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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

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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

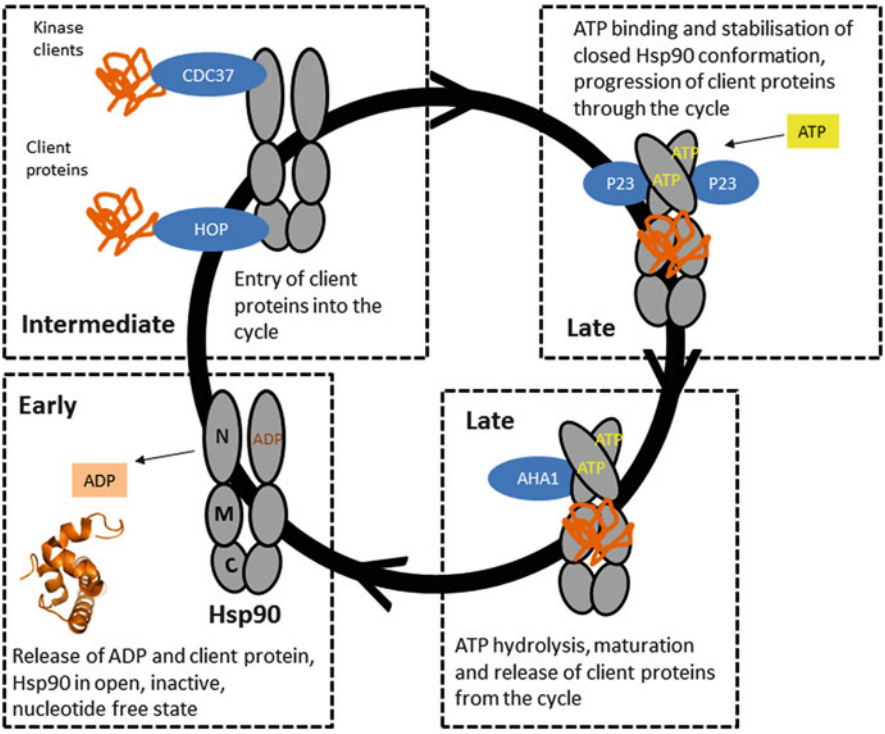

Fig. 1 Schematic diagram of the Hsp90 complex showing roles of co-chaperones Hop, Cdc37, p23 and Aha1 in terms of broad function. In the early phases of the cycle, the Hsp90 dimer is free of nucleotide, C-terminally dimerised and in the open conformation where N-terminal domains are separated. Hop and 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

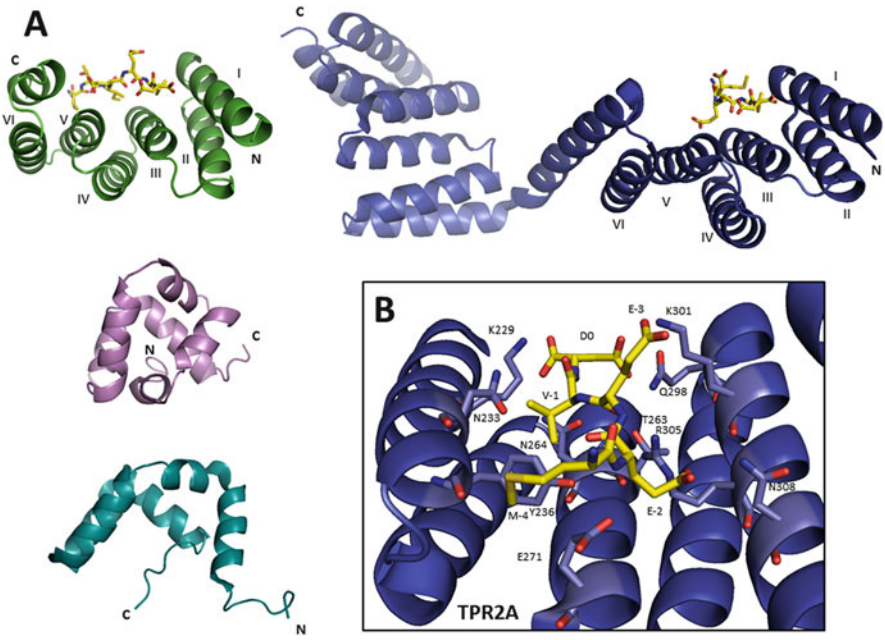

Fig. 2 Structural domains of Hop and interaction with C-terminal EEVD peptides. (**a**) Cartoon representations of TPR1 (1ELW, *green*, residues 2–116), TPR2AB (3UQ3, *dark blue*, residues 258–515), DP1 (2LLV, *pink*, residues 127–196) and DP2 (2LLW, *cyan*, residues 519–586) generated in PyMOL (DeLano 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

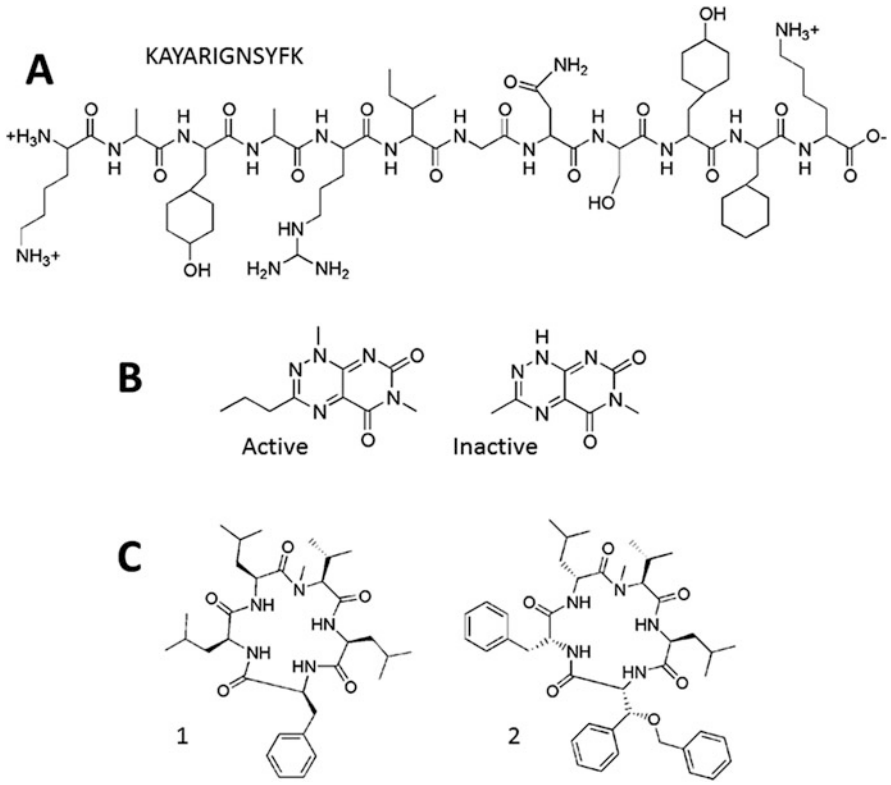

Fig. 3 Inhibitors of the interaction between Hsp90 and Hop. (**a**) Structure of the Antp-TPR peptide (KAYARIGNSYFK) that competes with Hop TPR2A for binding to Hsp90. (**b**) C9 (1,6-dimethyl-3-propylpyrimido[5,4-e][1,2,4]triazine-5,7-dione) and an inactive analogue. C9 binds to TPR2A and blocks the 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)

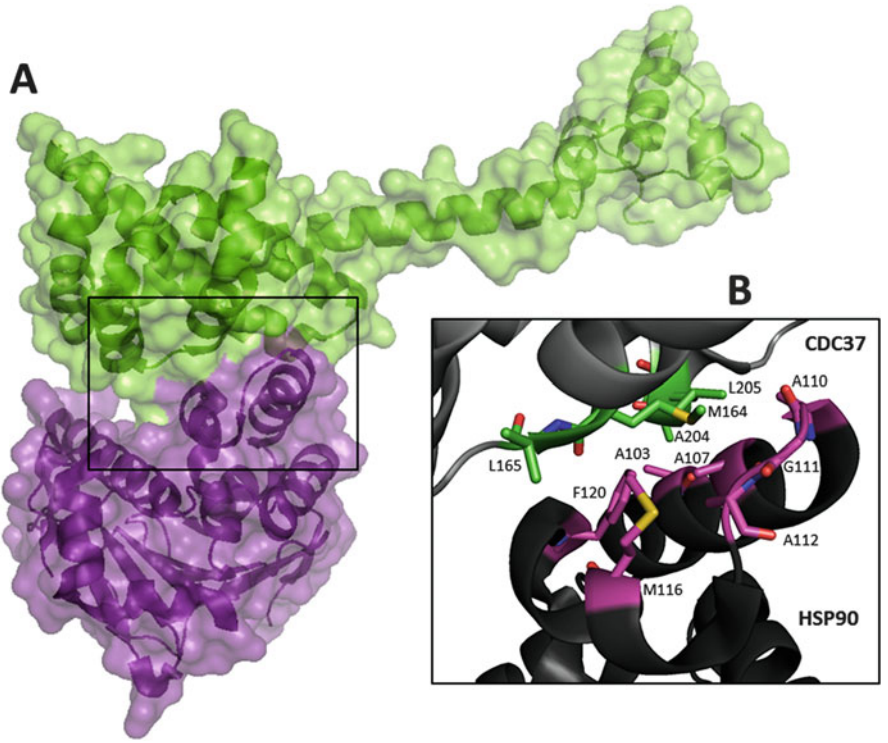

Fig. 4 Interaction of Hsp90 with the co-chaperone Cdc37. (**a**) Global view of the interaction of the N-terminal region of Hsp90 (*magenta*, residues 1–214) with the C-terminal region of Cdc37 (*green*, residues 125–378). The interaction interface is buried 1056 A of the molecular surface. (**b**) Residues 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

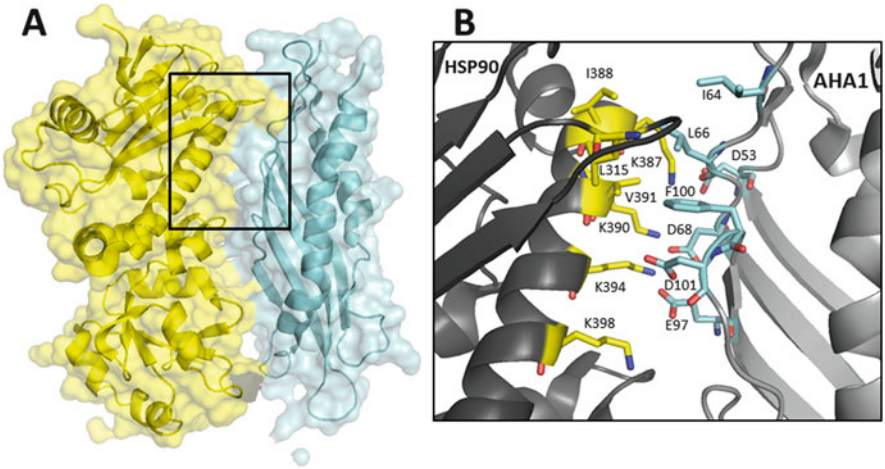

Fig. 5 Interaction of Hsp90 M domain and the N-terminal domain of the co-chaperone Aha1. (**a**) Global view of the interaction of the middle (M) domain of Hsp90 (*yellow*, residues 273–530) and the N-terminal region of Aha1 (*cyan*, residues 1–156, equivalent to Hch1) showing a large interaction interface 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].

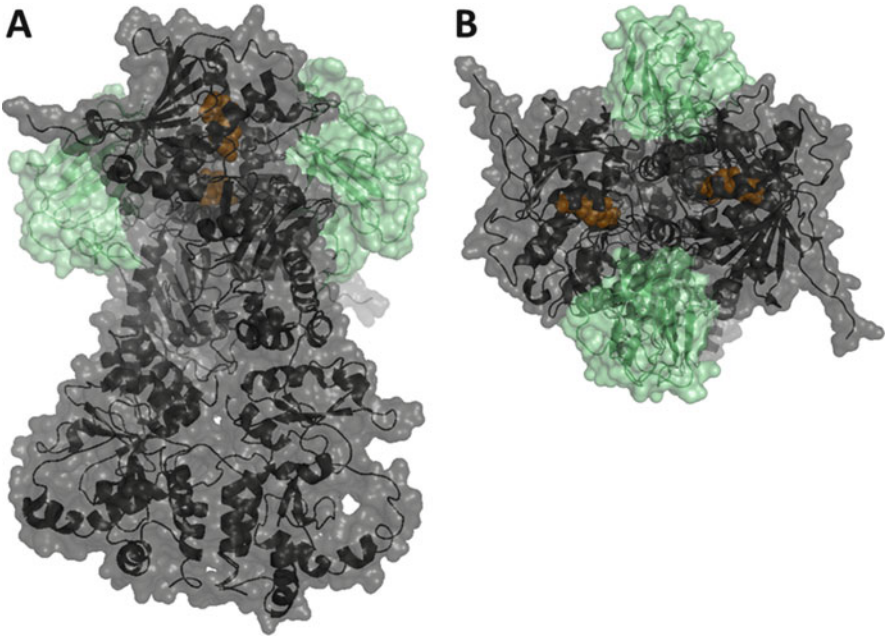

Fig. 6 Interaction of Hsp90 with the co-chaperone p23. Interaction of yeast Hsp90/Hsp82 (*grey*, residues 1–677 but with the sequence LQHMASVD replacing the charged linker at 221–255) with the yeast co-chaperone p23/Sba1 (*green*, residues 1–134). p23 interacts with the N-terminal domain of Hsp90 and 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

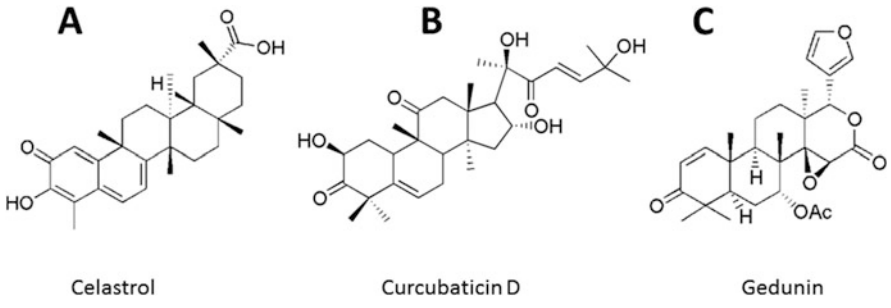

Fig. 7 Structural similarity between inhibitors celastrol, cucurbitacin D and gedunin. Celastrol and cucurbitacin D inhibit both Cdc37 and p23, while gedunin inhibits p23 and not Cdc37

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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