HSP90 inhibitors and cancer: Prospects for use in targeted therapies (Review)

ZI-NAN LI and YING LUO

Department of Pharmacy, Affiliated Hangzhou First People's Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310000, P.R. China

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Abstract. Heat shock protein 90 (HSP90) is a vital chaperone protein, regulating signaling pathways and correcting misfolded proteins in cancer cells by interacting with oncogenic client proteins and co-chaperones. The inhibition of HSP90 chaperone machinery has been demonstrated as a potential approach with which to inhibit tumor survival, proliferation, invasion and migration. Numerous HSP90 inhibitors have been reported and have exhibited value as cancer-targeted therapies by interrupting the ATPase activity of HSP90, thus suppressing the oncogenic pathways in cancer cells. These inhibitors have been classified into three categories: i) N-terminal domain (NTD) inhibitors; ii) C-terminal domain (CTD) inhibitors; and iii) isoform-selective inhibitors. However, none of these HSP90 inhibitors are used as clinical treatments. The major limiting factors can be summarized into drug resistance, dose-limiting toxicity and poor pharmacokinetic profiles. Novel HSP90-targeted compounds are constantly being discovered and tested for their antitumor efficacy in preclinical and clinical trials, highlighting the prospect of the use of HSP90 inhibitors as cancer-targeted therapies. Additionally, improved antitumor effects have been observed when HSP90 inhibitors are used in combination with chemotherapy, targeted agents, or immunotherapy. In the present review, the effects of HSP90 inhibitors on the management of the cancer process are discussed and previous and novel HSP90-based therapeutic strategies in cancer treatment are summarized. Furthermore, prospective HSP90-targeting candidates are proposed for their future evaluation as cancer treatments.

Correspondence to: Dr Ying Luo, Department of Pharmacy, Affiliated Hangzhou First People's Hospital, Zhejiang University School of Medicine, 261 HuanSha Road, Hangzhou, Zhejiang 310000, P.R. China
E-mail: luoying9009@163.com

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1. Introduction

Heat shock proteins (HSPs), also known as molecular chaperones, were discovered to be upregulated when cells were exposed to conditions of stress, including heat shock, chemical factors, and other pathological alterations (1). The HSP90 family, which are highly conserved molecules, are involved in the regulation of the folding of newly synthesized proteins, as well as in correcting incorrectly folded proteins and impeding the aggregation of incorrectly folded proteins (2). The HSP90 chaperone machinery plays a crucial role in protecting overexpressed and mutated proteins from misfolding and inducing their degradation where appropriate (3). Interactions between HSP90 and client proteins are essential processes in tumor survival, proliferation and migration (4,5). In total, >400 client proteins have been identified, with these proteins being involved in a wide range of important biological activities, including signaling cascades, DNA damage repair, protein transportation and hormone receptor activation (6). HSP90 consists of three domains: The N-terminal domain (NTD), the C-terminal domain (CTD) and the middle domain (MD). The NTD has an ATP binding site and a client protein binding site, the MD is vital for hydrolysis of ATP to ADP, and the CTD contains one site for protein dimerization and another for calmodulin binding; a charged linker domain connects the NTD to MD, contributing to maintaining the function, interaction and adaptability of the HSP90 chaperone (7-12). HSP90 activates and facilitates the activities of its client proteins through the ATPase cycle, which is completed by dimerization (13). The HSP90 family includes four isoforms that are present in different locations in cells. HSP90α and HSP90β are present in the cytoplasm and nucleus, GRP94 is present in the endoplasmic reticulum, and TRAP-1 is primarily located in the mitochondrion, but is also present in the endoplasmic reticulum (1,14-16). All four isoforms share

a high degree of sequence homology in their N-termini; thus, the ATP binding sites present in their N-termini are interesting target locations, and considerable research has been devoted to disrupting the molecular chaperone function through targeting this domain (7,17). A total of 18 inhibitors of HSP90 have been identified and have entered clinical trials (18,19). These inhibitors can be divided into five categories based on chemical structure: i) Natural products and their derivatives; ii) purine-based; iii) benzamide; iv) resorcinol-containing; and v) miscellaneous. None of these inhibitors are currently used as clinical treatments, due to their dose-limited toxicity and poor bioavailability (20). CTD inhibitors and the isoform-selective inhibitors that specifically bind to HSP90α, HSP90β, GRP94, or TRAP-1 have also been developed, attempting to improve their antitumor effects. In the present review, the present armamentarium of HSP90 inhibitors as a monotherapy in cancer management and the potential combination therapies of HSP90 inhibitors are discussed, along with other traditional clinical therapies, including chemotherapies, targeted agents, immunotherapy, and radiotherapy. Furthermore, prospective candidates for HSP90-targeting anti-neoplastic treatment are proposed.

2. Structure and biological function of HSP90

HSP90 is a crucial chaperone protein that functions to maintain the correct folding of client proteins. It regulates protein folding and degradation, several cell signaling pathways, cell proliferation and survival, and cell apoptosis via interacting with co-chaperones and client proteins (1). HSP90 consists of three distinct domains: i) The NTD, which includes an ATP binding site and the client protein binding site; ii) an MD that is responsible for the hydrolysis of ATP to ADP; and iii) a CTD, which is comprised of a protein dimerization site and a calmodulin-binding site; there is also a linker domain connecting the NTD to the MD, contributing to the maintenance of the functions, interactions, and adaptability of the HSP90 chaperone (7-12). The NTD, which is also referred to as the nucleotide-binding site, is necessary for the affinity between client proteins and HSP90, and for the chaperone cycle, due to the presence of the binding site for ATP which are crucial for the HSP90 ATPase activity (11,21). Thus, the NTD is considered a critical target in the development of inhibitors. The CTD is primarily involved in the dimerization of HSP90. There is also an ATP binding site that only opens when the ATP-binding site in the NTD is unavailable, making the C-terminal an allosteric regulator of the N-terminal ATPase activity (22,23). There are special motifs, including MEEVD or KDEL on CTD, which differ according to the different isoform types and the cellular localization (24,25).

Dimerization is necessary for HSP90 to function properly in cancer cells (2). HSP90 ATPase activity and the cycling between the closed and open conformations is vital in this process (26). Despite the different locations in cells, all the isoforms of HSP90 act in a similar manner in the ATPase cycle (27). HSP90 exists as flexible homodimers, being predominantly present in an open V-shaped conformation with N-terminus separating, termed the 'open conformation'. In the open conformation, the binding sites in the N-terminus are available to client proteins. ATP translocates over the specific sites in the NTD and induces the N-terminus to attach to the corresponding domain of the other homodimer, leading to closure of the V-shaped conformation. The dimerized N-domains are associated with the M-domains, forming a twisted and compacted conformation, which is termed 'closed conformation' (28,29). In the closed conformation, client proteins are confined within the chaperone (30). Following ATP hydrolysis to ADP and PP_i and release from the binding pocket, the NTDs dissociate, and HSP90 returns to its open conformation (Fig. 1) (14,31).

The HSP90 family includes four isoforms that are present at different locations in a cell. HSP90α and HSP90β are present in the cytoplasm and the nucleus, GRP94 is present in the endoplasmic reticulum, and TRAP-1 is primarily located in the mitochondrion, also being present in the endoplasmic reticulum (1,14-16). HSP90α and HSP90β are the most widely expressed isoforms and are primarily located in the cytoplasm (32). They are involved in cell signaling, energy metabolism and cell viability (1). Additionally, extracellular-secreted HSP90α (eHSP90α) has been demonstrated to play crucial roles in the invasion and migration of several types of cancer *in vitro* and *in vivo*, suggesting that blocking eHSP90α is a rational approach for cancer management (33-36). GRP94 functions as a 'quality supervisor' of secreted proteins and membrane proteins. GRP94 recognizes and binds to misfolded proteins, attempting to correct them, preventing their degradation in the cytoplasm (15,37). The inhibition of GRP94 results in the accumulation of misfolded proteins, which translocate to the cytoplasm and are marked for degradation. TRAP-1 is necessary for mitochondrial homeostasis, and has been revealed to be involved in regulating the mitochondrial redox state and curbing energy metabolism (38-40). All four isoforms share a high degree of sequence similarity in the N-terminus, thus the ATP binding sites on the N-terminus are important targeting spots that researchers have focused on to disrupt molecular chaperone function (7,17).

HSP90 is a key mediator of >200 proteins, termed 'HSP90 client proteins'. HSP90 facilitates protein-protein interactions and is involved in cell signaling and the responses to stress (27,41). Cancer cells use this mechanism to protect the mutated and overexpressed oncoproteins from misfolding and being degraded to ensure their survival and proliferation (42-44). Several HSP90 client proteins participate in signaling and other vital pathways that are of utmost importance for malignancy (45). These client proteins include receptor tyrosine kinases (HER2, EGFR, IGF-1R and MET), signaling proteins (AKT and SRC), transcription factors (HIF-1 and TP53) and cell cycle regulatory proteins (CDK4 and CDK6) (46-49). There are several co-chaperones, including p50/Cdc37, HSP90-organizing protein (HOP/Sti1, p23, Aha1 and HSP70), and a variety of immunophilins that require HSP90 to function (46,50,51). The HSP90 chaperone mechanisms and HSP90-client interactions are involved in establishing the acquired capabilities of cancer cells by regulating HSP90-dependent signal transduction (Fig. 2) (52). Thus, HSP90 inhibitors can effectively suppress the tumor-promoting signaling pathways and interrupt the functions of HSP90 in tumor cells.

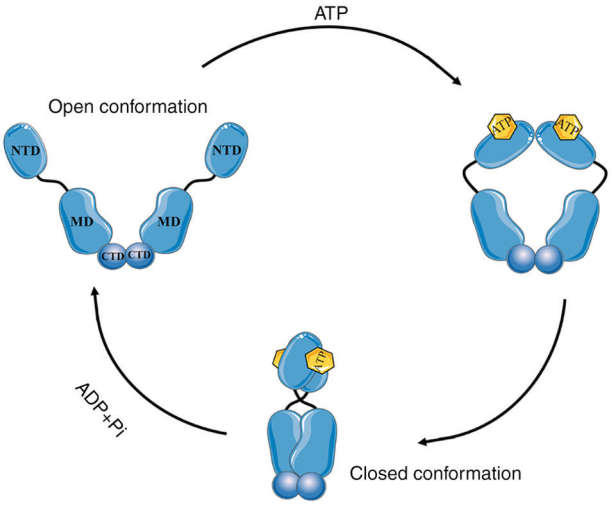

Figure 1. Cycling between the open conformation and closed conformation. HSP90 is predominantly an open V-shaped conformation with N-terminus separating. In open conformation, the binding sites on N-terminus are available to client proteins. ATP binds to specific sites in the NTD and induces the N-terminus to attach to the corresponding domain of the other homo-dimer, leading to the V-shaped conformation closed. Then N-domains are dimerized and associated with the M-domains, forming a twisted and compacted conformation. Following ATP hydrolysis to ADP and Pi is released from the binding pocket, the N-domains dissociate and HSP90 returns to its original open conformation. HSP90, heat shock protein 90; NTD, N-terminal domain; MD, middle domain; CTD, C-terminal domain.

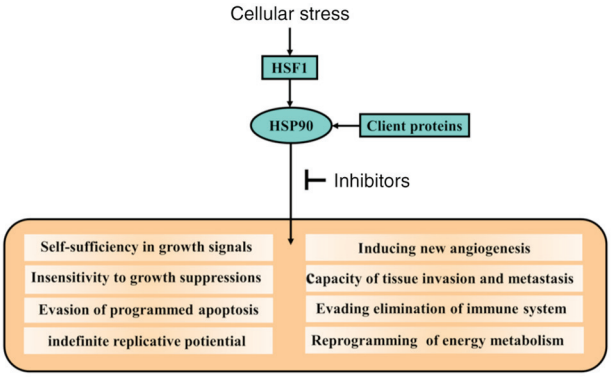

Figure 2. HSP90 is involved in establishing acquired capabilities of cancer cells. In cancer cells, HSF1 is released in response to cellular stress and subsequently upregulates the expression of HSP90. HSP90-client interactions triggers tumor-promoting signaling pathways which play essential roles in cancer cells. HSP90 inhibitors can suppresses all the signaling pathways concomitantly and interrupt the functions of HSP90 in cancer cells. HSP90, heat shock protein 90; HSF1, heat shock factor 1.

3. HSP90 as a druggable target in cancer therapy

HSP90 chaperone mechanisms in cancer cells differ considerably from those in normal cells (53). Firstly, in cancer cells, the rapid proliferation rate and reduced control in protein synthesis quality result in increased and constant cellular stress. In response to this situation, heat shock factor 1 (HSF1) is released and forms a trimer. The trimer translocates to the nucleus and upregulates HSP90 expression. Actually, increased levels of HSP90 improve the chances of cell survival and in maintaining function during tumorigenesis (3). Studies have demonstrated that the expression of HSP90 is 2- to 10-fold higher in cancer cells than in normal cells, and the overexpression of HSP90 has been found in numerous tumor cells including breast, lung, colorectal, ovarian, endometrial, esophageal, bone, urinary and prostate cancer (46,54-56). It has also been revealed that the high expression of HSP90 is associated with a poor prognosis in clinical treatment. HSP90 expression is a determining factor in the survival outcomes in hormone and protein kinase-dependent breast cancer (57). A previous study also revealed the association between a high level of HSP90 expression and a less favorable response to anti-neoplastic treatment (58). In another study, biostatisticians analyzed HSP90 expression levels from >4,000 breast cancer patients from 23 databases and overall survival data from >1,000 patients. It was concluded that upregulation of HSP90 and HSF1 was observed in breast cancer, resulting in a more aggressive profile of cancer and a poorer prognosis (59).

HSP90-client interactions and post-translational modifications also differ between tumor and healthy cells (4,5). Mutated oncoproteins expressed in cancer cells are involved in the uncontrolled growth and proliferation of cancer cells. The majority of these oncoproteins are also client proteins of HSP90 (60). Of note, >400 client proteins have been discovered to be involved in cell signaling by binding to HSP90 (6). Additionally, post-translational modifications regulate HSP90-client interactions (53). HSP90 inhibitors display a high affinity to HSP90 in tumor cells. Inhibitors can interrupt the function of HSP90 in cancer cells, while at the same dose, HSP90 is not inhibited in normal cells (53). The study by Kamal *et al* (61) revealed that HSP90 in cancer cells exists as a multichaperone complex. Complexes can promote the ATPase activity of HSP90 and promote the affinity of HSP90 to its inhibitor 100-fold. Moulick *et al* (62) also stated that there are biologically distinct HSP90 complexes present in tumor cells. Those HSP90 functions differ from its physiological roles and ensure tumor cell survival, by interacting with oncogenic proteins; however, the majority of HSP90 present in tumor cells is similar to that in normal cells. Notably, the higher the ATPase activity of HSP90 in tumors, the better the affinity is to HSP90 inhibitors, which is evidence supporting that transformation and malignancy do not just center around overexpression of HSP90 (61).

Cell surface HSP90 expression is considerably higher in tumor cells than in normal cells, highlighting HSP90 as a prospective target in anti-neoplastic treatment (57,63). To adapt to the reduced ATP-extracellular environment, extracellular HSP90 can function independently of ATP (64). Due to the consistent environmental stress in tumor cells, HSP90 is secreted consecutively, while HSP90 in healthy cells is secreted only under conditions of stress (65). A study revealed that blocking or neutralizing the secretion of HSP90 can inhibit the invasion and migration of cancer (35,66). A clinical study revealed that plasma concentrations of HSP90 are positively associated with tumor malignancy in cancer patients (33). The inhibition of extracellular HSP90 can be an attractive approach for preventing malignant tumor progression.

4. Old and new anti-neoplastic treatments targeting HSP90

HSP90 inhibitor monotherapies. Inhibitors targeting the N-terminal domain. Significant attention has been paid to

discovering inhibitors targeting the NTD of HSP90 in the last few decades (20). At present, there are 18 inhibitors of HSP90 that have been designed and have entered clinical trials (18,19). These inhibitors can be divided into five categories based on their chemical structures: i) natural products and their derivatives; ii) purine-based; iii) benzamide; iv) resorcinol-containing; and v) miscellaneous compounds (Table I).

Geldanamycin (GDA) was discovered as an HSP90 inhibitor in the early 1990s; its binding to the NTD interrupts ATPase activity and blocks the binding of the N-domain to client proteins, resulting in the degradation of oncoproteins and thus, in the abrogation of several cellular processes (67). It is worth mentioning that there is a reactive quinone in GDA which can also induce cell death independently of HSP90 inhibition (68). The toxicity and instability of GDA *in vivo* indicate that it has limited use in clinical trials (69). It has been demonstrated that the C-17 position of GDA is crucial concerning its function and toxicity. Based on this discovery, derivatives of GDA, including tanespimycin (17-allylamino-17-demethoxygeldanamycin; 17-AAG), retaspimycin hydrochloride (17-allylamino-demethoxygeldanamycin; 17-AAGH2; IPI-504), 17-amino-17-demethoxygeldanamycin (17-AG; IPI-493) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) were designed to correct the deficiency in HSP90-targeted treatment (67). 17-AAG has entered and has been evaluated in several phase II trials, where it has been demonstrated to exhibit a short-lived anti-HSP90 biological activity, and a poor solubility in water, resulting in poor bioavailability; these factors have limited the performance of further trials and other structural modifications (70-72). 17-AAGH2 was synthesized to improve the metabolic profile by eliminating the requirement for reduction (73). It is administered intravenously and is prepared by reducing the quinone portion of 17-AAG to hydroquinone using sodium hydrosulfite, forming hydrochloride salt (74). 17-AAGH2 monotherapy has exhibited good tolerance and antitumor activity in patients with gastrointestinal stromal tumors and in non-small cell lung cancer (NSCLC) (74,75). 17-AG (IPI-493) is a semi-synthetic benzoquinone ansamycin derivative, developed into an oral formulation that shows increased efficacy regarding HSP90 inhibition (76). 17-DMAG is a semi-synthetic derivative of geldanamycin. Compared with 17-AAG, 17-DMAG exhibits improved water solubility, bioavailability, reduced metabolism and antitumor efficacy (77). However, the majority of trials on this agent were postponed or terminated, and none of those derivatives entered phase III trials.

Purine-based inhibitors have a purine (or purine-like) scaffold with amine and aryl substituents. PU-3 is the first reported fully synthetic purine-based inhibitor of HSP90 (69). It displays antitumor efficacy by interrupting the proliferation and differentiation of breast cancer cells. Researchers designed derivatives by enhancing the purine chemical scaffold; in doing so, they synthesized new purine or purine-like compounds: BIIB021, BIIB028, MPC-3100, PU-H71 and Debio093 (78). BIIB021 alters the metabolic activity of GIST and improves the response rate in gastrointestinal stromal tumors. Additionally, it exhibits oral bioavailability and is well-tolerated in patients with no substantial hepatotoxicity observed. BIIB028 is an optimization of BIIB021, as the antitumor efficacy of BIIB021 was achieved with substantially high doses and the chemicals required for synthesis are toxic. Researchers found that the N-7 position on the purine scaffold is an optimal site for modification, selecting BIIB028 as the optimal candidate for a series of alkynol analogs. A clinical trial confirmed HSP90 inhibition and objective responses of BIIB028 in refractory metastatic or locally advanced solid tumors (79-82). MCP-3100 and Debio0932 also entered three trials but failed to move beyond phase II (69). PU-H71 has been reported to downregulate the expression of the oncoproteins involved in cell proliferation and cell apoptosis, whilst promoting the degradation of oncoproteins without exhibiting hematological, renal, or hepatic toxicity. These anticancer effects were also observed in triple-negative breast cancer cells, B-cell lymphomas and Ewing sarcoma. PU-H71 is now listed in six clinical trials (NCT03166085; NCT03373877; NCT01581541), and three of these (NCT03935555; NCT01393509; NCT01269593) are still active or recruiting, indicating that PU-H71 is the most promising purine-based inhibitor of HSP90 (83-85).

The only benzamide inhibitor, SNX-5422, was discovered in the pyrazole and was separated and purified using an ATP-affinity column. It has been investigated in multiple clinical trials demonstrating that SNX-5422 exhibited antiproliferative potency at low nanomolar concentrations and high oral bioavailability (86,87).

A resorcinol-containing inhibitor was identified using high-throughput screening (88). To optimize the solubility of this compound, researchers added substituents to it and synthesized the novel compound AUY922 (luminespib) (89), which demonstrated biological efficacy against tumor proliferation, invasion, and metastasis *in vivo* (90,91). STA-9090 (ganetespib) was synthesized by modifying the resorcinol scaffold. STA-9090 displayed improved performance in downregulating the expression of oncoproteins and the associated pathways than AUY922. STA-9090, having high biological activity and more optimal binding efficacy to HSP90, was discovered as part of a fragment-based drug design approach, combining NMR and X-ray crystallography (92). Notably, STA-9090 has entered nearly 40 trials and has thus far progressed to phase III trials, and it has been revealed to exhibit superior clinical potential regarding HSP90 inhibition (93). KW-2478 was discovered by Nakashima *et al* (94). The anti-proliferative and cell apoptosis-inducing effects were observed in KW-2478-treated tumor cells. KW-2478 interfered with the interactions between HSP90 and client proteins, such as fibroblast growth factor receptor3, proto-oncogene c-Maf, and cyclin D1, and depleted the transcriptional kinase Cdk9 and the translational inhibitor phosphorylated 4E-BP1 (94-96). KW-2478 was found to be well tolerated and displayed no dose-limiting toxicity in clinical trials (97).

Other HSP90 inhibitors have been identified, targeting the NTD. However, they are not classified into any of the four categories discussed above or do not present with publicly available structures. These compounds include FW-04-806, CH5164840 and XL888. FW-04-806 interferes with the interaction between HSP90 and Cdc37, disassociating the HSP90-Cdc37 complex compound and promoting the degradation of the associated client proteins (98). A decrease in cell viability and proliferation and an increase in programmed cell death were observed in HER2-positive breast cancer cells treated with FW-04-806 (98,99). CH5164840 is a macrocyclic

Table I. Inhibitors targeting the N-terminal domain.

Category	Inhibitor	Type	Clinical trial phase	Trial numbers
Natural products and their derivatives	GDA	1,4-Benzoquinone ansamycin antibiotic	N/A	N/A
	17-AAG	17-N-allylamino17-dimethoxygeldanamycin	II	NCT00117988; NCT00118248; NCT00118092
	17-AAGH2	Reduced quinone form of tanespimycin	II	NCT00276302; NCT00564928; NCT00431015
	17-AG	17-Amino-17-demethoxygel-danamycin	I	NCT00724425
	17-DMAG	17-Dimethylamino-17-dimthoxy-geldanamycin	II	NCT00088868
Purine-based	PU-3	Purine scaffold inhibitor	N/A	N/A
	BIIB021	2-Amino-6-halopurine purine-scaffold agent	II	NCT01004081
	BIIB028	Purine-scaffold agent	I	NCT00725933
	MPC-3100	3-N-carbon-purine	I	NCT00920205
	PU-H71	Purine-scaffold agent	I	NCT01393509; NCT03935555; NCT01269593
	Debio093	Purine-scaffold derivative	II	NCT01168752
Benzamide	SNX-5422	Pyrazole-contain benzamide	II	NCT01851096; NCT01635712
Resorcinol-containing	AUY922	Resorcinylic isoxazole amide	II	NCT01854034; NCT01922583; NCT01752400
	STA-9090	Resorcinolic triazolone	III	NCT01798485; NCT01348126
	KW-2478	Resorsinol containing synthetic agent	II	NCT01063907
Miscellaneous	FW-04-806	Bis-oxazolyl macrolide compound	N/A	N/A
	CH5164840	Macrocyclic 2-amino-6-arylpyrimidine	N/A	N/A
	XL888	Tropane-derived agent	I	NCT00796484

N/A, not available.

compound bearing a 2-amino-6-arylpyrimidine moiety; it has been shown to exhibit high oral bioavailability in mice (F=70.8%) and potential antitumor efficacy in colorectal cancer (CRC) (100). XL888 was designed by modifying 5-position amine substituent on the 4-carboxamido-2-methylbenzamide, resulting in the reduction of HSP90 client protein expression *in vitro*, and in presenting with antitumor effects *in vivo* (101).

Inhibitors targeting the CTD. There are three domains in HSP90, the C-terminus, N-terminus, and the middle domain. As discussed above, there is more research focus on the NTD domain of HSP90. Several compounds have been demonstrated as potential candidates for HSP90 inhibitors that target NTD. While HSF1 has been demonstrated to be a key factor of resistance to N-terminal inhibitors, it binds to HSP90 and forms homotrimers after N-terminal inhibition, thus inducing the pro-survival heat shock response (102). To overcome this 'resistance machinery', inhibitors targeting the CTD have been investigated. NTD inhibitors block HSP90 activity and several have entered clinical trials; however, there is also an ATP binding region in CTD (103-105). This indicates that both the C- and N-terminal regions function as co-chaperones and client proteins and subsequently regulate the associated biological activities; however, the mechanisms involved differ notably (106). N-terminus inhibitors competitively bind to ATP binding sites to abrogate ATPase activity, whereas C-terminus inhibitors disrupt the activities of co-chaperones containing TPR motifs, resulting in aberrant chaperone function. C-terminus inhibitors are emerging candidates for the development of novel cancer chemotherapeutics (27,107,108). Novobiocin is an inhibitor of the DNA gyrase ATP-binding site, and is able to interrupt HSP90 protein folding machinery,

leading to the hydrolysis of client proteins and the induction of the apoptosis of cancer cells. Novobiocin can selectively restrain the open-conformation HSP90 and block the progression of the open and close conformation cycle through a cascade of cumulative dynamic changes (105,107,109,110). KU-32 and KU-569 are derivatives of novobiocin. They bind to the C-terminus and lead to a structural shift in the chaperone, which can simultaneously enhance ATP binding and promote ATPase activity (109,111,112). LB76 is the first C-terminus inhibitor designed *de novo*, by interrupting co-chaperone binding. LB76 is derived from an HSP90 co-chaperone and selectively pulls down HSP90 from cell lysates. Further investigation confirmed that the identity of the binding region was a MEEVD motif in the C-terminus. LB76 restrains the protein-folding function of HSP90, thus blocking protein-protein interactions between HSP90 and co-chaperones (113,114). A *de novo* delivery system for LB76 produced by polymer nanoparticles and functioning by delivering LB76 into cells and releasing them in a pH-responsive manner, displayed improved inhibitory activity (115). The non-toxic profile and significant HSP90 inhibitory activity make LB70 a promising cancer-targeting candidate therapeutic. KU711 and KU757 target the C-domain of HSP90 in tumor-initiating cells that have stem cell-like properties. They exhibit anti-malignant potential in breast cancer stem cells and head and neck squamous cell carcinoma cancer stem cells by inhibiting invasion, EMT, and self-renewal (116,117). KU711 has been demonstrated to selectively inhibit HSP90 function in thyroid cancer stem cells and induce a potent antitumor response to cell growth, invasion, and migration (118). KU135, compared with the N-terminal inhibitor 17-AAG, displayed more potent anti-proliferative effects in human leukemic cells and most melanoma cells. 17-AAG functions by inducing cell cycle arrest whereas KU135 induces cell cycle arrest and cell apoptosis. The dual effects on tumor cells are in line with the expected effects of antitumor agents (119,120). KU363 was synthesized at the University of Kansas-Lawrence, and KU135 exhibited antiproliferative effects in different cancer models of bronchioalveolar carcinoma and epithelial lung carcinoma (121). It was demonstrated to induce cell apoptosis, and limit cell viability and proliferation in head and neck squamous cell carcinoma cells *in vitro* and *in vivo* (122). NCT-50 is a C-terminal-targeting hybrid compound of novobiocin and deguelin. It demonstrated a significant effect on inhibiting the viability, colony-forming ability, and angiogenic ability of NSCLC cells. NCT-50 overcomes the neurotoxicity observed with deguelin and displays more significant pro-apoptotic efficacy on NSCLC cells than either deguelin or novobiocin (123).

Isoform-selective inhibitors. As mentioned above, the mammalian HSP90 family is comprised of four isoforms, HSP90α, HSP90β, GRP94 and TRAP1. Among these, HSP90α and HSP90β are the most abundantly expressed isoforms and are primarily located in the cytoplasm (32). The extracellular form of HSP90α is key for cancer cell invasion and migration (124). GRP94 is present in the endoplasmic reticulum, and functions in 'quality control' of a small subset of proteins. It recognizes and binds to misfolded proteins, correcting, and refolding said proteins. GRP94 inhibitors block this process, resulting in the translocation of these misfolded proteins to the cytoplasm and hence their subsequent degradation (37). In addition, there are Ca^{2+} binding sites on GRP94 which play vital roles in tumor processes (125). TRAP1, located in the mitochondria, plays a crucial role in mitochondrial homeostasis. It is involved in the regulation of the organelle's redox state and in the disruption to the energy metabolism of cancer cells (1,38). Developing isoform-selective inhibitors allows for the alleviation of the challenges faced with the use of 'pan-inhibitors', permitting more targeted and personalized therapies.

HSP90α and HSP90β. For cancer cells, HSP90α- and HSP90β-selective inhibitors are optimal choices considering that they are the most abundantly expressed isoforms and they function by interacting with oncogenic client proteins (1,32). SNX-0723 is a promising candidate, due to its central nervous system permeability and selectivity to HSP90α and HSP90β both. The selectivity of SNX-0723 to cytosolic HSP90 isoforms is ~100-fold vs. GRP94 and ~300-fold vs. TRAP1. Additionally, its affinity to both HSP90α and HSP90β is similar (126,127). Previously, researchers identified a benzolactam-hydroindolone derivative of SNX-0723 that contains a cyclopentyl substituent, compound 31, that exhibited similar pharmacokinetics as SNX-0723, although with a reduced cellular toxicity and ~1,000-fold affinity for HSP90α and HSP90β vs. GRP94 and TRAP1 (128). TAS-116 was the first reported cytosolic-isoform selective inhibitor to enter clinical trials. In human xenograft mouse models, TAS-116 was found to induce HSP90 client protein degradation and reduce tumor burden (129). In another study, a total of 61 patients with advanced solid tumors participated in a clinical trial to identify the safety, maximum tolerated dose, and overall response rates of TAS-116 in monotherapy intervention (130). TAS-116 exhibited antitumor activity with acceptable adverse reactions was acceptable, suggesting further development of this HSP90 inhibitor.

GRP94. TAS-116 had an acceptable safety profile with notable antitumor activity, supporting the further development of this HSP90 inhibitor. GRP94-selective inhibitors were the first isoform-selective inhibitors being of utmost scientific interest. Using an assay to determine the differences in the N-terminal ATP-binding site between GRP94 and HSP90, it was eventually revealed that GRP94 could be selectively targeted (131). The GRP94-selective inhibitor, BnIm, was developed by replacing the cis-amide with a bioisosteric imidazole ring that mimicked the amide heteroatoms. No degradation of HSP90α/HSP90β client proteins was observed in BnIm-treated cancer cells, indicating its selectivity towards GRP94 vs. the cytosolic isoforms. Additionally, no cytotoxic effects were observed (132). In addition, researchers established the structure-activity relationships of the BnIm scaffold, and further enhancements were made by replacing the imidazole ring with other heterocycles and by modifying the benzyl appendage. This second generation of compounds demonstrated a two-fold higher affinity than BnIm and a 32-fold higher selectivity for GRP94 than HSP90α (133). PU-WS13 is a purine derivative that disrupts the cell surface oncoprotein HER2. This compound substantially decreases HER2 levels in overexpressing HER2 breast cancer cells (134). Compound 54 has a phenyl ring at the meta-position, an isopropyl appendage at the fourth carbon of the benzene ring, and a cyclohexanol with an amine linker at the ortho-position of the benzamide

scaffold, exhibiting >1,000-fold affinity to GRP94 than to HSP90α (135).

TRAP1. Pan-inhibitors of HSP90 have demonstrated negative efficacy with regard to its TRAP1 inhibitory effect due to its poor mitochondrial permeability, where TRAP1 is located. Shepherdin is the first peptidomimetic with the ability to permeate into the mitochondria and target TRAP1 (136). There is a highly positively charged moiety at the N-terminus of shepherdin which can interrupt mitochondrial integrity, making it swell and subsequently release cytochrome c (137). For mitochondrial penetration, investigators used the mitochondrial permeating TPP moiety to replace the corresponding ammonium group on PU-H71 to develop the novel new compound SMTIN-P01. SMTIN-P01 induced membrane depolarization and cytochrome c release, resulting in cytotoxicity to cancer cells (138). DN401 is the most selective TRAP1 inhibitor developed to date. Its selectivity for TRAP1 is >9-fold compared with the other isoforms. It was developed by modifying the pyridine ring to a pyrazolopyrimidine scaffold with a pyridinyl appendage (139).

HSP90 combination therapies. To date, although a few HSP90 inhibitors have been produced that demonstrate selective affinity to HSP90 and disrupt its biological activities, no HSP90 inhibitor has been applied in clinical cancer therapies, which indicates that the full prospects of HSP90 inhibitors have not been administered as a monotherapy in cancer treatment. The inhibitors that have entered clinical trials have had to be postponed or terminated due to their moderate effects. More importantly, resistance to HSP90 inhibitors has been demonstrated as the major reason for their limited effects as a monotherapy (140). HSF1 is a crucial factor for the underlying resistance to NTD inhibitors. It forms homotrimers after binding to the inhibited HSP90 and induces the pro-survival heat shock response (102). Additionally, the overexpression of the multidrug resistance efflux pump P-glycoprotein 1 overcomes the anticancer effects of benzoquinone-based HSP90 inhibitors (141). The overexpression of UDP glucuronosyltransferase 1A results in resistance to resorcinol-based HSP90 inhibitors (142). Based on these findings, combination therapy strategies have been investigated.

HSP90 inhibitors combined with chemotherapy. A previous study revealed that CRC cells treated with the resorcinol-containing inhibitor, AUY-922, exhibited a higher sensitivity to 5-FU-based chemotherapy both *in vitro* and *in vivo*, which supports the combined use of AUY-922 with 5-FU as a feasible therapeutic strategy (141). A phase I clinical study testing the efficacy of the combination therapy AUY-922 and capecitabine demonstrated that in 19 patients with advanced CRC, 63% of these patients demonstrated a partial response or stable disease when treated with the combination therapy (143). The administration of AUY922 in combination with doxorubicin resulted in increased levels of caspase-3 expression, a biomarker of mitochondrial apoptosis, as well as decreased levels of VEGF mRNA, an effect that was not observed in monotherapy treatments (144,145). The combination of irinotecan and 17-AAG was also assessed in a phase I study in patients with solid tumors. Of the 27 patients, a decrease in tumor volume was observed in 6 patients, 5 of 10 patients with p53-mutant tumor had stable disease and 2 of 6 patients with p53-wild-type tumor presented with stable disease (146). Additionally, the multifunctional nanoceria platform loaded with both doxorubicin and STA-9090 as a combination therapy was previously reported in NSCLC. The results reported >80% of NSCLC cell death levels within 48 h *in vitro*. STA-9090 synergizes with and enhances the therapeutic efficacy of doxorubicin, minimizing the potential cardiotoxicity of doxorubicin via reactive oxygen species (ROS) production. Furthermore, apoptosis, necrosis and migration assays supported the negative effects of STA-9090 on the proliferation and migration of cancer cells (147).

HSP90 inhibitors combined with targeted therapy. CH5164840 combined with the EGFR inhibitor, erlotinib, has demonstrated improved antitumor effects in EGFR-overexpressing xenograft models. Additionally, ERK signaling was suppressed by the combined application of erlotinib and CH5164840 *in vivo* (148). 17-AAG has been demonstrated as a positive factor for prognosis in breast cancer cells treated with the VEGF inhibitor, bevacizumab, in a preclinical study (149). The combination of AUY922 and erlotinib was tested in phase I trials that consisted of 18 patients with EGFR-mutant lung cancer. Partial responses were observed; however, the toxic effects were a major limiting factor (150). A combination of STA-9090 and Ziv-Aflibercept, an antiangiogenic agent, was evaluated in patients with advanced adenocarcinoma (three colon adenocarcinomas, one small bowel adenocarcinoma, and one rectal adenocarcinoma). Of 5 patients, 3 achieved stable disease, although dose-related toxicity was observed (151). 17-DMAG in combination with the anti-EGFR agent lapatinib overcame the acquired lapatinib resistance in an ER-positive HER2-overexpressing breast cancer cell line. Suppression of cell proliferation and HSP90 expression was observed in monotherapy and combination therapy, with the latter demonstrating a comparably increased synergistic effect (152). 17-AAG in combination with trastuzumab in HER2-positive breast cancer did not demonstrate suitable antitumor efficacy, whereas the combination therapy demonstrated antitumor efficacy in ALK-mutated lung cancers (50,153). 3-methyladenine (3-MA) is an autophagy inhibitor that selectively inhibits the P13K signaling pathway. The effect of combining 17-AAG with 3-MA evaluated in preclinical trials revealed that the combination therapy resulted in a notable increase in apoptosis and a lower level of autophagy vs. monotherapy (154). The HSP90 molecular chaperone mechanism regulates the Raf kinase signaling pathway; thus, the inhibition of HSP90 affects the Raf kinase signaling pathway. Based on this mechanism, a combination of 17-AAG and Raf kinase inhibitor sorafenib was evaluated in a phase I trial. Antitumor efficacy was observed in 9 out of 12 renal cancer patients and 4 out of 6 melanoma patients (155). A phase Ib trial of TAS-116 combined with nivolumab investigated the tumor response and corresponding adverse response at the same dose in CRC and other solid tumors patients. Positive tumor responses were observed and the optimum concentration for safety profiles and antitumor activity of TAS-116 was 160 mg (156). FW-04-806 is reported to promote the antitumor efficacy of lapatinib in inhibiting cell proliferation and inducing cell apoptosis, and in particular, in reducing HER3 levels which were increased by lapatinib to inhibit HER2. The results highlighted the potential of this combination therapy for

HER2-positive breast cancer (99). Bortezomib, a proteasome inhibitor, has demonstrated improved anticancer effects when combined with IPI-504, KW-2478 and PU-H71. The HSP90 inhibitors can overcome intrinsic and acquired resistance to the proteasome inhibitor in mantle cell lymphoma, multiple myeloma and Ewing sarcoma. In addition, the synergistic effect of HSP90 inhibitors and bortezomib is likely to reduce the toxic effects of HSP90 inhibitors, due to a reduction in the required doses (85,95,157,158).

HSP90 inhibitors combined with immunotherapy. In melanoma cells, HSP90 inhibitors have been demonstrated to be suitable candidates for increasing the sensitivity of tumor cells to T-cells out of a list of 850 bioactive drugs. In addition, the inhibition of HSP90 enhances the antitumor effects of anti-CTLA4 and anti-PD-1 therapy *in vivo* (159). Furthermore, several HSP90 clients such as mutated EGFR, rearranged ALK, HIF-1α and JAK2 have been revealed to play essential roles in regulating immune checkpoint blockade by promoting PD1 and PD-L1 expression (160). These results suggested that HSP90 inhibitors can be a complementary strategy to immune checkpoint blockade for cancer therapy. In a preclinical study, combining STA-9090 and the anti-PD-L1 antibody STI-A1015 demonstrated better efficacy in colon carcinoma and melanoma *in vivo* than monotherapies (160). The combination of 17-DMAG and agonists of EphA2 has also been found to improve the recruitment of therapeutic T-cells by reconditioning the tumor microenvironment, leading to an increase in antigen presentation and tumor cell recognition (161).

In addition to the three classifications of combination therapy discussed above, efforts have been made in de novo therapeutic strategies. Radiotherapy is one of the most frequently used cancer treatments. HSP90 client proteins, such as BRCA1, BRCA2, CHK1, DNA-PKcs, ATM, FANCA and the MRE11/RAD50/NBN complex are involved in DNA damage response pathways and have become a major cause of resistance to radiotherapy. Based on the preclinical study that demonstrated the favorable effect of AT13387 in combination with radiotherapy *in vitro* (162), an *in vivo* study was performed on mice and positive results were obtained: AT13387 increased sensitivity to radiotherapy, enhanced apoptosis, attenuation of migratory capacity, and a reduced DNA damage response were observed (163). A functional antioxidant nanomedicine composed of nanoceria encapsulated with a two-drug cocktail of lactonic sophorolipids, a constituent of natural sophorolipid known to inhibit histone deacetylase activity, and the HSP90 inhibitor, STA9090, were evaluated in NSCLC. The combination resulted in a marked reduction of cell viability and suppression of cell migration; nanoceria without any encapsulated drugs did not display any additional toxic burden (164). A novel multifunctional nano-platform for targeted delivery of heat, ROS, and 17AAG/17DMAG simultaneously was proposed for prostate cancer treatment (165). The common adverse effects of geldanamycin derivatives are hepatoxicity, renal failure, and gastrointestinal toxicities. Moreover, poor water solubility is a major limiting factor for its clinical use. In that study, nano-platforms were formulated to allow targeted delivery of HSP90 inhibitors, thus improving therapeutic efficacy whilst minimizing their off-site toxic effects (165).

Emerging anti-neoplastic strategies targeting HSP90. An abundance of HSP90 inhibitors have been discovered over the past decades, with certain inhibitors demonstrating excellent antitumor efficacy both *in vitro* and *in vivo* and entered clinical trials. However, limited clinical effects and insurmountable toxicity have forced these trials to be postponed or terminated. Phage display technology is the most commonly used and robust *in vitro* method to select specific peptides or antibodies against almost any antigen (166). Phage technology screens out the peptides required from a complex mixture pool of billions of displayed peptides on phages in a combinatorial library via the high affinity of peptides to phages with a specific target (167). Peptides that are applied as a targeting tool in cancer treatment may demonstrate advantages in high affinity, favorable absorbability, endogenous degradability and ease of synthesis, with fewer adverse reactions, improved safety profiles, and ease of modifications with a variety of linker chemistries (168,169). Peptide scFv47 was screened from commercially available Tomlinson I and J phage display libraries and was characterized as an HSP90α-selective binder. Experiments *in vitro* revealed the ability of scFv47 to bind specifically to HSP90α and inhibit ATPase in human breast cancer cells (170). In a recent study, a potentially druggable peptide with strongly selective binding to the N-terminal of HSP90 was screened using a T7-phage display system from an undisclosed-cryptand. This peptide was demonstrated to exhibit strong antibody-like affinity (KD, 62 nM) to the N-terminal of HSP90 driven by enthalpy and demonstrated HSP90-inhibitory biological activity by binding to the ATPase site in the NTD. Notably, it is the first reported strong NTD-specific homing peptide against HSP90 screened using a T7-phage display system from the library of an undisclosed-cryptand36 with two lariat arms (171). It is hypothesized that the HSP90-homing peptide obtained for target recognition is not the final achievement, these homing peptides may form the basis of novel antibody-based HSP90 targeted strategies for anticancer treatment.

5. Conclusions and future perspectives

Over the past few decades, several HSP90 inhibitors have been developed and entered clinical trials. Thus far, all the HSP90 inhibitors that have entered clinical trials target the NTD. However, toxicity and poor bioavailability prevent NTD-targeting inhibitors from being used clinically (20). Resistance to NTD inhibitors has been demonstrated to be another major contributor to the poor effects of NTD inhibitors (102). Studies have revealed the presence of ATP binding sites on CTD; this allows co-chaperones and unfolded client proteins to bind to either the CTD or the NTD (103,105). A deeper understanding of the four different isoforms provides a novel direction for the development of HSP90 inhibitors, allowing progression from the development of 'pan-inhibitors' and instead developing more specific treatments. Isoform-specific inhibitors can achieve antitumor effects with reduced toxicity compared with pan-inhibition. Targeting a specific isoform may be of additional value in each disease state, as compared to pan-inhibition. Furthermore, the results of clinical and preclinical investigations suggested that HSP90 inhibitors can enhance the efficacy of other anti-neoplastic treatments, including chemotherapies, targeted

agents, immunotherapy, and radiotherapy. Herein, the potential therapeutic strategies involving HSP90 targeting for the management of cancer were discussed. Certain peptide inhibitors that target HSP90 have been screened using phage display technology and revealed to exhibit high affinity to HSP90 *in vitro* and *in vivo*. Although additional studies are required before HSP90-targeting peptide drugs can be developed, novel antibody-based HSP90-targeting strategies based on these targeting peptides are prospective approaches for future cancer treatments.

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Competing interests

The authors declare that they have no competing interests.

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