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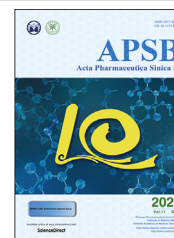

REVIEW

The disruption of protein–protein interactions with co-chaperones and client substrates as a strategy towards Hsp90 inhibition

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Abstract The 90-kiloDalton (kD) heat shock protein (Hsp90) is a ubiquitous, ATP-dependent molecular chaperone whose primary function is to ensure the proper folding of several hundred client protein substrates. Because many of these clients are overexpressed or become mutated during cancer progression, Hsp90 inhibition has been pursued as a potential strategy for cancer as one can target multiple oncoproteins and signaling pathways simultaneously. The first discovered Hsp90 inhibitors, geldanamycin and radicicol, function by competitively binding to Hsp90's N-terminal binding site and inhibiting its ATPase activity. However, most of these N-terminal inhibitors exhibited detrimental activities during clinical evaluation due to induction of the pro-survival heat shock response as well as poor selectivity amongst the four isoforms. Consequently, alternative approaches to Hsp90 inhibition have been pursued and include C-terminal inhibition, isoform-selective inhibition, and the disruption of Hsp90 protein–protein interactions. Since the Hsp90 protein folding cycle requires the assembly of Hsp90 into a large heteroprotein complex, along with various co-chaperones and immunophilins, the development of small molecules that prevent assembly of the complex offers an alternative method of Hsp90 inhibition.

Abbreviations: ADP, adenosine diphosphate; Aha1, activator of Hsp90 ATPase homologue 1; ATP, adenosine triphosphate; Cdc37, cell division cycle 37; CTD, C-terminal domain; Grp94, 94-kD glucose-regulated protein; Her-2, human epidermal growth factor receptor-2; hERG, human ether-à-go-go-related gene; HIF-1α, hypoxia-inducing factor-1α; HIP, Hsp70-interaction protein; HOP, Hsp70–Hsp90 organizing protein; HSQC, heteronuclear single quantum coherence; Hsp90, 90-kD heat shock protein; MD, middle domain; NTD, N-terminal domain; PPI, protein–protein interaction; SAHA, suberoylanilide hydroxamic acid; SAR, structure–activity relationship; SUMO, small ubiquitin-like modifier; TRAP1, Hsp75/tumor necrosis factor receptor associated protein 1; TROSY, transverse relaxation-optimized spectroscopy; TPR2A, tetratricopeptide-containing repeat 2A.

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1. Protein folding: An introduction

Proteins are a class of biomolecules that perform a variety of biological functions and include enzyme catalysis, the regulation of genes, cellular transport, and facilitating the cellular response to environmental signals/stresses.

The proper conformation of a protein is crucial to its function, and the question of how a protein transforms from a simple peptide sequence into a complex three-dimensional structure has been extensively studied for over half a century[1]. It was initially thought that protein folding is a thermodynamic process in which the protein's amino acid composition aids enthalpic interactions[2]. However, some proteins require the assistance of molecular chaperones to transform linear polypeptides into biologically active and three-dimensional proteins to carry out their biological function. Hartl[3] has defined molecular chaperones as "proteins that bind to and stabilize an otherwise unstable conformer of another protein—and, by controlled binding and release, facilitate its correct fate *in vivo*: be it folding, oligomeric assembly, transport to a particular subcellular compartment, or disposal by degradation." The existence of molecular chaperones was first suggested by Fohlman and colleagues[4] during their study with taipoxen, a sialo-glycoprotein neurotoxin that can be isolated from the venom of the Australian taipan (*Oxyuranus s. scutellatus*). Although their analysis found that taipoxen is comprised of three subunits (referred to as α, β, and γ), only the α subunit is toxic. Consequently, it was proposed that the role played by the other subunits is to increase α's stability and molecular recognition. Another early discovery included nucleoplasmins, which are chaperones that stabilize histones and ensure proper interactions with DNA during the assembly of chromatin[5]. Later studies confirmed that molecular chaperones are conserved across all kingdoms of life and exist as many different families, which are often related by molecular weight.

2. The 90-kiloDalton (kD) heat shock protein (Hsp90)

Cellular stress can disrupt the proteostatic equilibrium and result in lethal outcomes. Because of the dynamic nature between interior and exterior cellular environments, cells have evolved to express chaperones that promote survival in response to various cellular insults such as acidosis, oxidative stress, and hypoxia. One important family of molecular chaperones are the heat shock proteins, which were first observed as a cellular response to high temperature. The heat shock response was first observed when *Drosophila* salivary glands were exposed to elevated temperatures, dinitrophenol or salicylate, which resulted in chromosomal puffing[6–8]. Tissières and colleagues[9] correlated these puffs with the production of small proteins that would later come to be known as the heat shock proteins. The protein families are further subdivided by their molecular weight and include Hsp27, Hsp40, Hsp60, Hsp70, Hsp90, as well as other larger members.

The master regulator of the heat shock response is Hsp90, which is an ATP-dependent molecular chaperone that functions to fold nascent polypeptides into their active structures, as well as to facilitate the rematuration of protein aggregates/misfolded proteins and the processing of proteins *via* the ubiquitin–proteasome pathway. Hsp90 is among the most abundant proteins in the cell and comprises 1%–2% of a cell's total protein content, which is increased to 4%–6% under stressful conditions[10]. In humans, Hsp90 exists as four isoforms: Hsp90α, Hsp90β, the 94-kD glucose-regulated protein (Grp94), and the Hsp75/tumor necrosis factor receptor associated protein 1 (TRAP-1). Although Hsp90α and Hsp90β are differentially expressed, with Hsp90α being inducible and Hsp90β being constitutively expressed, they are both cytosolic; meanwhile, Grp94 and TRAP-1 are localized to the endoplasmic reticulum and mitochondria, respectively[11].

Hsp90 is a homodimer, and each monomer consists of four components; an N-terminal domain (NTD), a highly-charged linker region, a middle domain (MD), and a C-terminal domain (CTD)[10]. The N-terminal domain exhibits ATPase activity and is responsible for the generation of energy required for the proper folding of client protein substrates[12]. Unlike other ATP-dependent proteins like kinases, Hsp90 is a member of the gyrase, Hsp90, histidine kinase, MutL (GHKL) superfamily whose members contain an unusual Bergerat fold, a structural feature that forces ATP to bind in a "C-shaped" or bent conformation[13]. The middle domain contributes to ATPase activity by binding the γ-phosphate of ATP bound to the NTD[14]. In addition, the NTD facilitates the recognition and binding of client proteins and co-chaperones during the Hsp90-mediated protein folding cycle[14]. The CTD is responsible for homodimerization to yield the active form of Hsp90[15]. While the CTD also contains a nucleotide binding site, it does not hydrolyze ATP, but instead allosterically regulates the release of ADP from the NTD[16]. Thirdly, this domain contains a terminal MEEVD sequence that enables the docking of co-chaperones that contain a TPR (tetratricopeptide-containing repeats) domain[17].

Although the Hsp90 folding cycle (Fig. 1) has been extensively studied, it is not yet completely understood. However, it requires the assembly of a heteroprotein complex that utilizes both co-chaperones and ancillary proteins to promote the protein folding process. New polypeptides that are produced by the ribosome can form a complex with Hsp40 and Hsp70 to prevent aggregation, as well as the Hsp70-interacting protein (HIP)[18]. The Hsp70–Hsp90 organizing protein (HOP) associates with the complex[19] to aid the transfer of client proteins from Hsp70 to Hsp90[20]. Hsp40, Hsp70, HIP and HOP dissociate under some conditions and are replaced by various immunophilins, co-chaperones, and other partner proteins to form the Hsp90 heteroprotein complex[21]. When ATP binds to the NTD binding site, Hsp90 shifts to a closed conformation[22] and recruits p23 and the activator of Hsp90 ATPase homologue 1 (Aha1). Aha1 promotes the hydrolysis of ATP to provide the requisite energy necessary for the folding of the client protein, followed by a return to Hsp90's open conformation and regeneration of the homodimer[23].

3. Hsp90 as a target to treat cancer

In recent decades, Hsp90 gained widespread attention due to its potential as a target for the treatment of cancer. In 2000, Hanahan and Weinberg[24] proposed a list of six characteristics exhibited by

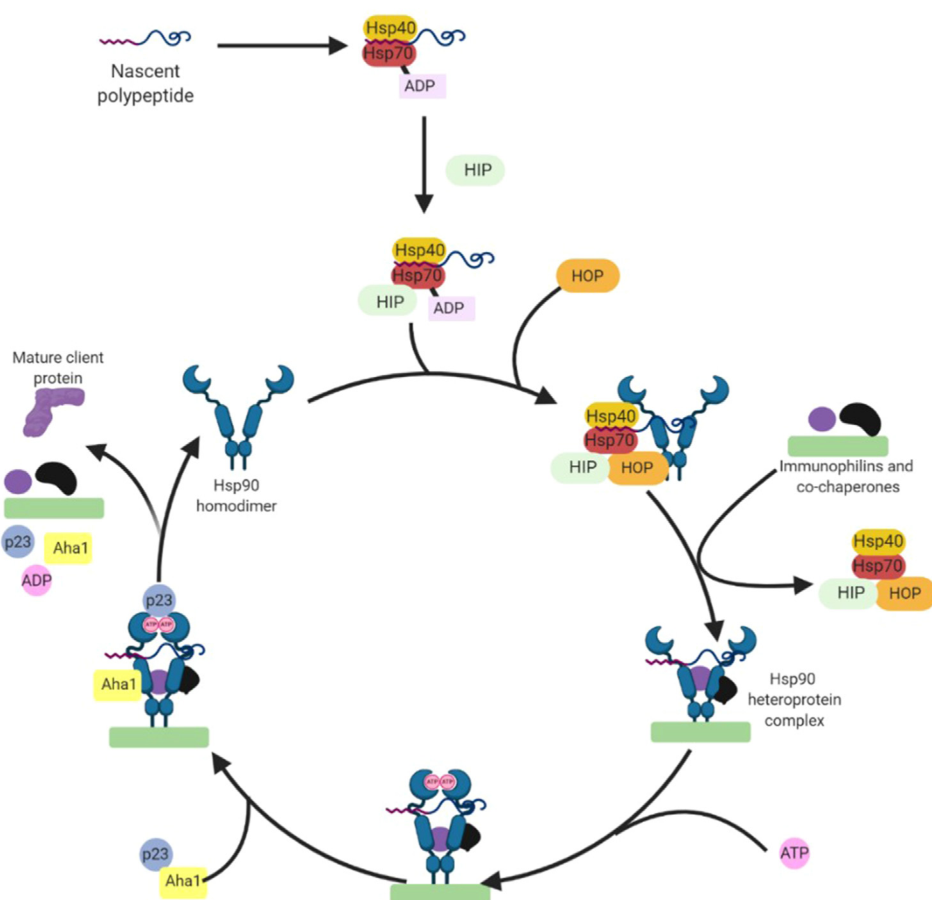

Figure 1 The Hsp90 protein folding cycle.

all cancers that include their ability to 1) produce their own growth signals, 2) exhibit insensitivity to anti-growth signals, 3) evade apoptosis, 4) increase angiogenesis, 5) induce telomerase levels, and 6) increase tissue invasion/metastasis. In 2011, they extended this list to include 7) the dysregulation of cellular energetics, 8) evasion of destruction by the immune system, 9) genome instability and mutations, and 10) tumor-promoting inflammation[25]. Since Hsp90 is responsible for the conformational maturation of ∼400 client protein substrates[26], many of these are transcription factors, receptors, kinases, or oncoproteins that are overexpressed and/or mutated in cancer, which creates an oncogenic addiction to Hsp90 (Table 1)[27–29]. Consequently, Hsp90 inhibition represents an attractive strategy to treat cancer as multiple oncoproteins and pathways are simultaneously impacted.

One of the challenges associated with the creation of an effective chemotherapy is the ability to selectively kill cancer cells while minimizing harm to normal tissue. Most cancer drugs achieve some selectivity by exploiting the high rate of malignant cell growth as compared to normal cells. Unfortunately, this also means that fast-replicating healthy cells are often affected, which can lead to undesired side effects. The high abundance of Hsp90 in all cell types raises an additional similar concern with selectivity; however, experimental evidence has demonstrated that Hsp90 inhibitors accumulate more abundantly in cancerous cells as compared to healthy tissue[30]. An explanation for this observation was reported by Kamal and colleagues[31], in which they suggest the Hsp90 heteroprotein complex to be exclusively found in tumor cells, while existing as an unassembled homodimer in

Table 1 A listing of Hsp90 clients implicated in Hanahan and Weinberg's hallmarks of cancer.

Hallmarks of cancer	Implicated Hsp90 client proteins	Ref.
Self-production of growth signals	Raf-1, Akt, Her-2, Mek, Bcr-Abl, Xpo1	27,28
Insensitivity to anti-growth signals	Plk, Wee 1, Myt1, Cdk4, Cdk6	27
Evasion of apoptosis	Akt, p53, c-Met, Apaf-1, survivin, WT1	27–29
Angiogenesis	Fak, Akt, HIF-1α, VEGFR, Flt-3, Tp73, Tbk1	27–29
Replicative senescence	Telomerase, FoxM1, Ntrk1, Ntrk2, Ntrk3	27–29
Tissue invasion/metastasis	c-Met, HIF-1α, Prmt5, Ikbka, Nuak2, MMP2	27–29
Dysregulation of cellular energetics	Arnt, Arrb1, Arrb2, Hmga1	27,28
Evasion of the immune response	Irak3	27,28
Genome instability and mutations	Mafg, Nek8, Nek9, Nek11	27,28
Tumor-promoting inflammation	IkbkA, IkbkB, IkbkG, IL-6, IL-8	27,28

normal tissue. Furthermore, the heteroprotein complex that exists in cancer cells is more active and exhibits a higher level of ATPase activity, which ultimately leads to a ∼200-fold higher affinity for inhibitors and/or ATP. Together, these cancer-derived data provide a foundation to develop Hsp90 inhibitors that can selectively target the molecular chaperone within this large therapeutic window.

One of the first strategies to target Hsp90 was the construction of molecules that compete with ATP for binding to the NTD. The first two Hsp90 inhibitors discovered were the natural products, geldanamycin and radicicol, which served as the basis for a number of compounds that underwent clinical evaluation. Unfortunately, none have been FDA-approved and most have failed[32,33]. The most likely explanation is that these compounds inhibit all four Hsp90 isoforms similarly, which is likely to manifest negative side effects that include cardiac, gastrointestinal, and ocular toxicities[34]. Specifically, formation of a functional hERG channel is heavily dependent on Hsp90α[35], which highlights why compounds that target Hsp90α may cause cardiotoxicity. Furthermore, inhibition of Hsp90α may also induce ocular toxicity as it was recently reported that Hsp90α-deficient mice experienced retinal degradation, which led to blindness[36]. All four isoforms share at least 85% sequence identity in their NTD ATP-binding sites, with Hsp90α and Hsp90β exhibiting ∼95% sequence identity and differing by only two amino acids within the nucleotide-binding site[37]. However, Khandelwal and coworkers[37] proved that even a difference of only two amino acids can be exploited to develop isoform-selective inhibitors.

A major disadvantage associated with the use of N-terminal inhibitors is induction of the pro-survival heat shock response[29]. When an inhibitor binds the NTD binding site, heat shock transcription factor 1 dissociates from Hsp90, trimerizes, undergoes phosphorylation, and enters the nucleus to promote expression of the heat shock proteins to facilitate cell survival[38]. Although complications related to dosing and toxicity have hindered the advancement of Hsp90 N-terminal inhibitors in the clinic, the same heat shock response that is detrimental to cancer, may prove advantageous for the treatment of neurodegenerative diseases such as Alzheimer's, Parkinson's and multiple sclerosis[39]. Neckers and colleagues[40] discovered an alternative to N-terminal inhibition in 2000 *via* novobiocin, a coumarin antibiotic that induced the degradation of the oncogenic client proteins v-Src, Raf-1, and Erb 2 *via* inhibition of the CTD binding site. Because such binding does not induce the heat shock response[41], the development of C-terminal inhibitors has become an alternative strategy for Hsp90 inhibition. Unfortunately, their Hsp90 C-terminal binding site remains poorly characterized and unconfirmed[42].

4. Disruption of Hsp90 protein−protein interactions (PPIs)

While isoform-selective and C-terminal inhibition are promising strategies to target Hsp90, a third option is to prevent assembly of the functional heteroprotein complex. The Hsp90 protein folding cycle involves numerous proteins that associate with Hsp90 at different stages and appear to be dependent upon the substrate. In addition, Hsp90 is subject to post-translational modifications like phosphorylation, sumoylation and *S*-nitrosylation, which further attenuate its activity or affinity for clients/partner proteins[43]. Therefore, the design of inhibitors that disrupt Hsp90 PPIs represents a novel approach to treat cancer by "fine-tuning" Hsp90 inhibition instead of eliminating it. The following section highlights PPIs of interest, the molecules that have been discovered or developed to interfere with those interactions, and the results from such studies.

4.1. *Hsp90 and activator of Hsp90 ATPase homologue 1 (Aha1)*

Aha1 (Fig. 2) is a major co-chaperone whose interactions with Hsp90 are important for client protein maturation, as it enhances Hsp90's inherently low ATPase activity. Although the precise mechanism through which this stimulation occurs remains unknown, Oroz and colleagues[44] used NMR studies to demonstrate that the increased activity results from the binding of Aha1's N-terminus with Hsp90's MD, while Hsp90's N-terminal domains remain flexible to allow their dimerization and the binding of ATP. More recently, cryo-electron microscopy studies performed by Liu and colleagues[45] yielded multiple Hsp90–Aha1 complexes and provided mechanistic insights into Hsp90's stimulation by Aha1. From these models, they proposed that the Aha1 NTD is recruited to the Hsp90 MD to induce a semi-closed state of the molecular chaperone. Steric clashes with the Aha1 CTD causes the Hsp90 NTD to undock from the MD, which leads to ATP binding, as well as rotation and dimerization of the two NTDs. Aha1 then rearranges to promote the asymmetric hydrolysis of both ATP molecules[45].

4.1.1. *A12 and A16*

In 2017, Ihrig and Obermann[48] reported the disclosure of small molecules that disrupt interactions between Aha1 and Hsp90. Using an Alpha screening assay, they identified A12 and A16 (**1** and **2**, Fig. 3) from an initial pool of 16 compounds. As demonstrated by an iodide efflux assay, both compounds manifested IC_{50} values of 0.3 μmol/L at stabilizing CFTRΔF508, a mutated chloride channel whose degradation is implicated in cystic fibrosis. When used in combination with VX-809, a drug that promotes CFTRΔF508 trafficking to the cell surface[47], A12 and A16 exhibited synergy, suggesting that disruption of the Aha1–Hsp90 interaction could represent a viable treatment for cystic fibrosis[48].

4.1.2. *Hsp90–Aha1 modulators*

During the same year, Stiegler and colleagues[49] used a FRET-based assay to identify molecules that modulate Hsp90–Aha1 interactions. Their scientific investigation led to the identification of 6 Hsp90–Aha1 modulators (HAMs, **3**−**8**, Fig. 3), three of which inhibited Aha1's stimulation of Hsp90 ATPase activity while the others enhanced it. The best inhibitor, HAM-1, resulted in 93 ± 1% inhibition at saturating concentrations with an apparent K_D of 24 μmol/L, while NMR studies demonstrated it to bind the Hsp90 NTD and impair interactions with Aha1's C-terminus[49].

4.1.3. *TL-2-8*

Another molecule that disrupts Hsp90–Aha1 interactions was discovered by Liu and coworkers[50], who demonstrated that TL-2-8 (**9**, Fig. 3), a derivative of quercetin, reduced the expression of PLK1, HSF1, Cdk 1, and cyclin D1 in MDA-MB-231 and MDA-MB-468 cancer cells in a concentration-dependent manner. The overexpression of Aha1 was found to rescue all four client proteins, which verifies the role played by Aha1 to accelerate protein folding.

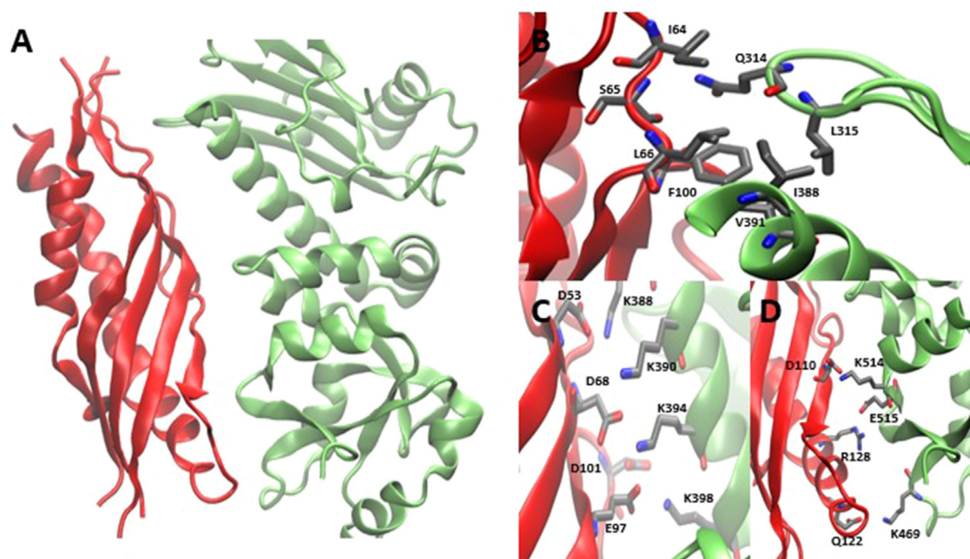

Figure 2 PPIs between Hsp90 middle domain and Aha1 (A) Co-crystal structure of Hsp90 (green) and Aha1 (red). (B) Hydrophobic interface between Hsp90 and Aha1, mediated by hydrogen bonding between Q314 of Hsp90 and the backbone of I64 and S65 on Aha1. (C) Hydrogen bonding and ionic interactions between Hsp90 lysines and Aha1 aspartic/glutamic acids. (D) Ion-pair interactions between K469, K514, and E515 on Hsp90 and D110, Q122, and R128 on Aha1 (PDB: 1USU[46]).

4.1.4. SEW84

SEW84 (**10**, Fig. 3) was recently identified by Singh and colleagues[51] as an inhibitor of Aha1-stimulated Hsp90 ATPase activity during a quinaldine red ATPase assay (IC_{50} = 0.3 μmol/L). $^{1}H-^{15}N$-TROSY-HSQC spectroscopy revealed that SEW84 binds the Aha1 CTD with a K_D of 1.74 μmol/L, thereby disrupting PPIs with Hsp90. SEW84's reported *in vitro* activities include inhibition of both wild-type and mutated variants of the androgen receptor, which is implicated in prostate cancer, as well as the clearance of phosphorylated-tau in HEK293 cells. The latter was also observed in rat cortical neurons and a transgenic mouse model, wherein similar results prevailed. Structure−activity relationship (SAR) analyses performed on SEW84 highlighted the importance of the trifluoromethyl and hydrazinecarbanothiamide moieties for activity, while *meta*- and *para*-substituents on the phenyl ring modulate activity[51].

4.2. Hsp90 and cell division cycle 37 (Cdc37)

Cdc37 (Fig. 4), also known as p50, is a cell cycle protein that is also a major Hsp90 co-chaperone due to its participation in the folding of approximately 300 client proteins, many of which are kinases[26]. There are many structural features of Cdc37 that help to stabilize these substrates and facilitate their transfer to Hsp90, and the proposed mechanism is as follows. First, Cdc37 is phosphorylated by casein kinase 2 at Ser-13, which enables it to recognize and associate with client kinases[52]. The resulting complex then binds Hsp90, after which protein phosphatase 5 dephosphorylates Cdc37, which stabilizes the client protein and enables transfer to Hsp90[53,54].

4.2.1. Celastrol A

Celastrol A (**11**, Fig. 5) is a pentacyclic quinone methide triterpene natural product isolated from *Tripterygium wilfordii* Hook F. It has been used in traditional Eastern medicine for inflammation and autoimmune disorders and has been investigated as a potential treatment for various inflammatory diseases and cancers. In 2008, Zhang and coworkers[56] performed *in silico* molecular dynamics simulations and discovered a potential binding site on Hsp90 that blocked several key interactions with Cdc37. *In vitro* and *in vivo* studies demonstrated that the administration of celastrol to cells decreased levels of Akt and Cdk4 by 80% and 70%, respectively. In addition, celastrol exhibited antiproliferative activity (IC_{50} = 3 μmol/L) and induced apoptosis. It also displayed *in vivo* efficacy in a transgenic mice model of pancreatic cancer.

In a follow-up study, the same group investigated the mechanism by which celastrol disrupts Hsp90–Cdc37 interactions, and because it was found to protect Hsp90 from trypsin degradation, they determined that the molecule binds to Hsp90's CTD[57]. However, HSQC NMR studies performed by Sreeramulu and coworkers[58] suggested the quinone portion of celastrol to act as a Michael acceptor for cysteine residues present in Cdc37, while its three saturated rings participate in hydrophobic interactions. Jiang and colleagues[59] synthesized 23 ester and amide derivatives, and the most active derivative, CEL20 (**12**, Fig. 5) manifested an IC_{50} value of 4.71 ± 0.14 μmol/L against Panc-1 cells. Additional studies on this natural product involved the preparation of chimeras between celastrol and cinnamic acid[60]/ferulic acid[61]. The most potent derivatives obtained from these studies were **13** and **14** (Fig. 5), which were found to disrupt Hsp90–Cdc37 PPIs more effectively than their parent compound. Hsp90 client substrates Akt and Cdk4 were degraded and a G_0/G_1 cell cycle arrest was observed along with the induction of apoptosis in A549 cancer cells. Celastrol's precise mechanism of action remains unclear, but such work demonstrates that the natural product and its derivatives exhibit utility as both a starting point for drug discovery and as a biological probe to further interrogate the nature of Hsp90–Cdc37 PPIs.

4.2.2. DCZ3112

In 2016, Zhao and colleagues[62] synthesized a triazine derivative known as X66 (**15**, Fig. 5) and reported its antitumor activity and

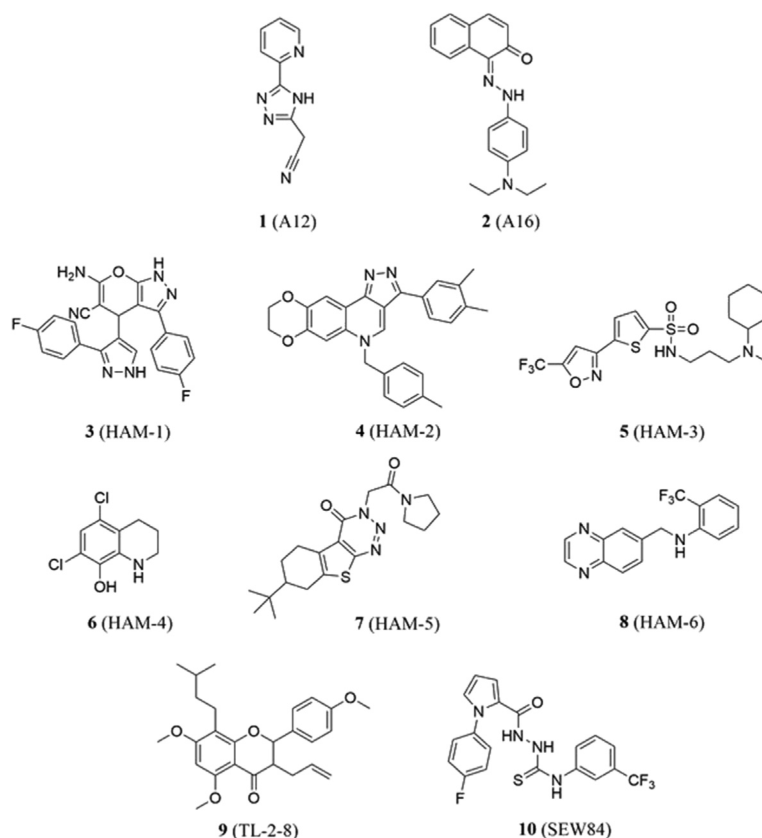

Figure 3 Disruptors of Hsp90–Aha1 PPIs.

mechanism of action *via* binding to the Hsp90 NTD (IC_{50} values against SkBr3, BT-474, A549, K562, and HCT-116 cell lines were 8.9, 7.1, 7.5, 8.6 and 6.7 μmol/L, respectively). In a subsequent study, the same group found the structurally related analog, DCZ3112 (**16**, Fig. 5), disrupts Hsp90–Cdc37 interactions. DCZ3112 also induced cell cycle arrest and apoptosis in Her-2 positive breast cancer cells, which was observed when DCZ3112 was used individually or in combination with the anti-Her-2 antibodies, trastuzumab or pertuzumab. Furthermore, DCZ3112 was shown to overcome resistance to either antibody. Molecular docking studies suggest the compound operates *via* competitive binding to the Hsp90 NTD to displace Cdc37[63].

4.2.3. DDO-5936

In 2019, Wang and colleagues[64] used molecular dynamics simulations and mutagenesis studies to discover a novel binding interaction between Glu-47 and Gln-133 on Hsp90 and Arg-167 on Cdc37. Based on computational and biophysical assays, **18** (Fig. 5) was identified as a disruptor of Hsp90–Cdc37 PPIs *via* binding to Hsp90 (K_D = 21.1 μmol/L). Chemical optimization of this molecule led to DDO-5936 (**19**, Fig. 5), which exhibited improved solubility and binding (K_D = 7.41 μmol/L). DDO-5936 was found to disrupt Hsp90–Cdc37 PPIs against numerous cancer cell lines, and led to antiproliferative activity, G_0/G_1 cell cycle arrest, and the degradation of Hsp90 kinase clients in HCT116 cells without induction of the heat shock response. This activity was translated *in vivo*, as the administration of DDO-5936 in a HCT116 tumor xenograft mouse model led to reductions in the volume and growth of tumors, while manifesting little toxicity to normal tissues[64]. Although identification of a binding site enables DDO-5936 to stand out among the Hsp90–Cdc37 PPI disruptors, its efficacy and drug-like properties are less than ideal. Replacement of the pyrrolidine with a piperazine yielded derivative **20** (Fig. 5) and resulted in improved binding affinity (K_D = 0.50 μmol/L), inhibitory activity, stability in plasma and liver microsomes, and ultimately, oral activity[65].

4.2.4. Derrubone

Derrubone (**17**, Fig. 5) is an isoflavenoid natural product that is isolated from the Indian tree, *Derris robusta*[66], though its biological properties weren't fully revealed until 2007 when a luciferase refolding assay indicated that it was a potent inhibitor of Hsp90, resulting in an IC_{50} value of 0.23 ± 0.04 μmol/L. That same study found that derrubone induces the degradation of Her-2 in SkBr3 cells as well as the degradation of Raf-1, Akt, and ERα in MCF-7 cells in a dose-dependent manner. It also prevented geldanamycin from disrupting the Hsp90–Cdc37–HRI client protein complex, suggesting that derrubone stabilizes the heteroprotein complex and hinders progression through the protein folding cycle[67]. In a subsequent study, the same group synthesized derrubone analogues, which revealed a requirement for the prenyl side chain and substitution at the 3′-aryl position for activity[68]. In 2010, Mays and colleagues[69] synthesized novobiocin–derrubone chimeras that suggested the parent molecules exhibit different modes of binding. In 2014, Khalid and Paul[70] performed docking studies and identified residues that form the Hsp90 C-terminal binding site, which led to the identification of leucine residues. At present, little work has continued toward elucidation of derrubone's precise mechanism of action.

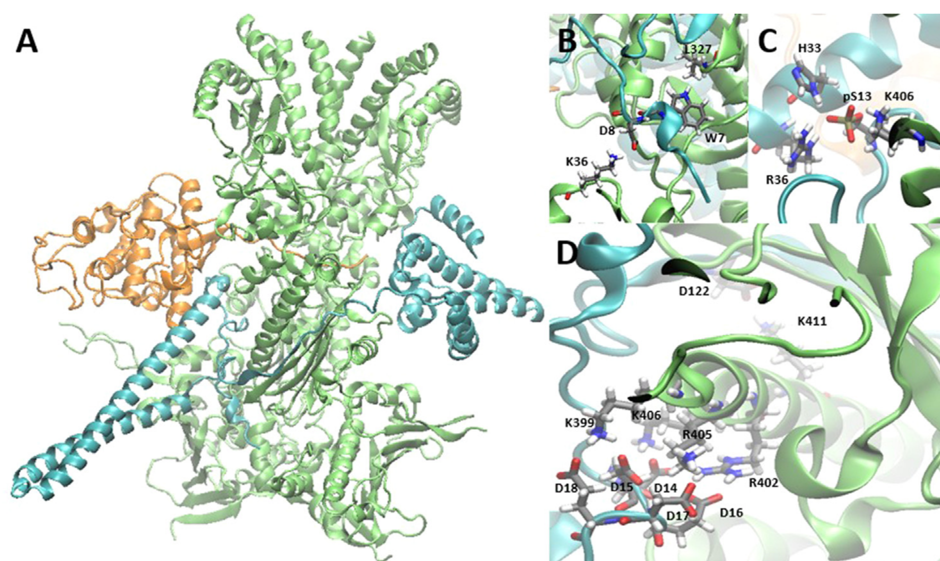

Figure 4 PPIs between Hsp90 and Cdc37. (A) Co-crystal structure of Hsp90 (green), Cdc37 (cyan), and client kinase Cdk4 (gold). (B) Structural motif resembling Hsp90–p23 PPIs. (C) The pS13 of Cdc37 facilitates both the protein's own stability and an interaction with K406 of Hsp90. (D) Numerous ionic interactions between Cdc37 aspartates and Hsp90 lysines/arginines (PDB ID: 5FWP[55]).

4.2.5. FW-04-806

FW-04-806 (also known as conglobatin, **21**, Fig. 5) is a bis-oxazolyl natural product isolated from *Streptomyces* FIM-04-806. Preliminary studies showed it to inhibit the growth of chronic myelocytic leukemia with an IC_{50} of 6.66 μg/mL[71]. An affinity-based screen found FW-04-806 to bind the Hsp90 NTD, but not alter ATP binding or Hsp90's ATPase activity. Pull-down experiments confirmed FW-04-806 disrupts Hsp90's interactions with Cdc37 and client proteins. *In vitro* studies revealed FW-04-806 could induce the degradation of Her-2, p-Her-2, Raf-1, Akt, and p-Akt levels in both SkBr3 and MCF-7 cells. *In vivo* studies revealed FW-04-806 exhibited efficacy in tumor xenograft models[72]. Further research showed that FW-04-806, either alone or in combination with the EGFR/Her-2 tyrosine kinase inhibitor, lapatinib, is effective against Her-2 positive breast cancer cells[73].

4.2.6. Gambogic acid

(−)-Gambogic acid (**22**, Fig. 5) is a natural product found in *Garcinia hanburyi* (Hook F), a plant that has been commonly used in Southeast Asia for its medicinal properties. Like celastrol A, gambogic acid was among the natural products identified as an Hsp90 inhibitor based on the results from a luciferase refolding assay. It was found to disrupt Hsp90–Cdc37 interactions as supported by the depletion of Cdc37-dependent client proteins. Surface plasmon resonance and molecular docking studies suggested it to bind the Hsp90 NTD without affecting ATP binding[74]. Zhang and colleagues[75] also reported that gambogic acid could down-regulate TNF-α/NF-κB signaling pathway in HeLa cells, which leads to apoptosis.

4.2.7. Kongensin A

Kongensin A (**23**, Fig. 5) is a diterpenoid natural product that was first isolated from the plant, *Croton kongensis*. Kongensin A first gained attention as an Hsp90 inhibitor during a high throughput screen that sought to identify compounds that exhibit anti-necroptotic activity. Li and coworkers[76] elucidated the mechanism of action in which it forms a covalent linkage with Cys-420 in the Hsp90 MD, which disrupts interactions with Cdc37. Ultimately, this mechanism blocks RIP3-dependent necroptosis and induces apoptosis. While their work was performed in cancer cells, the authors propose that kongensin A could be used to treat inflammation, atherosclerosis, and/or ischemia–reperfusion injury[76].

4.2.8. Pep-1

In addition to small molecules, peptidomimetics that imitate residues on the partner protein represents another strategy to develop PPI disruptors that take advantage of the large, shallow surface areas that are common with PPIs. Using a combination of molecular dynamics simulations and MM-PBSA analyses, Wang and coworkers[77] rationally designed a series of oligopeptides based on the Hsp90–Cdc37 interface. Their best molecule, Pep-1 (Ac-KHFGMLRRWDD-NH_2), was found to block Hsp90–Cdc37 association by binding to the Hsp90 NTD with a calculated K_D of 6.90 ± 0.9 μmol/L. It also inhibited Hsp90's ATPase activity and exhibited an IC_{50} of 3.0 ± 0.7 μmol/L. Two years later, the same group optimized Pep-1, which led to the truncated derivative Pep-5 (Ac-HFGMLRR-NH_2). Using a pull-down assay, Pep-5 was shown to disrupt the Hsp90–Cdc37 PPI, and isothermal titration calorimetry measured a slightly lower K_D of 5.99 ± 0.8 μmol/L[78]. This was the first report of a synthetic polypeptide that was capable of disrupting Hsp90–Cdc37 interactions, and thus providing another opportunity to target the molecular chaperone.

4.2.9. Platycodin D

Platycodin D (**24**, Fig. 5) is a saponin isolated from the Chinese herb Platycodonis Radix, and has exhibited immunoregulatory, anti-atherogenic, and anticancer activities[79,80]. It has also been shown to prevent cell adhesion, migration, invasion, and proliferation against numerous cancers[81]. In 2016, Li and colleagues[82] proposed that Hsp90 was a potential target for the natural product, as decreased levels of the Hsp90-dependent clients, EGFR and Her-2, were observed upon the administration of platycodin D. *In silico* modeling studies suggest the natural product to hydrogen

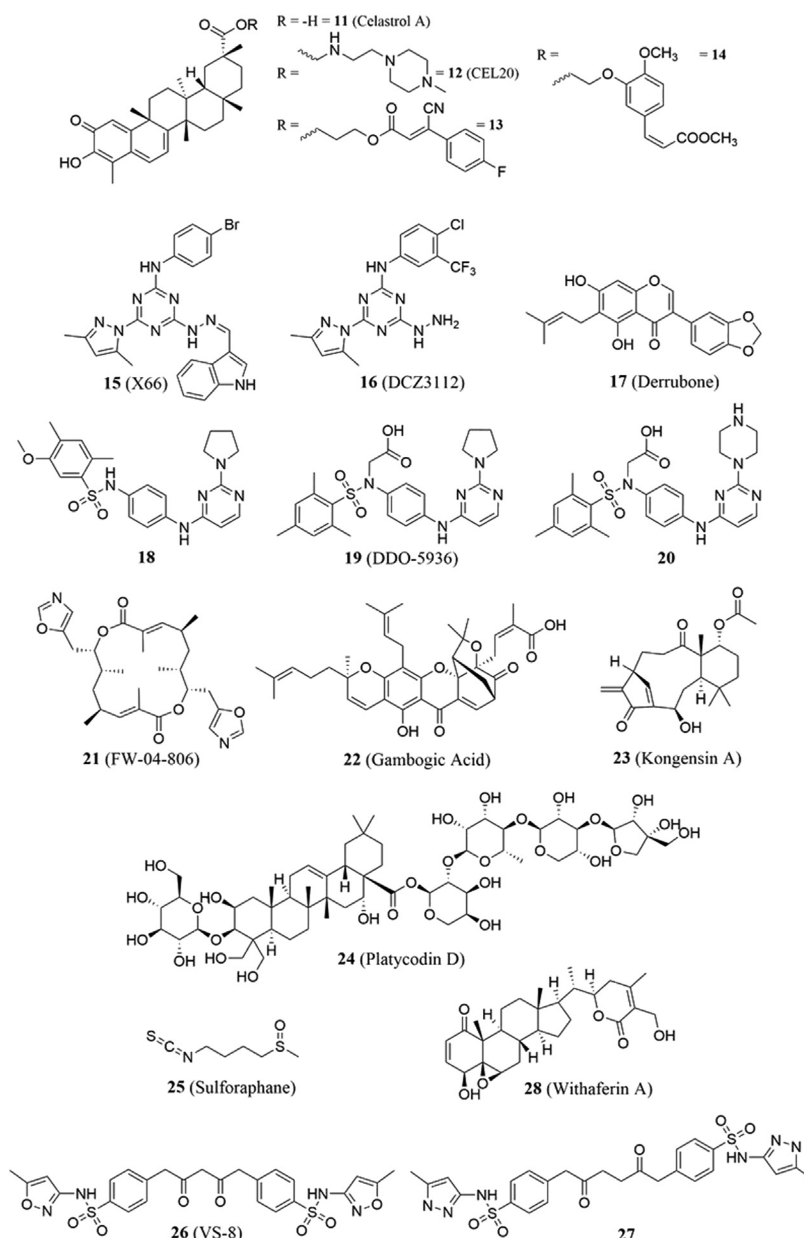

Figure 5 Disruptors of Hsp90–Cdc37 interactions.

bond with residues on both proteins (Arg-32 and Phe-200 on Hsp90 and Asp-169 and Asp-170 on Cdc37), which was supported *via* immunoprecipitation assays. Using platycodin D in combination with the mTOR inhibitor, everolimus, the authors found the molecule to sensitize non-small cell lung cancer (NSCLC) cells to everolimus. Based on these data, the mechanism of action involves the modulation of Hsp90, which results in activation of the EGFR/IGF1R/Akt signaling pathway that normally renders mTOR inhibitors ineffective[83]. Additional studies to further deconvolute the mechanism of action for platycodin D are currently underway.

4.2.10. *Sulforaphane*

Sulforaphane (**25**, Fig. 5) is an antioxidant isothiocyanate present in broccoli and other cruciferous vegetables that has been studied as a potential treatment for pancreatic cancer[84] and NSCLC[85]. The first report of Hsp90 as a potential target of sulforaphane occurred in 2009 when Gibbs and coworkers[86] reported that inhibition of histone deacetylase 6 led to Hsp90 hyperacetylation, which in turn, destabilized the androgen receptor. A more direct effect on Hsp90 was reported by Li and colleagues[87] when they used sulforaphane in combination with the geldanamycin derivative, 17-allylamino-17-demethoxygeldanamycin (17-AAG). Their data revealed that sulforaphane was able to sensitize Mia Paca-1 and Panc-1 pancreatic cells to treatment with 17-AAG, which led to enhanced degradation of Hsp90 client proteins *in vitro* and increased efficacy in pancreatic cancer xenograft mouse models. Immunoprecipitation assays were also used to confirm that sulforaphane disrupted Hsp90–Cdc37 complex formation. The same researchers investigated the nature of this interaction *via* NMR studies and noted sulforaphane caused significant chemical shifts to several isoleucines on Hsp90, including Ile-74, Ile-75, Ile-43, and Ile-125. Interactions with the former two were further

confirmed *via* an LC–MS analysis which identified the Hsp90 NTD residues Ile-72 to Arg-81 (IDIIPNPQER) to be covalently labeled by sulforaphane. Meanwhile, shifts in the latter two were attributed to allosteric effects, which is significant since Ile-125 resides within the interface between the two proteins[88].

4.2.11. VS-8

In 2017, Wang and colleagues[89] sought to discover disruptors of Hsp90–Cdc37 PPIs, which led to the development of a pharmacophore model, analog synthesis, and *in vitro* evaluation. Based on these efforts, VS-8 (**26**, Fig. 5) was identified by its ability to bind Hsp90 (K_D = 80 μmol/L) and inhibit PPIs (IC_{50} = 77 μmol/L). Truncation of the central linker and replacement of the isoxazoles with *N*-methylpyrazoles produced **27** (Fig. 5). Not only did **27** exhibit better activity than VS-8 (K_D = 40 μmol/L, IC_{50} = 27 μmol/L), but it also exhibited antiproliferative activity against MCF-7, SkBR3, and A549 cancer cells (IC_{50} = 26, 15, and 38 μmol/L, respectively) and induced the degradation of Hsp90 clients Akt and Cdk4 in SkBR3 cells. Although celastrol remained superior in these evaluations, VS-8 and **27** represent the first synthetic disruptors of this PPI.

4.2.12. Withaferin A

Withaferin A (**28**, Fig. 5) is a steroidal lactone found in *Withania somnifera* and exhibits both antitumor and antiangiogenic activities[90,91]. Withaferin A has been shown to inhibit NF-κB, resulting in the induction of apoptosis[90,92]. In 2010, Yu and colleagues[93] found that the administration of withaferin A to pancreatic cells resulted in decreased proliferation and increased apoptosis *via* Hsp90 client protein degradation. A pull-down assay demonstrated that withaferin A binds the Hsp90 CTD *via* interactions with cysteine residues. Coimmunoprecipitation studies revealed that withaferin A disrupted the Hsp90–Cdc37 complex in a dose-dependent manner. Molecular docking and molecular dynamics simulations performed by Grover and coworkers[94] suggested that withaferin's disruption of the complex is thermodynamically favored. Initial SAR studies on withaferin A performed by Yokota and coworkers[95] highlighted the importance of the unsaturated lactone and the 4-hydroxy-5,6-epoxy-2-en-1-one moiety for activity. Further SAR studies performed by Gu and colleagues[96] confirmed the importance of the epoxide ring for its reactivity towards cysteine residues.

4.3. Hsp90 and F_1F_0-ATP synthase

F_1F_0-ATP synthase is a protein that is embedded in the eukaryotic inner mitochondrial membrane and generates ATP *via* the electrochemical proton gradient produced from oxidative phosphorylation. Previously, it had been shown to complex with Hsp60, but co-immunoprecipitations by Papathanassiu and coworkers[97] discovered that the synthase also associates with Hsp90.

4.3.1. Cruentaren A

Cruentaren A (**29**, Fig. 6) is a benzolactone macrolide that can be isolated from the myxobacterium, *Byssovorax cruenta*[98], and exhibits both antifungal[99] and anticancer activities. The latter was reported by Kunze and coworkers[99], after demonstrating that cruentaren A inhibited the growth of various cancers with IC_{50} values ranging from 0.1 to 1.0 ng/mL. Further studies by the same researchers determined that cruentaren A's mechanism of action involves selective inhibition of the catalytic F_1 subunit[100]. In 2014, Hall and coworkers[101] discovered that cruentaren A also modulated the Hsp90 protein folding machinery. Incubation of the natural product with MCF-7 cancer cells induced a dose-dependent decrease in the Hsp90 client proteins pAkt, Her-2 and Raf, as well as a disruption of F_1F_0–ATP synthase–Hsp90α interactions that led to an increased nuclear localization of Hsp90α[101]. These data offer another approach to inhibit the Hsp90 heteroprotein complex without inducing the pro-survival heat shock response.

4.3.2. Efrapeptins

Efrapeptins are a family of naturally occurring peptides that are known to inhibit the synthesis of ATP[102]. As part of the work demonstrating that F_1F_0-ATP synthase associates with Hsp90, Papathanassiu and coworkers[97] utilized efrapeptins D and E as probes (**30** and **31**, Fig. 6) to assess this interaction. Not only did the peptides induce degradation of the complex, but they also induced the degradation of the Hsp90-dependent client proteins caspase-3, and p53[97]. *In vitro*, the peptides exhibited antiproliferative activities, and produced IC_{50} values ranging from 6 nmol/L–3.4 μmol/L. Synergy was also observed when these efrapeptins were used in combination with 2-deoxyglucose as a complementary method to inhibit glycolysis. However, *in vivo* studies using MCF-7 and MDA-MB-231 xenograft models found that the efrapeptins inhibited tumor growth on their own, while an antagonistic effect was observed in the combination studies. Suppression of Hsp90 chaperone activity is believed to be responsible for the paradoxical results; however, it is not uncommon for *in vivo* results to differ from *in vitro* studies[103].

4.4. Hsp90 and human epidermal growth factor receptor-2 (Her-2)

Her-2 is a receptor-like glycoprotein and member of the ErbB family of receptor tyrosine kinases whose overexpression is commonly observed in highly advanced and metastatic cancers[104]. Consequently, it has become a promising target for the treatment of breast and ovarian cancers, which has resulted in currently available therapeutics such as lapatinib and herceptin. Several studies have revealed the role played by Hsp90 to stabilize Her-2[105,106] *via* interactions between Hsp90 and Her-2's kinase domain[107]. Furthermore, Sidera and coworkers[108] discovered a novel interaction between cell-surface Hsp90 and Her-2's extracellular domain that creates an additional opportunity to develop therapeutic that target this interaction.

4.4.1. Emodin azide methyl anthraquinone derivative (AMAD)

In 2008, Yan and colleagues[109] extracted an emodin AMAD (**32**, Fig. 7) from the giant knotweed rhizome and demonstrated that it induced apoptosis in MDA-MB-453 and Calu-3 cells. Three years later, they determined that emodin AMAD disrupts Hsp90–Her-2 PPIs *via* hydrophobic, electrostatic, and hydrogen bonding interactions with each protein's nucleotide binding site, ultimately leading to Her-2 proteasomal degradation[110]. In addition, the administration of AMAD to MDA-MB-453 breast cancer cells induced G_0/G_1 cell cycle arrest as evidenced by reduced expression of the cell cycle proteins c-Myc, cyclin D1, Cdk4, and pRb[111].

4.4.2. Nefilnavir

Nefilnavir (**33**, Fig. 7) is an FDA-approved protease inhibitor that is used to treat HIV[112]. In addition to its antiviral activity, nefilnavir has been found to disrupt the PI3K and Akt signaling

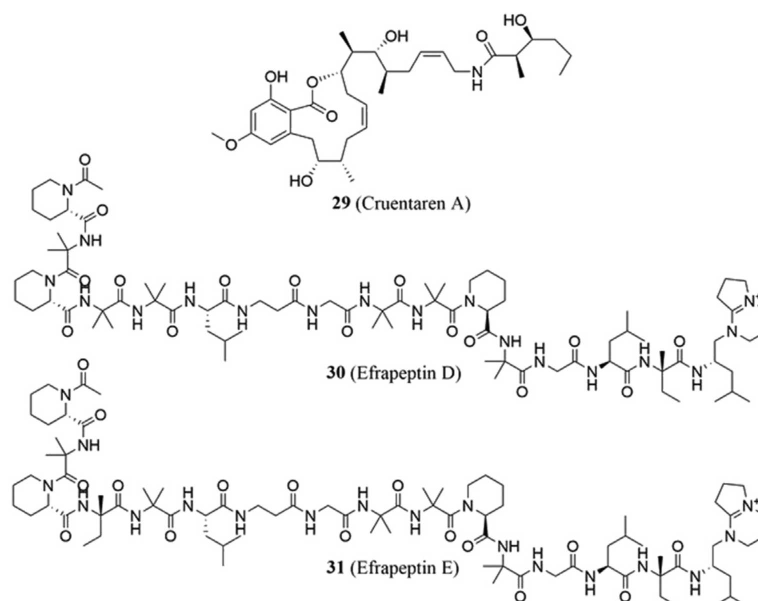

Figure 6 Disruptors of Hsp90–F_1F_0-ATP synthase PPIs.

pathways, prompting investigation into its potential application as a chemotherapeutic[113,114]. In 2012, the molecule emerged as a hit from a screen of the Johns Hopkins Drug Library to identify genotype-selective anti-breast cancer drugs. From this screen, it was demonstrated that nefilnavir could selectively inhibit the growth of Her-2 positive breast cancers *in vivo* and cells resistant to trastuzumab and/or lapatinib *in vitro*[115]. However, the work of Soprano and coworkers[116] suggested that the molecule may exhibit its anticancer activity *via* the production of reactive oxygen species.

Subsequent studies by Shim and colleagues[115] indicated that nelfinavir binds the Hsp90 CTD, but in a manner different than novobiocin. This was further supported *via* molecular docking studies performed by Arodola and Soliman[117] who proposed the potential repurposing of other HIV protease inhibitors as novel anticancer compounds as well.

4.5. Hsp90 and hypoxia inducing factor 1α (HIF-1α)

Hypoxia inducing factor (HIF) is one of the major transcription factors deployed in response to hypoxia, wherein it promotes the transcriptional activation of genes associated with angiogenesis, oxygen consumption, increased rates of glycolysis, and metastasis[118]. The protein consists of a constitutively expressed β subunit and an oxygen-sensitive α subunit[119], the latter of which is stabilized and regulated by Hsp90[120].

4.5.1. Bisphenol A

Bisphenol A (BPA, **34**, Fig. 8) is commonly used in the manufacturing of plastics but has also gained notoriety in recent years as an endocrine disruptor[121]. Initial studies by Kubo and coworkers[122] identified HIF-1α as a target of BPA, which results in its dissociation from Hsp90 and subsequent degradation. SAR studies found that although increased hydrophobicity on either side chain increased HIF-1α degradation, branched alkyl derivatives failed to exhibit activity[123]. Interestingly, BPA and its derivatives promoted HIF-1α degradation *via* the lysosome, rather than the ubiquitin–proteasome pathway[123].

4.5.2. Deguelin

Deguelin (**35**, Fig. 8) is a rotenoid that can be extracted from members of the Fabaceae (legume) family and displays chemoprotective behavior against skin and breast tumor models[124]. In addition, it has been shown to induce cell cycle arrest and apoptosis in malignant human bronchial epithelial cells and NSCLC[125], the latter study of which involved disruption of the PI3K/Akt signaling pathway. Oh and coworkers[126] reported that deguelin targets Hsp90, interacts with its N-terminal ATP binding site and induces the ubiquitin-mediated degradation of client proteins, including HIF-1α and pAkt. Chang and coworkers[127] provided evidence that deguelin binds the Hsp90 CTD. Although deguelin has been reported to be well-tolerated and exhibit no adverse effects after a 4- or 19-week treatment[128], Caboni and coworkers[129] observed that subcutaneous administration in rats caused them to develop a Parkinson's-like syndrome.

Research has been conducted to investigate structure−activity relationships for deguelin. In 2012, Chang and colleagues[127] synthesized several derivatives of deguelin and reported that both methoxy groups, the *cis* conformation, oxygenation at carbon-7, and the 2,2-dimethyl-2*H*-chromene moiety are essential for activity. Truncation of the B and/or C rings led to compounds that exhibited comparable activity to the parent compound. Two derivatives, **36** and **37** (Fig. 8), were the most efficient at suppressing both HIF-1α and angiogenesis[127]. Further studies on the synthesis and biological evaluation of truncated derivatives have gained additional attention in recent years[130−132]. Two derivatives

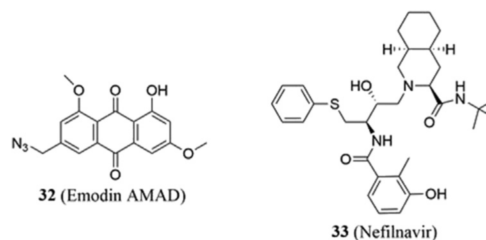

Figure 7 Disruptors of Hsp90–Her-2 PPIs.

that emerged from these studies are SH-1242 and L80 (**36** and **38**, Fig. 8). SH-1242 is a derivative with truncation to both the B and C rings. In 2014, Jo and coworkers[133] reported that SH-1242 was able to reduce hypoxia-mediated retinal neovascularization in a diabetic mouse model *via* destabilization of HIF-1α. Since then, SH-1242 has undergone pre-clinical and pharmacokinetic studies[134,135]. L80 is a derivative that contains a C ring truncation. In 2015, Lee and coworkers[136] reported the synthesis and evaluation of L80 against NSCLC. Their results showed that L80 suppressed proliferation, angiogenesis, metastasis and displayed reduced toxicity to healthy cells as compared to the natural product[136]. Co-precipitation and molecular docking studies support L80 to bind the Hsp90 CTD[136]. L80 has also been found to inhibit metastasis in triple negative breast cancer[137]. There is also a report of a novobiocin–deguelin chimera that exhibits cytotoxic and antiangiogenic properties against NSCLC[138], suggesting that their binding sites may be near one another or overlap.

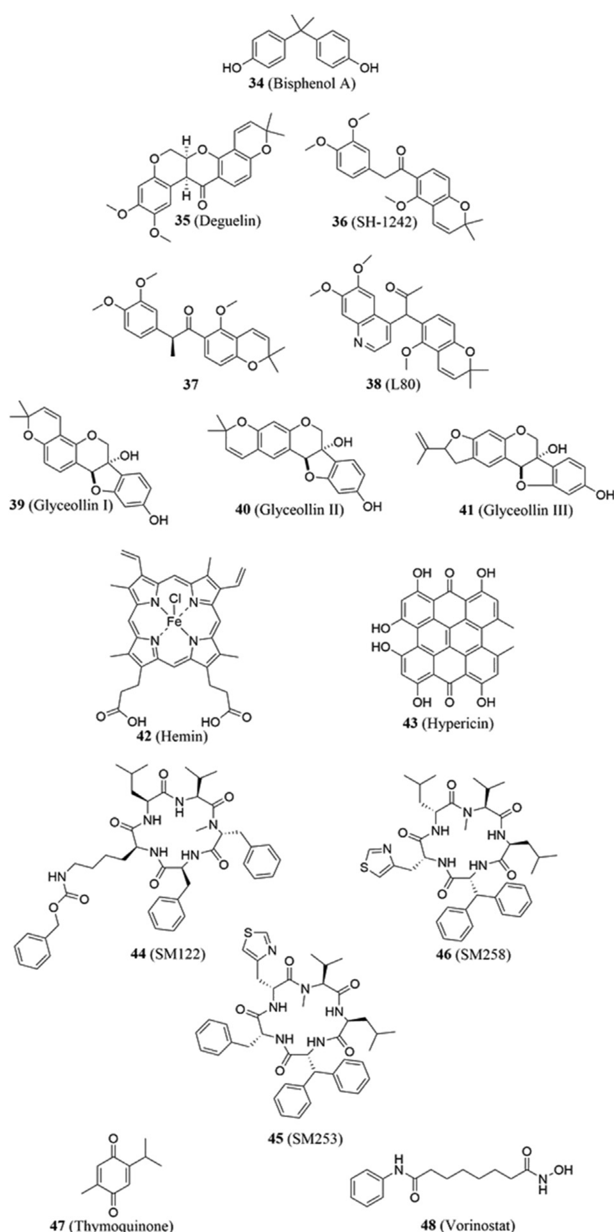

Figure 8 Disruptors of Hsp90–HIF-1α PPIs.

4.5.3. *Glyceollins*

Glyceollins (**39**−**41**, Fig. 8) are a family phytoalexins found in soybeans that have been shown to exhibit anticancer activity[139−141]. In 2014, Lee and colleagues[142] investigated the glyceollins' mechanism of action and observed decreased levels of HIF-1α in MKN1, SNU668, and MDA-MB-321 cells after administration. They proposed two potential mechanisms; 1) inhibition of the PI3K/Akt/mTOR signaling pathway or 2) binding to the Hsp90 NTD binding site[142]. Hsp90 binding was supported by immunoprecipitation studies and molecular docking. *In vivo*, the natural products were shown to reduce the tumor size in a xenograft mouse model for lung cancer[142].

4.5.4. *Hemin*

Hemin (**42**, Fig. 8) is an iron-containing porphyrin that has been used to treat porphyria attacks[143]. However, a handful of studies have suggested that these molecules exhibit protective effects against mutagenesis and carcinogenesis *via* the promotion of oxidative stress[144,145]. In 2012, Lee and coworkers[146] discovered that hemin and other protoporphyrins could induce the proteasomal degradation of HIF-1α by interfering with its binding to Hsp90. This inhibitory activity resulted in a reduction of angiogenesis and the suppression of HCT116 cell proliferation and migration[146]. *In vitro* immunoprecipitation studies confirmed disruption, but determined that the addition of ATP could reverse this observation, suggesting that the porphyrins interact with Hsp90's nucleotide binding site[146]. While further studies are needed to determine how the porphyrin ring structure and chelated metal impact inhibitory activities, the data demonstrate protoporphyrins as novel inhibitors of Hsp90 PPIs.

4.5.5. *Hypericin*

Hypericin (**43**, Fig. 8) is a perihydroxylated perylene quinone whose anticancer properties are believed to occur *via* the disruption of multiple signaling pathways related to tumor proliferation[147] and angiogenesis[148]. In 2004, Blank and coworkers[149] reported that hypericin inhibited murine breast and squamous cell carcinoma tumor metastasis *in vivo*. In addition, they observed that hypericin promoted poly-ubiquitinylation of Hsp90[149], while Barliya and coworkers[150] demonstrated that poly-ubiquitinylation negatively impacted its interactions with HIF-1α, as Hsp90 was unable to transport HIF-1α to the nucleus.

4.5.6. *SM compounds*

The SM compounds (SM122, SM253, and SM258, **44**−**46**, Fig. 8) are three sansalvamide A analogues developed by Kataria and colleagues[152] as Hsp90 C-terminal inhibitors that disrupt Hsp90–HIF-1α PPIs without inducing the heat shock response. The molecules had previously been shown to bind the Hsp90 CTD[151] and were subsequently evaluated against hypoxic colorectal, prostate, and breast cancers. Those studies demonstrated that the compounds reduced HIF-1α mRNA levels, which resulted in decreased expression. As anticipated, the compounds also induced apoptosis and decreased angiogenesis without induction of the heat shock response[152].

4.5.7. *Thymoquinone*

Thymoquinone (**47**, Fig. 8) is a natural product that is present in black cumin. While it has been reported to exhibit diverse

activities such as anti-microbial and anti-diabetic activities[153], it also inhibits angiogenesis, proliferation, and the metastasis of cancer cells[154]. Because of HIF-1α′s implication in those processes, Lee and coworkers[155] hypothesized that thymoquinone's mechanism of action involved HIF-1α. Thymoquinone induced a decrease in HIF-1α levels in hypoxic renal cancer cells *via* a proteasome-mediated pathway[155], leading researchers to suspect that thymoquinone disrupted interactions between HIF-1α and Hsp90. This was confirmed when a combination of thymoquinone and geldanamycin did not lead to a further reduction in HIF-1α levels[154]. Subsequent studies with Caki-1 and A498 cancer cells demonstrated thymoquinone's ability to selectively target hypoxic cancer cells over normal tissue[155].

4.5.8. Vorinostat

Vorinostat (suberoylanilide hydroxamic acid (SAHA), **48**, Fig. 8) is a histone deacetylase inhibitor that is approved to treat cutaneous T-cell lymphoma[156]. It has been shown to inhibit hypoxia signaling pathways along with a decrease in HIF-1α levels; however, its precise mechanism of action remained unclear[157–159]. In 2017, Zhang and colleagues[160] proposed that SAHA increases the amount of acetylated Hsp90, which leads to a lower affinity for client proteins, while simultaneously inhibiting chaperone function. Co-immunoprecipitation experiments demonstrated that SAHA decreased the affinity of HIF-1α for both Hsp90 and the nuclear karyopherin importin, as well as inducing its ubiquitinylation and proteasomal degradation.

4.6. Hsp90 and the Hsp70–Hsp90 organizing protein (HOP)

The Hsp70–Hsp90 organizing protein (HOP) is an essential component of the Hsp90-mediated protein folding cycle as it bridges both proteins and ensures the transfer of nascent polypeptides from Hsp70 to Hsp90. The CTD of Hsp90 contains a pentapeptide MEEVD sequence that is responsible for binding proteins with tetratricopeptide repeat (TPR) domains. The interaction between Hsp90 and HOP occurs within its TPR2A domain (Fig. 9). It was determined that high affinity between these two proteins is maintained by a combination of electrostatic, hydrogen bonding, and hydrophobic interactions, which gives rise to a "carboxylic clamp"[161,162]. The necessity of this PPI for proper Hsp90 activity provides yet another opportunity to inhibit the molecular chaperone.

4.6.1. 7-Azapteridines

7-Azapteridines (**49**–**54**, Fig. 10) are compounds identified as novel Hsp90–HOP PPI inhibitors during a high-throughput Alpha Screen developed by Yi and Regan[164]. The screen initially yielded 149 compounds that manifested IC_{50} values $\leq$10 μmol/L, while follow-up assays eliminated the false positives to ultimately reveal three molecules[164]. **49** was chosen for further investigation, and fluorescence polarization and isothermal titration calorimetry confirmed it to disrupt PPIs by binding to HOP's TPR2A domain with an apparent K_D of 16 μmol/L. Overall, the compounds induced the death of BT474 cells; however, they displayed modest selectivity over non-malignant MCF-12F cells. The 7-azapteridines also led to a reduction of Her-2 levels in BT474 and SkBR3 cells after 6 h, but this led to an increase in Her-2 levels after 12 h. Further studies are needed to resolve the mechanism of action manifested by these novel inhibitors.

In 2011, the same researchers identified **50** and discovered its antimigratory and antiproliferative activity against breast cancer cell lines, including the highly drug-resistant MDA-MB-468 and MDA-MD-231 cells, manifesting IC_{50} values of 2 and 1.75 μmol/L, respectively[165]. When used in combination with the known N-terminal inhibitors 17-AAG, PU-H71 or NVP-AUY922, the cytotoxic activity exhibited by **50** was enhanced. Similarly, the administration of **50** also led to a decrease in the levels of Hsp90-dependent client proteins Cdc37, Cdk4, Raf-1, JNK1/2, p38 and HSF-1, but did not alter Hsp70 mRNA expression, indicating that it did not induce the heat shock response.

4.6.2. Celastrol A

In 2010, Zhang and colleagues[166] reported that celastrol (**55**, Fig. 10) regulates multiple transcription factors in a dose-dependent manner, but to a different extent in MCF-7, HepG2, and THP-1 cells. Co-immunoprecipitations studies with MCF-7 whole cell lysates revealed a reduction in HOP bound to Hsp90, suggesting that celastrol can disrupt the stability of other client proteins as well[166].

4.6.3. Peptide-based disruptors

In 2011, Horibe and coworkers[167] synthesized a peptidomimetic based on the TPR2A domain of HOP (KAYARIGNSYFK). The inclusion of a lysine and arginine at the first and fifth positions, respectively, recreated a crucial hydrogen bonding interaction with the Hsp90 MEEVD sequence. The peptide, referred to as the "Antp-TPR peptide," was found to bind Hsp90 with a K_D of 4.43 μmol/L and exhibited antiproliferative activity against breast, pancreatic, renal, gastric, lung, and prostate cancer cell lines manifesting IC_{50} values in the range of 19.4–65.9 μmol/L. Furthermore, "Antp-TPR peptide" exhibited efficacy *in vivo* against BXPC3 pancreatic xenograft mouse models, which resulted in a significant decrease in tumor size.

Another peptide inhibitor reported by Gupta and coworkers[168] was developed *in silico*. The authors performed molecular docking studies between Hsp90α and HOP to identify residues that are most likely to participate in PPIs. A series of ten peptides was rationally designed and then docked to Hsp90α, with the best (PEP7) exhibiting a strong predicted docking energy. PEP7 (INSAYKFKYARG), which required further refinement due to its potential to form amyloid plaques, yielded 5 derivatives. PEP73 (INSAYKLKYARG) was tentatively the best compound identified based upon docking scores. However, the results have yet to be validated *in vitro*, and there have been no follow-up studies reported thus far.

4.6.4. Y-632

Y-632 (**56**, Fig. 10) is a pyrimidine that was identified as an Hsp90 inhibitor by Wang and coworkers[169] in 2016. Y-632 induced the proteasome-mediated degradation of several Hsp90-dependent client proteins in SkBr3, A-431, MCF-7, and SNU-5 cancer cell lines, which led to induction of G_0/G_1 cell cycle arrest and apoptosis[169]. Y-632's mechanism of action was discovered after it failed to inhibit the ATPase activity of Hsp90, and surface plasmon resonance studies demonstrated that it doesn't bind the chaperone directly[169]. The first indication that Y-632 disrupted Hsp90–HOP interactions came during a luciferase refolding assay and immunoprecipitation experiments in which HOP was the only co-chaperone downregulated[168]. Glutathione reversed the anticancer activities manifested by Y-632, suggesting that Y-632 disrupts Hsp90 PPIs with HOP *via* the oxidation of cysteines, and this is supported by the presence of its α,β-unsaturated amide moiety that can serve as a Michael acceptor. In addition, Y-632

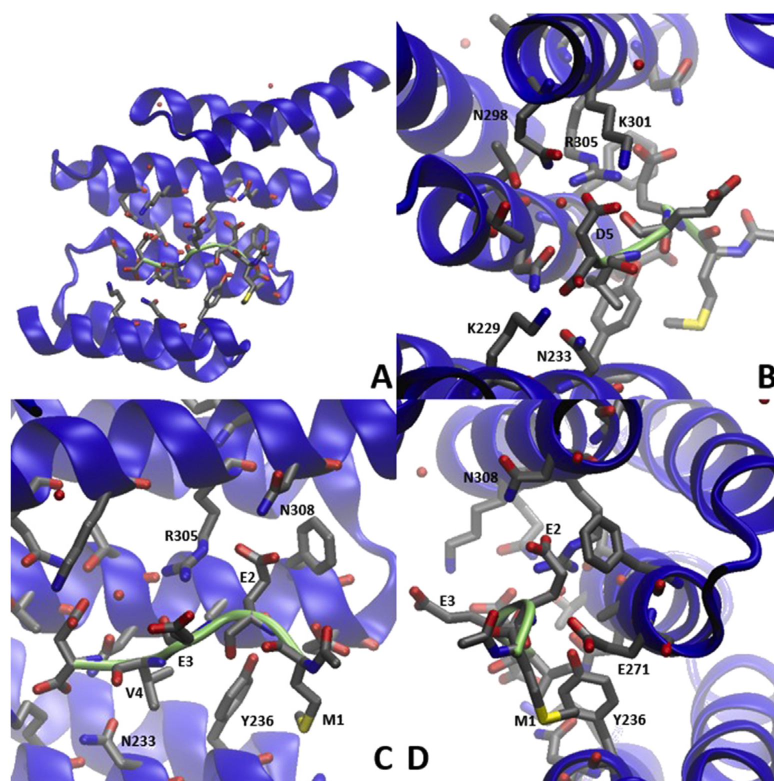

Figure 9 PPIs between Hsp90 and HOP. (A) Co-crystal structure of HOP (blue) with the Hsp90 C-terminal MEEVD sequence (green). (B)–(D) Depictions of the electrostatic, hydrogen bonding, and hydrophobic interactions between residues from the viewpoints of the MEEVD C-terminus, a 90° rotation, and the MEEVD N-terminus, respectively (PDB: 1ELR[163]).

was shown to be effective against imatinib-resistant cells and to inhibit their growth.

4.7. *Hsp90 and survivin*

Survivin is a member of the inhibitors of apoptosis family and is universally overexpressed in cancer[170]. In addition to its ability to prevent apoptosis, it also serves as a major mitotic regulator[171]. Because of its overexpression and diverse function, survivin has been implicated to confer chemotherapy and radiotherapy resistance in cancer cells[172]. Fortugno and coworkers[173] were the first to report that survivin binds Hsp90; however, it does not depend upon Hsp90 to achieve its conformational maturity, but instead, relies upon Hsp90 to stabilize its fully assembled form. The interface between the two resides at the Hsp90 NTD and survivin region comprised of Lys-79–Lys-90. However, mutagenesis studies reinforced the importance of His-80 and Cys-84 to maintain these interactions[174]. Consequently, these residues serve as the basis for molecules that were specifically designed to disrupt this PPI.

4.7.1. *17-DMCHAG*

17-(6-(3,4-Dimethoxycinnamamido)hexylamino)-17-demethoxy-geldanamycin (17-DMCHAG, **57**, Fig. 11) is a geldanamycin analogue prepared by Wang and coworkers[175] that was evaluated for anticancer activity against prostate cancer cells. 17-DMCHAG selectively induced apoptosis in malignant cells *versus* normal RWPE-1 cells and inhibited cellular migration of metastatic cancer cells. Co-immunoprecipitation studies and Western blots revealed 17-DMCHAG to promote Hsp90 client protein degradation. However, the compound stood out by its abilities to potently disrupt androgen receptor signaling and to downregulate survivin levels, suggesting that 17-DMCHAG may disrupt interactions with Hsp90. Unlike geldanamycin and its first derivatives, 17-DMCHAG does not appear to induce the heat shock response, and instead, suppressed the tumor growth of DU-145 and LNCaP xenografts without organ damage or toxicity.

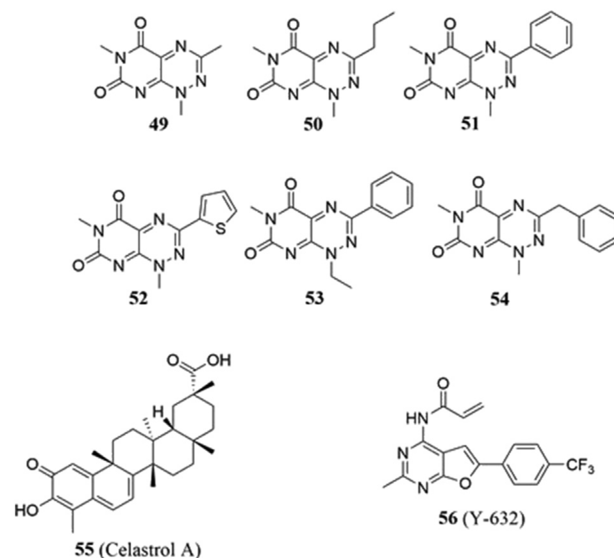

Figure 10 Disruptors of Hsp90–HOP PPIs.

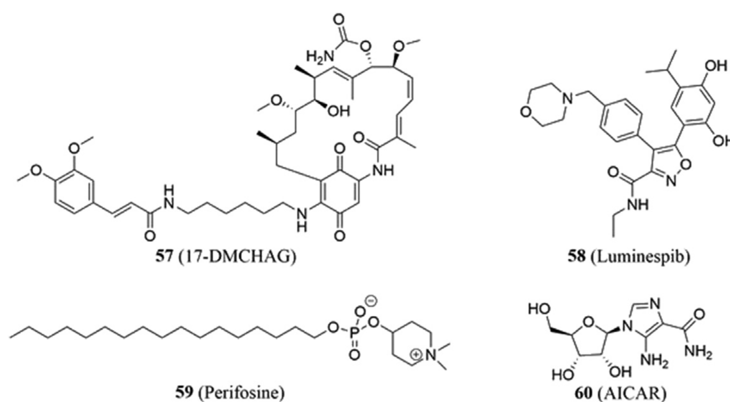

Figure 11 Disruptors of Hsp90–survivin PPIs.

Overall, these data provide further support for 17-DMCHAG as a potential treatment for prostate cancer.

4.7.2. *Luminespib (NVP-AUY922)*

Luminespib (NVP-AUY922, **58**, Fig. 11) is an isoxazole-based inhibitor of Hsp90 ATPase activity that is currently being investigated in at least 13 clinical trials[176,177]. Liu and coworkers[178] sought to determine its effectiveness against papillary thyroid carcinoma (PTC). *In vitro* studies using IHH4 and K1 PTC cells demonstrated that luminespib inhibited cell proliferation and induced cell death *via* apoptosis[178]. It was also demonstrated that luminespib lowered survivin levels *via* disruption of the Hsp90–survivin complex.

4.7.3. *Perifosine*

Perifosine (**59**, Fig. 11) is an alkyl phospholipid drug candidate that has been investigated in clinical trials as a treatment for multiple cancers, including neuroblastoma[179], colorectal cancer[180], and metastatic melanoma[181]. While its primary mechanism involves interactions with the plasma membrane and the inactivation of Akt[182], it has been proposed to display activity through other pathways as well. In 2013, Yao and coworkers[183] administered the molecule to osteosarcoma cells and found that perifosine inhibited the growth, promoted apoptosis, and sensitized cells to doxorubicin. In addition to Akt inhibition, the authors noted that perifosine induced the degradation of survivin, which co-immunoprecipitation studies indicate is due to interference with the Hsp90–survivin complex.

4.7.4. *Shepherdin and AICAR*

Shepherdin (KHSSGCAFLIVK) is a peptidomimetic and one of the first disruptors of Hsp90–survivin PPIs identified. The molecule represents the minimum sequence required for survivin to bind Hsp90 and was designed by Plescia and colleagues[174] to recreate this interaction. *In vitro* studies revealed shepherdin to selectively target carcinomas while leaving human fibroblasts unaffected. Shepherdin also reduced *in vivo* tumor size in both PC3 and MCF-7 xenograft mouse models without any detrimental side effects or toxicity. It was reported that shepherdin also suppressed ATP binding to Hsp90, promoted the degradation of other Hsp90 clients, and induced cell death *via* both apoptopic and non-apoptopic pathways. Such data suggests that shepherdin exhibits anticancer activity through multiple pathways. Shepherdin has since been assessed against numerous cancers including retinoblastoma[184], leukemia[185], and gallbladder carcinoma[186].

In 2006, the same researchers discovered 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR, **60**, Fig. 11), a small molecule that behaves similarly to shepherdin. AICAR was developed following a molecular docking and dynamics screen[187]. *In silico* modeling suggested that AICAR binds the Hsp90 N-terminal ATP binding site, but in a manner different than other Hsp90 N-terminal inhibitors. Such binding was confirmed *via* an ELISA screen. Like shepherdin, AICAR inhibits Hsp90 function, which led to the selective induction of apoptosis in cancer cell lines and the degradation of survivin along with other client proteins. *In silico* modeling and NMR studies suggested that both shepherdin and AICAR depend upon their imidazole rings for establishing hydrophobic interactions with a nonpolar patch of Hsp90 that is comprised of Ala-55, Ile-96, and Met-98[188]. Despite its potential as a lead compound for inhibitor development, no medicinal chemistry studies pertaining to AICAR have been reported. Instead, AICAR has been used as a probe for biological investigation of various pathways[189,190].

4.8. *Hsp90 and p23*

p23 is one of the many co-chaperones that associates with Hsp90 during the protein folding cycle. Upon transfer of the client protein to Hsp90 from the Hsp40/Hsp70/HIP/HOP complex, p23 is recruited to Hsp90 to inhibit Hsp90's ATPase activity and to stabilize its interactions with the client substrate. Ali and colleagues[23] were among the first to solve a co-crystal structure of the Hsp90–p23 complex in yeast, indicating that p23 binds to a cavity formed by the NTDs of the Hsp90 dimer in an ATP-dependent manner (Fig. 12). However, NMR studies by Martinez-Yamout and coworkers[191] suggest that Aha1, which is known to bind Hsp90's MD[46], competes with p23 to achieve this. The precise location of p23 binding to Hsp90 thus remains unclear. p23 has been implicated to play a key role in metastasis and advanced malignancy[192], and therefore, disruption of Hsp90–p23 PPIs has become an attractive therapeutic strategy.

4.8.1. *Ailanthone*

Ailanthone (**61**, Fig. 13) is a quassinoid natural product isolated from the Chinese tree of heaven (*Ailanthus altissima*)[193] that exhibits anticancer activity against numerous cancer types, including bladder[194], leukemia[195], and hepatocellular carcinoma[196]. Though it has been reported to induce G_0/G_1 cell cycle arrest[197] and interfere with various oncogenic and tumor-suppressant miRNAs[198,199], the work of He and colleagues[200] discovered that ailanthone inhibits p23. During their studies of castration-resistant

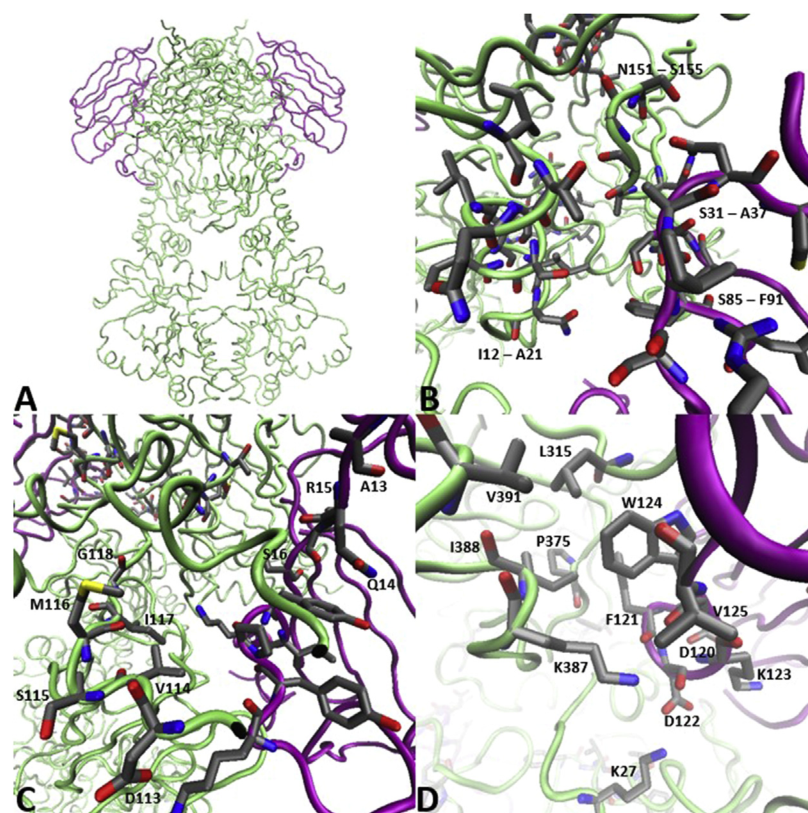

Figure 12 PPIs between Hsp90 and p23. (A) Co-crystal structure of yeast Hsp90 (green) and p23/Sba1 (purple). (B)–(D) Residues within the Hsp90 NTD and MD that interact with p23/Sba1 (PDBID: 2CG9[23]).

prostate cancer, the authors found that ailanthone inhibited tumor growth and metastasis *via* degradation of the androgen receptor. This degradation was caused by ailanthone's ability to prevent p23's binding to Hsp90, which was further supported when p23 knockdown reduced ailanthone inhibition and p23 overexpression rescued ailanthone-hindered proliferation[200]. Molecular docking studies suggest a potential binding site for ailanthone on p23 in a pocket that is formed by residues Trp-8, Pro-87, Arg-93, Lys-95, Ser-100, and Val-101. This proposed binding site differs from that ascribed to celastrol[201], suggesting that the two might display synergistic activities against p23.

4.8.2. Celastrol A

Despite uncertainty regarding the mechanism of action manifested by celastrol (**62**, Fig. 13) and its ability to promote the degradation of Hsp90-dependent client proteins, its ability to disrupt p23 has been well-defined. p23 was first identified as a target of celastrol by Chadli and coworkers[201] who reported that the compound induced fibrillization of the co-chaperone. This aggregation was supported by $^{1}H-^{15}N$ heteronuclear single quantum coherence spectroscopy and detection of the fibrils *via* electron microscopy[201]. Although it has been hypothesized that celastrol reacts with cysteine residues to form a covalent linkage[202], mutagenic studies with p23 indicate they are not required for formation of the fibrils[201]. Instead, the NMR spectra of the mutants suggest celastrol to bind the same hydrophobic pocket on p23 as Hsp90.

4.8.3. Cucurbitacin D

Cucurbitacins are a family of triterpenoids that are isolated from the fruit of the Cucurbitaceae family and other related species and are known to display a variety of biological activities, including anticancer and antimetastatic properties[203]. In 2015, Hall and coworkers[204] isolated cucurbitacin D (**63**, Fig. 13) and its isomer, 3-*epi*-isocucurbitacin D, from *Cucurbita texana* and synthesized derivatives to assess their biological properties. With few exceptions, all the evaluated cucurbitacins manifested IC_{50} values in the nanomolar range against MCF-7 breast cancer cells. The proposed mechanism of action involved a reaction between the electrophilic α,β-unsaturated carbonyl and nucleophilic residues. The compounds induced the degradation of Hsp90 client proteins in a dose-dependent manner and without induction of the heat shock response, indicating that they act through a mechanism different than Hsp90 N-terminal inhibition. However, co-immunoprecipitation experiments suggest that only cucurbitcain D demonstrated its antiproliferative activity through disruption of the Hsp90–p23–Cdc37 complex.

4.8.4. CP9

CP9 (*N*-(5-methylisoxazol-3-yl)-2-[4-(thiophen-2-yl)-6-(trifluoromethyl)pyrimidin-2-ylthio]acetamide, **64**, Fig. 13) was found to disrupt Hsp90–p23 interactions in a high throughput screen developed by Chan and colleagues[205]. A competitive binding assay determined that CP9 binds in a manner similar to 17-AAG and displayed antiproliferative activity, as well as enabled glucose metabolism and thymidine kinase function across multiple cancer cell lines, while exhibiting no significant effect on normal embryonic mouse fibroblasts. In addition, similar results were obtained in a 293 T xenograft mouse study. SAR studies on CP9 yielded the derivative A17 (**65**, Fig. 13), which exhibits superior activity and is believed to result from increased hydrophobicity.

4.8.5. Docosahexaenoic acid (DHA)

Docosahexaenoic acid (**66**, Fig. 13) is an omega-3 polyunsaturated fatty acid found in fish oil that has been proposed to improve outcomes in cancer patients either on its own[206] or as a supplement to chemotherapy and/or radiotherapy regiments[207]. To determine how DHA elicits its anticancer effects, Mouradian and coworkers[208] administered DHA to A549 lung and BT-474 breast cancer cells, which resulted in decreased cellular ATP and disruption of Hsp90–p23 interactions. It was also observed that DHA decreased levels of the Hsp90 clients, HIF-1α and Her-2. These data indicate that DHA manifests its activity through regulation of the Hsp90 heteroprotein complex and its ATPase activity, while also highlighting the potential influence of a patient's diet on treatment outcome.

4.8.6. Gedunin

Gedunin (**67**, Fig. 13) is a tetranortriterpenoid natural product that can be isolated from the mahogany family of plants and has been reported to exhibit antiproliferative activity against colon[209] and ovarian cancer cell lines[210]. Patwardhan and colleagues[211] sought to determine whether the compound could serve as an inhibitor of Hsp90. *In vitro* and *in vivo* analyses demonstrated that gedunin could target p23 and inhibit its ability to chaperone citrate synthase and various steroid receptors. In addition, gedunin caused destabilization of the Hsp90–p23 complex, induced apoptosis in cancer cells, and enabled cleavage of p23 by caspase-7[211]. Molecular docking studies identified a potential binding site for gedunin, which highlighted Thr-90 and Lys-95 as critical for hydrogen bonds, while Ala-94 mediated a hydrophobic interaction. The proposed binding site was supported *via* mutagenic studies; however, deletion of the p23 C-terminal residues also support its role to stabilize this interaction.

SAR studies on this compound resulted in the synthesis of 19 derivatives. Although none of the derivatives were more potent than the natural product, important information about the structure was still obtained[212]. The 7-position was found to be sensitive to steric bulk, but not electronic properties. In addition, the α,β-unsaturated carbonyl of the A-ring does not serve as a Michael acceptor. In 2018, Pinkerton and coworkers[213] reported a synthesis that allows for diversification of the BCD ring system of gedunin, and through this route, they generated molecules which were found equipotent as gedunin at inhibiting p23 function.

4.8.7. Luminespib (NVP-AUY922)

In addition to the disruption of PPIs between Hsp90 and survivin, luminespib (**68**, Fig. 13) has been found to destabilize the Hsp90–p23 complex. While luminespib has received attention as a potential treatment for a variety of cancers, Jensen and colleagues[214] investigated it specifically for activity against breast cancer. *In vitro* results found that luminespib potently inhibited the growth of multiple breast cancer cell lines and manifested an average GI_{50} of 5.4 nmol/L, while growth inhibition of several breast tumors

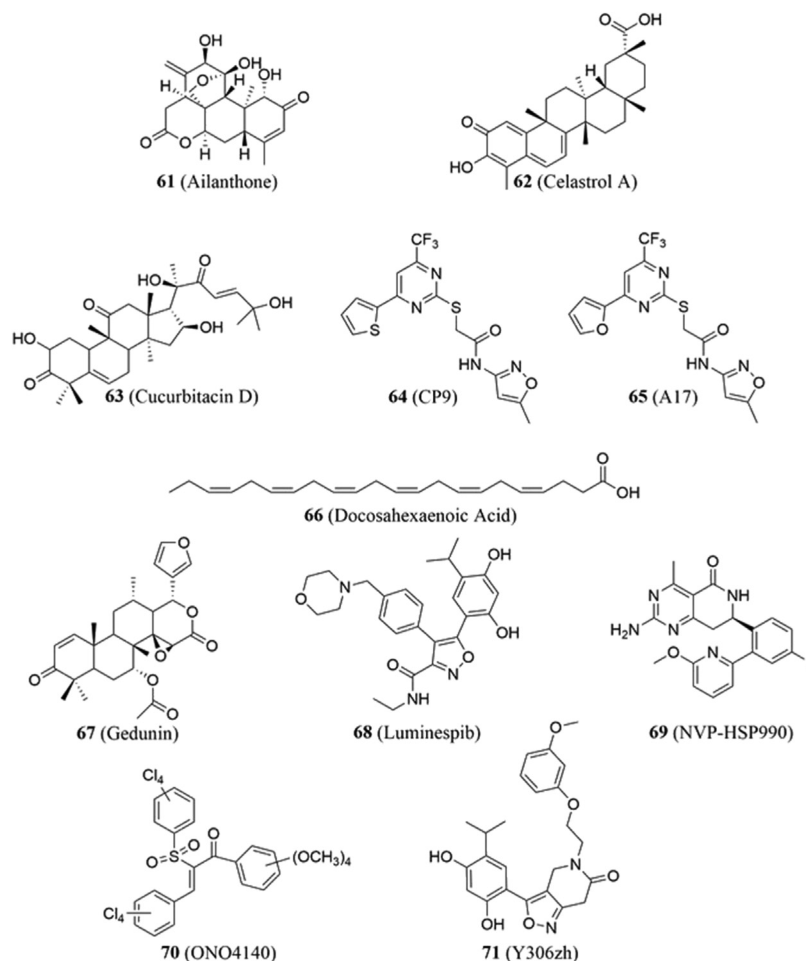

Figure 13 Disruptors of Hsp90–p23 PPIs.

was reported to have an average GI_{50} value of 191 nmol/L. Unfortunately, one of the tumors exhibited drug resistance. Immunoprecipitation studies supported the hypothesis that the molecule interferes with the Hsp90–p23 complex and provides support for the potential use of NVP-AUY922 for the treatment of breast cancer.

4.8.8. *NVP-HSP990*

NVP-HSP990 (**69**, Fig. 13) is a novel dihydropyridopyrimidinone Hsp90 inhibitor that binds the N-terminal ATP binding site. In 2012, Menezes and colleagues[215] found that it inhibited Hsp90α, Hsp90β, and Grp94 by manifesting IC_{50} values of 0.6, 0.8 and 8.5 nmol/L, respectively. In addition, such inhibition resulted in degradation of the Hsp90–p23 complex in both a time- and dose-dependent manner[215]. *In vivo* studies showed that NVP-HSP990 suppressed the growth of various cancer cell lines, and this translated well against gastric, breast, AML, and NSCLC xenograft models. In addition, NVP-HSP990 did not exhibit hepatotoxicity, suggesting minimal chance for drug−drug interactions. SAR studies performed by McBride and coworkers[216] determined the importance of the *para*-fluorine on ring C to maintain interactions with the Hsp90 binding pocket. Derivatives wherein the *ortho*-pyridine was replaced with either a pyrazine or a thiazole exhibited comparable activity, but the parent molecule exhibited a more favorable half-life[216]. A phase I clinical trial has indicated that NVP-HSP990 is well-tolerated, although neurological toxicities may limit the maximum-tolerated dosage to 50 mg per week[217].

4.8.9. *ONO4140*

ONO4140 (**70**, Fig. 13) is a novel Hsp90 inhibitor that was identified by Eachkoti and colleagues[218] during a luciferase refolding screening assay. ONO4140 exhibited low GI_{50} values against BT-474 (invasive ductal breast carcinoma), DU-145 (prostrate carcinoma), and K562 (Bcr-Abl positive CML) cell lines. ONO4140 also induced the degradation of Hsp90-dependent client proteins, Her-2 and Akt, and induced the expression of Hsp70, which provided evidence for the mechanism of action for ONO4140 as Hsp90 inhibition. At higher concentrations, ONO4140 disrupted the Hsp90–p23 PPI as confirmed by co-immunoprecipitation experiments.

4.8.10. *Y306zh*

Y306zh (**71**, Fig. 13) is a novel Hsp90 inhibitor identified by Xue and colleagues[219] as a potential treatment for pancreatic cancer. During the elucidation of the mechanism of action, it was determined that Y306zh blocks Hsp90–p23 association by competing with ATP for binding to the NTD, while manifesting an IC_{50} of 85 nmol/L[219]. *In vitro* analysis found Y306zh to induce G_2/M cell cycle arrest in pancreatic cell lines and inhibit tumor growth in a Mia-paca 2 xenograft mouse model without affecting normal cells.

5. Conclusions and perspectives

Hsp90 is an ATP-dependent molecular chaperone whose primary function is to maintain cellular proteostasis by folding $\sim$400 client substrates, restoring damaged/denatured proteins, solubilizing protein aggregates, and promoting protein turnover *via* the ubiquitin–proteasome pathway. Because many of its client proteins are implicated in the development and progression of cancer, Hsp90 inhibition has been pursued as a chemotherapeutic strategy to disrupt multiple oncogenic pathways simultaneously.

The abundance of Hsp90, its higher affinity for ATP, and its enhanced ATPase activity in cancer cells relative to normal tissue support the development of inhibitors that selectively target malignant cells. Despite evidence that demonstrates the effectiveness of Hsp90 N-terminal inhibitors to treat cancer *in vitro* and *in vivo*, there are no FDA-approved Hsp90 inhibitors. The first Hsp90 inhibitors discovered were geldanamycin, radicicol and their derivatives, which have in many cases failed during clinical evaluation due to unanticipated issues related to dosing and toxicity. Unfortunately, NTD-inhibition results in induction of the heat shock response, which leads to the overexpression of pro-survival proteins that contradict inhibitory activity. The latter appears to be related to the inhibitors' lack of selectivity among the four isoforms. Potential solutions include C-terminal or isoform-selective inhibition, however, each of them comes with their own challenge. For example, the binding pocket for C-terminal inhibitors has not been fully elucidated, and the high sequence identity among the isoforms in the NTD makes selective inhibition very difficult.

A third approach to modulate the Hsp90 machinery involves disruption of protein−protein interactions between Hsp90 and various co-chaperones/client proteins. Some co-chaperones such as HOP, p23 and Aha1 are essential for progression through the protein folding cycle; and therefore, disruption of these interactions represents an alternative approach toward Hsp90 inhibition. Roughly 400 client proteins rely upon Hsp90 to attain conformational maturity, and this process also requires the recruitment of other immunophilins and co-chaperones as well. Since co-chaperones associate with Hsp90 at different stages during the protein folding cycle, they can be exploited to target a more refined set of substrates. For instance, the folding of kinase clients is facilitated by Cdc37, and consequently, disruption of Hsp90−Cdc37 PPIs are likely to prove effective for the treatment of kinase-driven cancers. Other proteins like survivin and HIF-1α are not clients, but depend upon Hsp90 for stability. As a result of Hsp90 inhibition, they are degraded and ultimately, can lead to cell death.

The discovery and development of small molecule inhibitors has been the primary approach toward Hsp90 inhibition, although peptidomimetics of certain PPIs have also been pursued. Much like early Hsp90 inhibitors, many of these molecules have proven to be efficacious *in vitro* and *in vivo* and remain at various stages of the drug development process. Many of these inhibitors are derived from natural products, although some were discovered from high throughput screens. Other compounds are known Hsp90 inhibitors whose biological outcomes are better understood; however, the exact mechanism manifested by some of these compounds remains undetermined.

Although Hsp90 inhibition has been heavily pursued as a promising approach to treat cancer for the last decade, the molecular chaperone remains a challenge. In this article, the disruption of Hsp90 PPIs is presented and provides insights into the potential promise of targeting Hsp90 PPIs as a viable and complementary approach to Hsp90 N-terminal inhibition.

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Author contributions

Brian Blagg proposed the idea and revised/edited the manuscript. Michael Serwetnyk wrote the manuscript.

Conflicts of interest

The authors have no conflict of interest to declare.

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