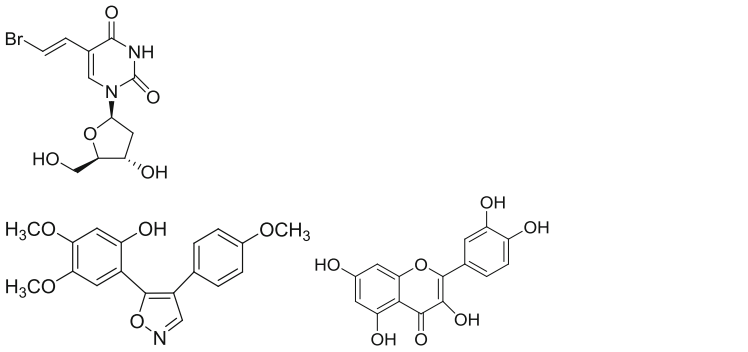
siRNAs are double-stranded RNAs that are processed via the conserved RNA interference pathway in cells and ultimately bind to complimentary mRNA molecules and result in specific degradation of that mRNA [49]. siRNA's interference in the production of Hsp27 expression has a profound impact on cancer cell growth. Treating prostate cancer cells with an Hsp27-specific siRNA sequence caused a dose-dependent and sequence-specific downregulation of Hsp27. Furthermore, this treatment significantly induced apoptosis over controls and suppressed cancer cell growth [50].

4.3 Small-Molecule Hsp27 Protein Regulators

Several small-molecule inhibitors have been developed to inhibit the expression of Hsp27. Hsp27 expression inhibitors include: (a) Bromovinyl-deoxyuridine (RP101, Fig. 5) [51], (b) 5-(5-ethyl-2-hydroxy-4-methoxyphenyl)-4-(4-methoxyphenyl) isoxazole (KRIBB, Fig. 5) [52] and (c) quercetin (Fig. 5).

RP101 binds to the mRNA of the *HSPB1* gene, which encodes for the Hsp27 protein, thereby inhibiting translation to the Hsp27 protein 48. KRIBB3 is a microtubule inhibitor and exerts its anti-proliferative activity by inhibiting tubulin polymerisation 49. KRIBB3 also reportedly binds to Hsp27 and inhibits Hsp27's role in cell migration, possibly by inhibiting the Hsp27–F-actin interaction, thereby impacting microtubule formation by destabilising F-actin.

Quercetin belongs to a class of natural products of high pharmacological potency called flavonoids [53]. Flavonoids were first discovered in 1936 by Hungarian



RP101 KRIBB3 Quercetin

Fig. 5 Small-molecule inhibitors that reduce Hsp27 protein production in the cell

scientist Albert Szent-Gyorgi [54]. They are low molecular weight natural products present in wide variety of fruits, legumes, herbs and vegetables [55]. Quercetin, the most abundant flavonoids, consists of three rings and five hydroxyl groups (Fig. 5) [56]. Quercetin has generated scientific interest because of its potential beneficial effects on human health, including antioxidant, anticancer, anti-inflammatory, antiviral and antimicrobial activities [57]. Naturally occurring derivatives of quercetin were also found to be biologically active [58, 59]. It is believed that anticancer and other biological properties of quercetin are related to its modes of action [60, 61]. Quercetin's anticancer mechanism appears to be complex, and there are several proposed mechanisms that are thought to happen simultaneously. Specifically, quercetin acts as an antioxidant scavenger [62], as an anti-proliferation molecule [63] and as an anti-angiogenesis [63], and it inhibits protein kinases [64, 65].

In 1990, quercetin was reported to reduce the expression levels of heat shock proteins Hsp90, Hsp70, Hsp47 and Hsp28 [66] and to regulate their expression in leukaemia cells [67]. Quercetin acts by inhibiting the expression of phosphorylated Hsp27, which leads to apoptosis in drug-resistant oral squamous cell carcinoma (OSCC). When used in combination with cisplatin-based chemotherapy, quercetin produces significant inhibition of tumour growth in OSCC [68]. It also produces effective results that control tumour growth by reducing Hsp27 protein levels when used in combination with traditional chemotherapy [69].

4.4 Peptide Aptamers

Synthesis and testing of small peptide sequences that contain Hsp27 residues 141–175 [70] placed as aptamers in the rigid scaffold of thioredoxin A led to the identification of several small peptide sequences that reduced Hsp27 protein levels in the transfected cells. Two sequence-specific aptamers that reduced Hsp27 protein levels contained portions of the 34 amino acid region between 141 and 175 residues of Hsp27; specifically these sequences were Q-L-S-G-W-V-G-R-C-L-N-I-N and Y-L-L-R-R-L-C-C, respectively (Fig. 6). When treating HeLa cells depleted of Hsp27 (via aptamer transfection) with cisplatin, there was a 20% increase in apoptosis over cells that were not treated with the aptamers. Treatment of aptamer-transfected cells with doxorubicin, another common chemotherapeutic drug, produced a 50% increase in cancer cell apoptosis compared to doxorubicin treatment of equivalent control cells expressing Hsp27.

Treating xenograft mouse models (head and neck squamous cell carcinoma, cell line SQ20B) with either aptamer led to a highly reduced tumour size compared to control mice, which had no aptamer treatment. Specifically, after 9 weeks of aptamer treatment, the tumour volume in the mice was 4-fold below control (200 mm^3 versus 800 mm^3) [70]. Tumour staining using Ki67 on control versus tumour tissue showed that there was a change in the proliferation process, which produced the reduced tumour size in the aptamer-treated samples. These results

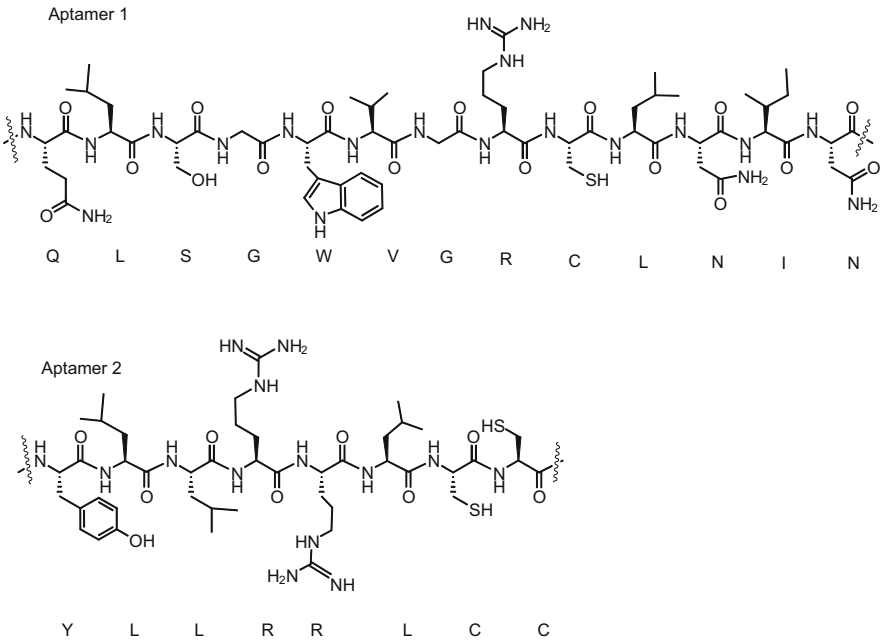


Fig. 6 The two peptide aptamers, placed within the rigid structure of thioredoxin A, which reduce Hsp27 protein production when transfected into cells

support the hypothesis that Hsp27 plays a key role in tumour growth and proliferation. Since Hsp27 is overexpressed in cancer cells compared to normal cells [71], reducing its function in cancer cells will likely be an exciting new approach to chemotherapy.

5 Conclusions and Future Directions

Herein we described the structure and function of Hsp27, its role in cancer, cardiac and neuronal diseases and molecules used to regulate the Hsp27 protein levels in the cell. Hsp27 has emerged as a vital protein where it plays a supportive role to numerous proteins critical to cell growth, prevention of apoptosis and protein homeostasis. Although the current inhibitors demonstrate efficacy for treating cancer, they only control protein levels rather than inhibit their function. Thus, there is a need to develop direct Hsp27 protein inhibitors. In addition, molecules that facilitate Hsp27 function perhaps via promoting dimer formation or increasing the levels of the protein could play a critical role in reducing Alzheimer's, Huntington's or ALS disease.

References

1. Concannon CG, Gorman AM, Samali A (2003) On the role of Hsp27 in regulating apoptosis. Apoptosis 8:61–70. doi:10.1023/A:1021601103096
2. Ciocca DR, Adams DJ, Edwards DP, Bjercke RJ, McGuire WL (1983) Distribution of an estrogen-induced protein with a molecular weight of 24,000 in normal and malignant human tissues and cells. Cancer Res 43:1204–1210
3. Hickey E, Brandon SE, Potter R, Stein G, Stein J, Weber LA (1986) Sequence and organization of genes encoding the human 27 kDa heat shock protein. Nucleic Acids Res 14:4127–4145
4. Sun Y, MacRae TH (2005) Small heat shock proteins: molecular structure and chaperone function. Cell Mol Life Sci 62:2460–2476
5. Jovcevski B, Kelly M, Rote A, Berg T, Gastall HY, Benesch J, Aquilina JA, Ecroyd H (2015) Phosphomimics destabilize Hsp27 oligomeric assemblies and enhance chaperone activity. Chem Bio 22:186–195
6. Horwitz J (1992) Alpha-crystallin can function as a molecular chaperone. Proc Natl Acad Sci USA 89:10449–10453
7. Pauli D, Tonka CH, Tissieres A, Arrigo AP (1990) Tissue-specific expression of the heat shock protein HSP27 during *Drosophila melanogaster* development. J Cell Biol 111:817–828. doi:10.1083/jcb.111.3.817
8. Hastie AT, Everts KB, Zangrilli J, Shaver JR, Pollice MB, Fish JE, Peters SP (1997) HSP27 elevated in mild allergic inflammation protects airway epithelium from H_2SO_4 effects. J Physiol Lung Cell Mol Physiol 273:L401–L409
9. Lebherz-Eichinger D, Ankersmit JH, Hacker S, Hetz H, Kimberger O, Schmidt ME, Reiter T, Horl HW, Haas M, Krenn GC, Roth AG (2012) HSP27 and HSP70 serum and urine levels in patients suffering from chronic kidney disease. Clin Chim Acta 413:282–286
10. Lo HW, Hsu SC, Xia W, Cao X, Shih JY, Wei Y, Abbruzzese JL, Hortobagyi GN, Hung MC (2007) Epidermal growth factor receptor cooperates with signal transducer and activator of transcription to induce epithelial-mesenchymal transition in cancer cells via up-regulation of TWIST gene expression. Cancer Res 67:9066–9076
11. McConnell JP, McAlpine SR (2013) Heat shock proteins 27, 40, and 70 as combinational and dual therapeutic cancer targets. Bioorg Med Chem Lett 23:1923–1928
12. Vander Heide RS (2002) Increased expression of HSP27 protects canine myocytes from simulated ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 282:H935–H941. doi:10.1152/ajpheart.00660.2001
13. Yang C, Wang H, Zhu D, Hong CS, Dmitriev P, Zhang C, Li Y, Ikejiri B, Brady RO, Zhuang Z (2015) Mutant glucocerebrosidase in Gaucher disease recruits Hsp27 to the Hsp90 chaperone complex for proteasomal degradation. Proc Natl Acad Sci U S A 112:1137–1142
14. Mendillo ML, Santagata S, Koeva M, Bell GW, Hu R, Tamimi RM, Fraenkel E, Ince TA, Whitesell L, Lindquist S (2012) HSF1 drives a transcriptional program distinct from heat shock to support highly malignant human cancers. Cell 150:549–562
15. Santagata S, Hu R, Lin NU, Mendillo ML, Collins LC, Hankinson SE, Schnitt JS, Whitesell L, Tamimi RL, Lindquist S, Ince TA (2011) High levels of nuclear heat-shock factor 1 (HSF1) are associated with poor prognosis in breast cancer. PNAS 108:18378–18383
16. Whitesell L, Lindquist S (2009) Inhibiting the transcription factor HSF1 as an anticancer strategy. Expert Opin Ther Targets 13:469–478
17. Lelj-Garolla B, Mauk AG (2012) Roles of the N- and C-terminal sequences in Hsp27 self-association and chaperone activity. Protein Sci 21:122–133
18. Lambert H, Charette JS, Bernier FZ, A G, Landry J (1999) Hsp27 multimerization mediated by phosphorylation-sensitive intermolecular interactions at the amino terminus. J Biol Chem 14:9378–9385
19. Hayes D, Napoli V, Mazurkie A, Stafford FW, Graceffa P (2009) Phosphorylation dependence of Hsp27 multimeric size and molecular chaperone function. J Biol Chem 284:18801–18807

20. McDonald ET, Bortolus M, Koteiche HA, Mchaourab HS (2012) Sequence, structure, and dynamic determinants of Hsp27 (HspB1) equilibrium dissociation are encoded by the N-terminal domain. Biochemistry 51:1257–1268
21. Rogalla T, Ehrnsperger M, Preville X, Kotlyarov A, Lutsch G, Ducasse C, Paul C, Wieske M, Arrigo AP, Buchner J, Gaestel M (1999) Regulation of Hsp27 oligomerization, chaperone function, and protective activity against oxidative stress/tumor necrosis factor α by phosphorylation. J Biol Chem 274:18947–18956
22. Shashidharamurthy R, Koteiche HA, Dong J, McHaourab HS (2005) Mechanism of chaperone function in small heat shock proteins: dissociation of the HSP27 oligomer is required for recognition and binding of destabilized T4 lysozyme. J Biol Chem 280:5281–5289
23. Baranova EV, Weeks SD, Beelen S, Bukach OV, Gusev NB, Strelkov SV (2011) Three-dimensional structure of α-crystallin domain dimers of human small heat shock proteins HSPB1 and HSPB6. J Mol Biol 411:110–122
24. Wang X, Chen M, Zhou J, Zhang X (2014) HSP27, 70 and 90, anti-apoptotic proteins, in clinical cancer therapy (review). Int J Oncol 45:18–30
25. Hessling M, Richter K, Buchner J (2009) Dissection of the ATP-induced conformational cycle of the molecular chaperone Hsp90. Nat Struct Mol Biol 16:287–293
26. Li J, Soroka J, Buchner J (2012) The Hsp90 chaperone machinery: conformational dynamics and regulation by co-chaperones. Biochim Biophys Acta 1832:624–635
27. Elmore S (2007) Apoptosis: a review of programmed cell death. Toxicol Pathol 35:495–516
28. Guay J, Lambert H, Gingras-Breton G, Lavoie JN, Huot J, Landry J (1997) Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. J Cell Sci 110:357–368
29. Lavoie JN, Hickey E, Weber LA, Landry J (1993) Modulation of actin microfilament dynamics and fluid phase pinocytosis by phosphorylation of heat shock protein 27. J Biol Chern 268:3420–3429
30. Dominguez R, Holmes KC (2011) Actin structure and function. Ann Rev Biophys 40:169–186
31. Gorman AM, Heavey B, Creagh E, Cotter TG, Samali A (1999) Antioxidant-mediated inhibition of the heat shock response leads to apoptosis. FEBS Lett 445:98–102
32. Chauhan D, Li G, Hideshima T, Podar K, Mitsiades C, Mitsiades N, Catley L, Tai YT, Hayashi T, Shringarpure R, Burger R, Munshi N, Ohtake Y, Saxena S, Anderson KC (2003) Hsp27 inhibits release of mitochondrial protein Smac in multiple myeloma cells and confers dexamethasone resistance. Blood 102:3379–3386
33. Samali A, Robertson JD, Peterson E, Manero F, Van Zeijl L, Paul C, Cotgreave IA, Arrigo AP, Orrenius S (2001) Hsp27 protects mitochondria of thermotolerant cells against apoptotic stimuli. Cell Stress Chaperones 6:49–58
34. O'Neill PA, Shaaban AM, West CR, Dodson A, Jarvis C, Moore P, Davies MP, Sibson DR, Foster CS (2004) Increased risk of malignant progression in benign proliferating breast lesions defined by expression of heat shock protein 27. Br J Cancer 90:182–188
35. Kang SH, Kang KW, Kim KH, Kwon B, Kim SK, Lee HY, Kong SY, Lee ES, Jang SG, Yoo BG (2008) Upregulated HSP27 in human breast cancer cells reduces Herceptin susceptibility by increasing Her2 protein stability. BMC Cancer 8(286)
36. Cornford PA, Dodson AR, Parsons KF, Desmond AD, Woolfenden A, Fordham M, Neoptolemos JP, Ke Y, Foster CS (2000) Heat shock protein expression independently predicts clinical outcome in prostate cancer. Cancer Res 60:7099–7105
37. Shiota M, Bishop JL, KM N, Zardan A, Takeuchi A, Cordonnier T, Beraldi E, Bazov J, Fazli L, Chi K, Gleave M, Zoubeidi A (2013) Hsp27 regulates epithelial mesenchymal transition, metastasis, and circulating tumor cells in prostate cancer. Cancer Res 73:3109–3119
38. Marinova DM, Slavova YG, Trifonova N, Kostadinov D, Maksimov V, Petrov D (2013) Stress protein Hsp27 expression predicts the outcome in operated small cell lung carcinoma and large cell neuroendocrine carcinoma patients. JBUON 18(4):915–920

39. Oba M, Yano S, Shuto T, Suico AM, Euma A, Kai H (2008) IFN-γ down-regulates Hsp27 expression and enhance hyperthermia-induced tumor cell death in vitro and tumor suppression in vivo. Int J Oncol 32:1317–1324
40. Wua J, Jianga S, Ding Z, Liu L (2013) Role of heat shock protein 27 in cardiovascular disease. J Biochem Pharmacol Res 1:43–50
41. Jin C, Phillips VL, Williams MJ, Van Rij AM, Jones GT (2014) Plasma heat shock protein 27 is associated with coronary artery disease, abdominal aortic aneurysm and peripheral artery disease. SpringerPlus 3(635)
42. Zhang X, Min X, Li C, Benjamin JI, Qian BI, Zhang X, Ding Z, Gao X, Yao Y, Ma Y, Cheng Y, Liu L (2010) Involvement of reductive stress in the cardiomyopathy in transgenic mice with cardiac-specific overexpression of heat shock protein 27. Hypertension 55:1412–1417
43. Chiti F, Dobson CM (2006) Protein misfolding, functional amyloid, and human disease. Annu Rev Biochem 75:333–366
44. Dobson CM (2001) The structural basis of protein folding and its links with human disease. Philos Trans R Soc Lond B Biol Sci 356:133–145
45. Muchowski PJ, Wacker JLW (2005) Modulation of neurodegeneration by molecular chaperones. Nat Rev Neurosci 6:11–22
46. Shimura H, Miura-Shimura Y, Kosik KS (2004) Binding of tau to heat shock protein 27 leads to decreased concentration of hyper-phosphorylated tau and enhanced cell survival. J Biol Chem 279:17957–17962
47. Perrin V, Regulier E, Abbas-Terki T, Hassig R, Brouillet E, Aebischer P, Luthi-Carter R, Deglon N (2007) Neuroprotection by Hsp104 and Hsp27 in lentiviral-based rat models of Huntington's disease. Mol Ther 15:903–911
48. Baylot V, Andrieu C, Katsogiannou M, Taieb D, Garcia S, Giusiano S, Acunzo J, Iovanna J, Gleave M, Garrido C, Rocchi P (2011) OGX-427 inhibits tumor progression and enhances gemcitabine chemotherapy in pancreatic cancer. Cell Death Dis 2(e221)
49. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391:806–811
50. Liu XX, Rocchi P, Qu FQ, Zheng SQ, Liang ZC, Gleave M, Iovanna J, Peng L (2009) PAMAM dendrimers mediate siRNA delivery to target Hsp27 and produce potent antiproliferative effects on prostate cancer cells. ChemMedChem 4:1302–1310
51. Heinrich JC, Tuukkanen A, Schroeder M, Fahrig T, Fahrig R (2011) RP101 (brivudine) binds to heat shock protein Hsp27 (HSPB1) and enhances survival in animals and pancreatic cancer patients. J Cancer Res Clin Oncol 137:1349–1361
52. Shin KD, Yoon YJ, Kang YR, Son KH, Kim HM, Kwon BM, Han DC (2008) KRIBB3, a novel microtubule inhibitor, induces mitotic arrest and apoptosis in human cancer cells. Biochem Pharmacol 75:383–394
53. Di Carlo G, Mascolo N, lzzo AA, Capasso F (1999) Flavonoids: old and new aspects of a class of natural therapeutic drugs. Life Sci 65:337–353
54. Rusznyak S, Szent-Gyorgyi A (1936) Vitamin P: flavonols as vitamins. Nature 136:27
55. Peterson J, Dwyer J (1998) Flavonoids: dietary occurrence and biochemical activity. Nutr Res 18:1995–1998
56. Rossi M, Rickles LF, Halpin WA (1986) The crystal and molecular structure of quercetin: a biologically active and naturally occurring flavonoid. Bioorg Chem 14:55–69
57. Maalik A, Khan FA, Mumtaz A, Mehmood A, Azhar S, Atif M, Karim S, Altaf Y, Tariq I (2014) Pharmacological applications of quercetin and its derivatives: a short review. Trop J Pharm Res 13:1561–1566
58. Boumendjel A, Di Pietro A, Dumontet C, Barron D (2002) Recent advances in the discovery of flavonoids and analogs with high-affinity binding to P-Glycoprotein responsible for cancer cell multidrug resistance. Med Res Rev 22:512–529
59. Materska M (2008) Quercetin and its derivatives: chemical structure and bioactivity-a review. Pol J Food Nutr Sci 58:407–413

60. Nijveldt RJ, Van Nood E, Van Hoorn DE, Boelens PG, Van Norren K, Van Leeuwen PA (2001) Flavonoids: a review of probable mechanisms of action and potential applications. Am J Clin Nutr 74:418–425
61. Pratheeshkumar P, Sreekala C, Zhang Z, Budhraja A, Ding S, Son YO, Wang X, Hitron A, Hyun-Jung K, Wang L, Lee JC, Shi X (2012) Cancer prevention with promising natural products: mechanisms of action and molecular targets. Anticancer Agents Med Chem 12:1159–1184
62. Afpinasev IB, Dokozhko AI, Brodakii AV, Kostyuk VA, Potapovitchs AI (1989) Chelating and free radical scavenging mechanisms of inhibitory action of Rutin and Quercetin in lipid peroxidation. Biochem Pharmacol 38:1763–1769
63. Fotsis T, Pepper MS, Aktas E, Breit S, Rasku S, Adlercreutz H, Wahala K, Montesano R, Schweigerer L (1997) Flavonoids, dietary-derived inhibitors of cell proliferation and in vitro angiogenesis. Cancer Res 57:2916–2921
64. Hou DX, Kumamoto T (2010) Flavonoids as protein kinase inhibitors for cancer chemoprevention: direct binding and molecular modeling. Antioxid Redox Signal 13:691–719
65. Russo M, Palumbo R, Mupo A, Tosto M, Iacomino G, Scognamiglio A, Tedesco I, Galano G, Russo GL (2003) Flavonoid quercetin sensitizes a CD95-resistant cell line to apoptosis by activating protein kinase. Oncogene 22:3330–3342
66. Hosokawa N, Hirayoshi K, Nakai A, Hosokawa Y, Marui N, Yoshida M, Sakai T, Nishino H, Aoike A, Kawai K, Nagata K (1990) Flavonoids inhibit the expression of heat shock proteins. Cell Struct Funct 15:393–401
67. Elia G, Santoro MG (1994) Regulation of heat shock protein synthesis by quercetin in human erythroleukaemia cells. J Biochem 300:201–209
68. Chen SF, Nieh S, Jao SW, Liu CL, Wu CH, Chang YC, Yang CY, Lin YS (2012) Quercetin suppresses drug-resistant spheres via the p38 MAPK–Hsp27 apoptotic pathway in oral cancer cells. PLoS One 7, e49275
69. Badziul D, Jakubowicz-Gil J, Paduch R, Głowniak K, Gawron A (2014) Combined treatment with quercetin and imperatorin as a potent strategy for killing HeLa and Hep-2 cells. Mol Cell Biochem 392:213–227
70. Gibert B, Hadchity E, Czekalla A, Aloy MT, Colas P, Rodriguez-Lafrasse C, Arrigo AP, Diaz-Latoud C (2011) Inhibition of heat shock protein 27 (HspB1) tumorigenic functions by peptide aptamers. Oncogene 30:3672–3681
71. Fanelli MA, Cuello Carrion FD, Dekker J, Schoemaker J, Ciocca DR (1998) Serological detection of heat shock protein Hsp27 in normal and breast cancer patients. Cancer Epidemiol Biomarkers Prev 7:791–795

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