Top Med Chem (2016) 19: 1–20
DOI: 10.1007/7355_2015_93

Published online: 2 August 2015

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

Contents

J. McConnell, Y. Wang, and S. McAlpine (✉)
School of Chemistry, University of New South Wales, Kensington, NSW 2052, Australia
e-mail: s.mcalpine@unsw.edu.au

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

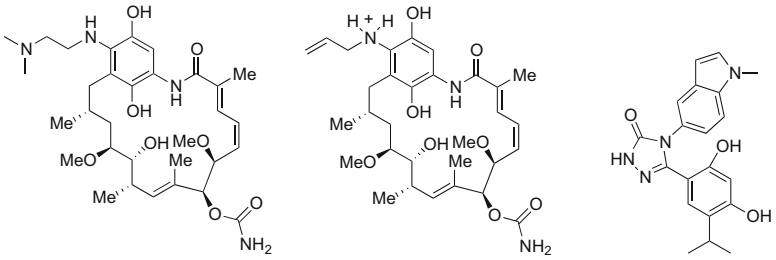

Fig. 1 Structure of Hsp90 inhibitors that have had moderate success as single-agent therapeutics in the clinic. 17-DMAG, IPI-504, and ganetespib

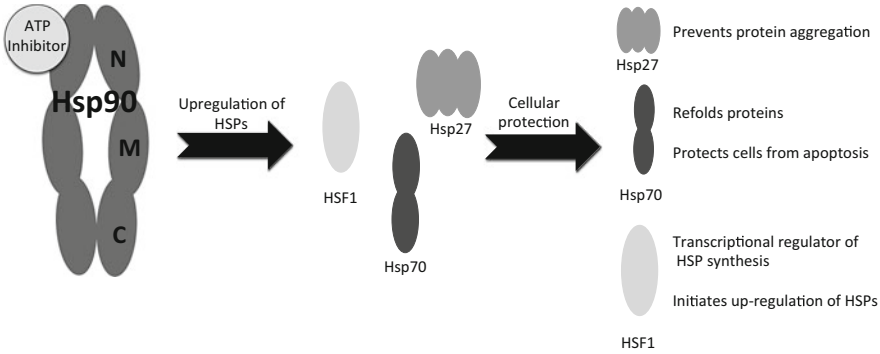

Fig. 2 Depiction of the cytoprotective consequences of ATP binding site Hsp90 inhibitors. All N-terminal ATP binding site inhibitors induce elevated levels of heat shock proteins leading to cellular protection. The key players and their role in cellular protection are depicted here

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

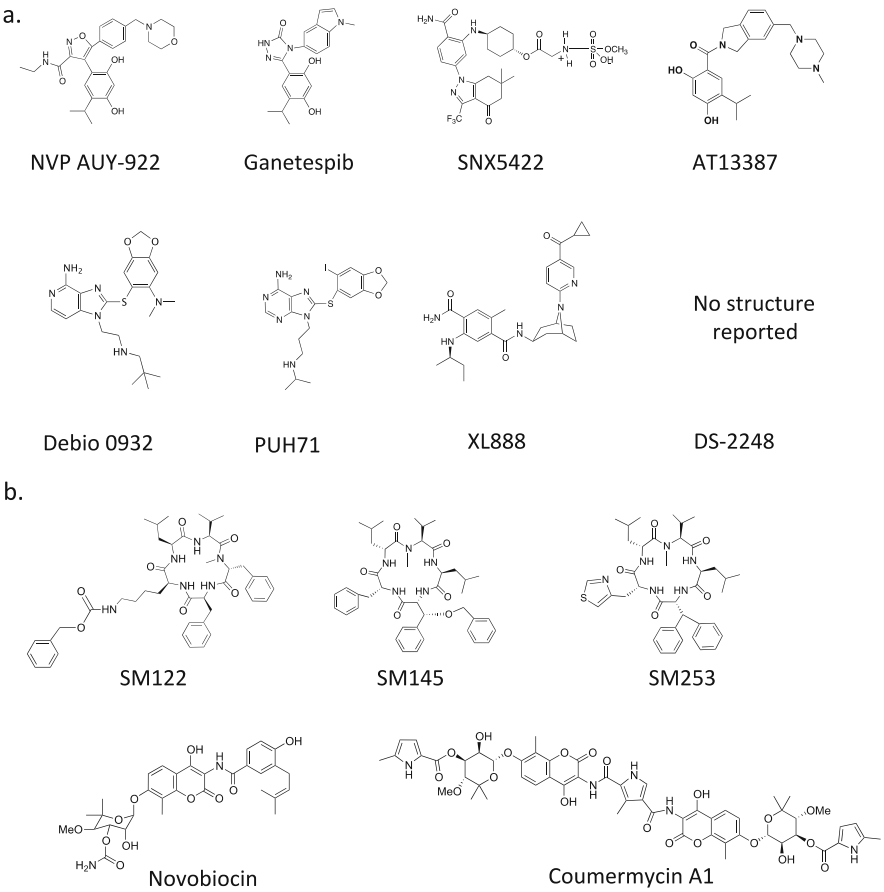

Fig. 3 (**a**) Structures of current Hsp90 inhibitors in clinical trials, all targeting the ATP binding site at the N-terminus. (**b**) Structures of molecules that modulate the C-terminus of Hsp90 inhibitors (SM122, SM145, and SM253; novobiocin and coumermycin A1)

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

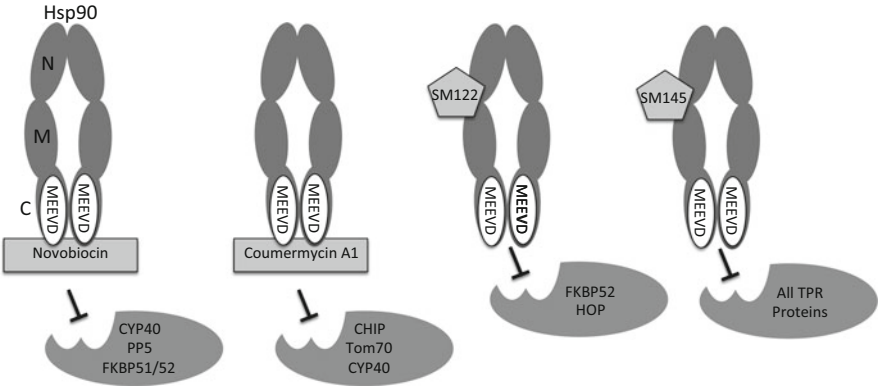

Fig. 4 Diagram depicting C-terminal-binding compounds novobiocin and coumermycin A1 as well as N-middle-binding compounds SM122 and SM145 blocking the interaction of various TPR-containing proteins with the C-terminus of Hsp90

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].

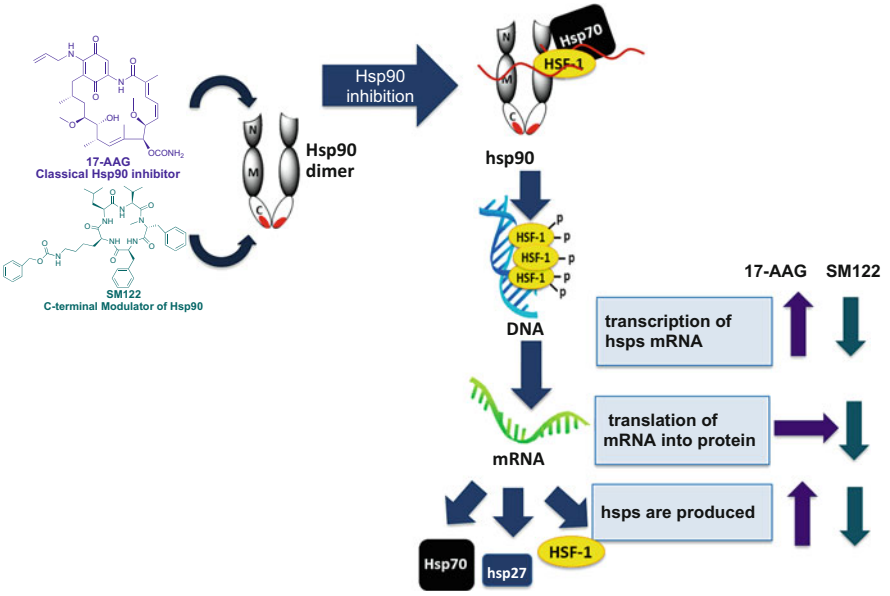

Fig. 5 N-terminal and C-terminal Hsp90 inhibition cause differing mechanistic results. Two Hsp90 inhibitors, 17-AAG and SM122, were evaluated at several points during the heat shock response pathway. The mRNA levels of Hsp70 and Hsp27 increased when cells were treated with 17-AAG, whereas mRNA production 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

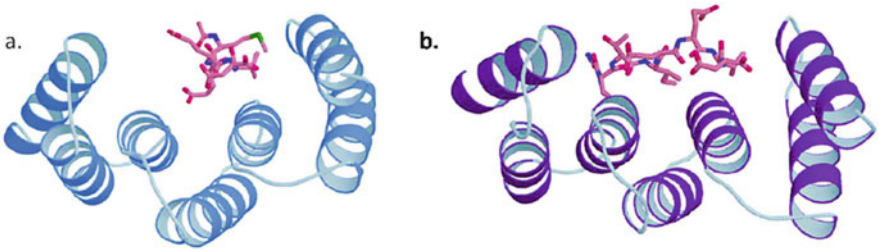

Fig. 6 Two TPR domains of HOP in complex with peptides. (**a**) TRP2A domain of HOP bound to the MEEVD sequence of Hsp90. (**b**) TRP1 domain of HOP bound to the last 12 amino acids of Hsp70. Figure taken from [71]

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

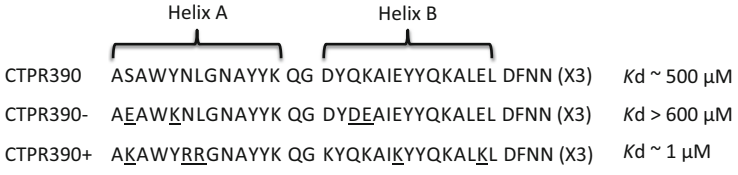

Fig. 7 Sequence of CTPR390 and the charged derivatives (CTPR390+/-) along with their *K*d values for binding to Hsp90

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.

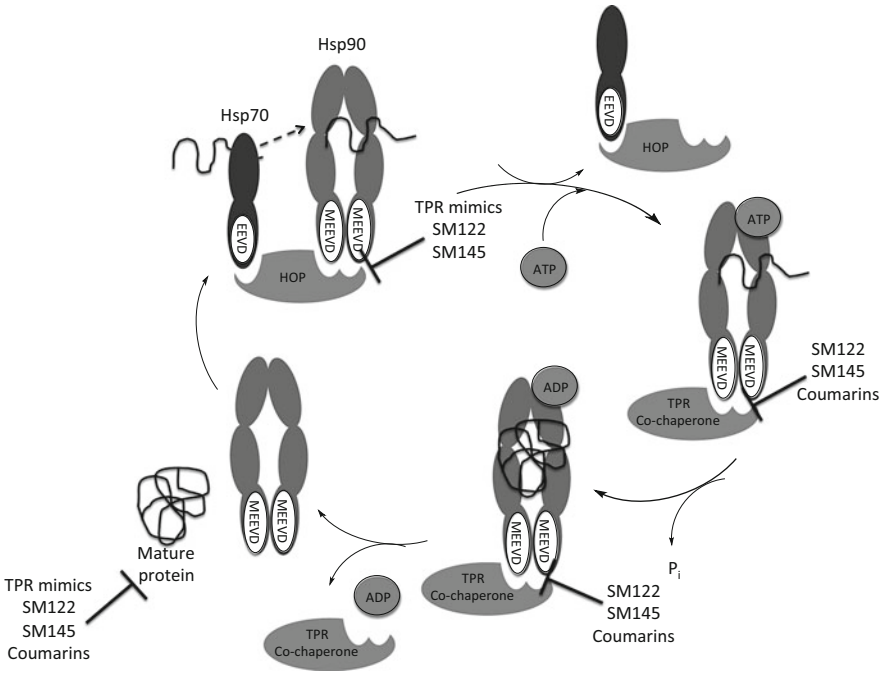

Fig. 8 Depiction of Hsp90-mediated protein folding and where the C-terminal-modulating molecules disrupt this pathway. In the normal protein-folding pathway, an unfolded protein is transferred to Hsp90 by Hsp70 via HOP. However, the TPR mimics and SM series inhibitors disrupt the interaction of Hsp90 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.

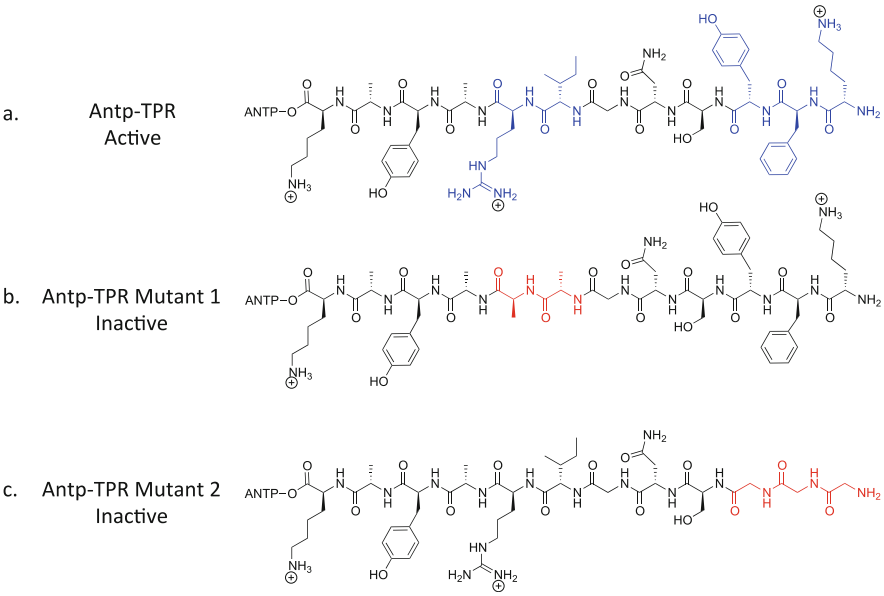

Fig. 9 (**a**) Sequence of the Antp-TPR peptide with residues essential for activity in *blue*. (**b**, **c**) Sequence of mutated versions of Antp-TPR used to determine important amino acid residues (mutated amino acids in *red*)

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.

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