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New developments in Hsp90 inhibitors as anti-cancer therapeutics: mechanisms, clinical perspective and more potential

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Abstract

The molecular chaperone Hsp90 (heat shock protein 90) is a promising target in cancer therapy. Preclinical and clinical evaluations of a variety of Hsp90 inhibitors have shown antitumor effect as a single agent and in combination with chemotherapy. Current Hsp90 inhibitors are categorized into several classes based on distinct modes of inhibition, including i) blockade of ATP binding, ii) disruption of cochaperone/Hsp90 interactions, iii) antagonism of client/Hsp90 associations and iv) interference with post-translational modifications of Hsp90. The different functions of Hsp90 isoforms and the *isoform* selectivity of drugs need further investigation. The correlation of *cell surface* Hsp90 with cancer metastasis and the emerging involvement of Hsp90 inhibition in cancer stem cells have become exciting areas that could be exploited. Therefore, the aim of this review is (1) to summarize the up-to-date knowledge of mechanistic studies and clinical prospect of currently available Hsp90 inhibitors, (2) to enhance our perspectives for designing and discovering novel Hsp90 inhibitors, and (3) to provide an insight into less-understood potential of Hsp90 inhibition in cancer therapy.

Keywords

Hsp90; inhibitor; cancer therapy; client protein; cochaperone; hsp70; cdc37; 17-AAG; tanespimycin; derrubone; shepherdin; tubocapsenolide A; celastrol

1. Introduction

1.1. Why Hsp90?

The molecular chaperone Hsp90 (heat shock protein 90) was initially identified as one of the highly conserved heat shock proteins involved in the stress response (Ritossa, 1996; Westerheide and Morimoto, 2005). Hsp90 is a highly abundant protein, constituting about 1–2% of total proteins under non-stress conditions in most tissues (Welch, 1991; Welch and Feramisco, 1982). It possesses common molecular chaperone functions, i.e., assisting protein

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folding and preventing aggregation of non-native proteins (Wiech et al., 1992). Over the past several years, the application of global analysis has extended Hsp90 clientele to more than 200 proteins, covering almost all the cellular processes (Falsone et al., 2005; McClellan et al., 2007; Zhao et al., 2005). For instance, these client proteins include transmembrane tyrosine kinases (Her-2, EGFR), metastable signaling proteins (Akt, Raf-1 and IKK), mutated signaling proteins (p53, v-Src), chimeric signaling proteins (Bcr-Abl), cell cycle regulators (Cdk4, Cdk6), and steroid receptors (androgen, estrogen, and progesterone receptors) (Kamal et al., 2004). Many of these client proteins are mutated and/or overexpressed in cancers (Pearl et al., 2008) (Didelot et al., 2007).

Because early studies have shown that the function of cytoplasmic Hsp90 is essential for *normal* cell viability and growth (Borkovich et al., 1989), it was difficult, if not impossible, to consider Hsp90 as a potential therapeutic target. However, since geldanamycin (GA) was demonstrated to possess potent anti-cancer effects through inhibiting Hsp90 (Supko et al., 1995; Whitesell et al., 1994), a great deal of efforts have been devoted to this area and a diversity of Hsp90 inhibitors have either been identified or synthesized (Schulte, 1998; Whitesell et al., 1994; Cheung et al., 2005).

The feasibility of targeting Hsp90 for cancer therapy is well supported:

- **i.** First, Hsp90 is involved in the maturation and stabilization of a wide range of *oncogenic* client proteins crucial for oncogenesis and malignant progression (Kamal et al., 2004; Powers and Workman, 2007; Whitesell and Lindquist, 2005), making cancer cells particularly dependent on proper Hsp90 function (Chiosis and Neckers, 2006).
- **ii.** The harsh environmental conditions found in tumors such as hypoxia, low pH, and bad nutritional status may tend to destabilize proteins, making them even more dependent on Hsp90 activity (Solit and Chiosis, 2008). The extraordinary reliance of tumor cells on Hsp90 is consistent with a report that Hsp90 comprises as much as 4–6% of total proteins in tumor cells in contrast with the 1–2% in normal cells (Chiosis and Neckers, 2006).
- **iii.** Another explanation for tumor selectivity of Hsp90 inhibitors comes from the observation that in cancer cells Hsp90 predominantly exists as *multi*-chaperone complex with unusually high affinity for ATP and drug, whereas in normal cells most Hsp90 is present in an uncomplexed or latent state (Chiosis and Neckers, 2006). Hsp90 derived from tumor cells has an approximately 100-fold higher binding affinity for 17-AAG than does Hsp90 isolated from normal cells (Kamal et al., 2003).
- **iv.** Finally, tumor-specific accumulation has been observed for a number of Hsp90 inhibitors, such as 17-AAG, 17-DMAG, IPI-504, radicicol derivatives and purine-scaffold inhibitors (Chiosis and Neckers, 2006). Thus, this selectivity may not be due to the structural or physicochemical properties of a specific class of compounds, but rather to properties of Hsp90 itself (Solit and Chiosis, 2008).

Although the mechanisms underlying the tumor selectivity of Hsp90 inhibitors are not fully understood, Hsp90 has become validated as a potential target in cancer therapy. Furthermore, preclinical and clinical evaluation of a plethora of Hsp90 inhibitors have already shown promising results as a single agent and/or in combination with chemotherapy (Solit and Chiosis, 2008).

1.2. How does Hsp90 work?

Based on the crystal structures of yeast Hsp90 (Ali et al., 2006) and Grp94 (Hsp90 isoform in mammalian endoplasmic reticulum) (Dollins et al., 2007), Hsp90 exists as a homodimer, each

monomer consisting of three highly conserved domains: an N-terminal ATP-binding domain (25 kDa), a middle domain (35 kDa) and a C-terminal dimerization domain (12 kDa) (Pearl and Prodromou, 2006). In eukaryotes, the N-terminal and middle domains are connected by a charged linker (Terasawa et al., 2005). The N-terminus of Hsp90 contains a specific ATP binding pocket (Dutta and Inouye, 2000). The major role of the middle domain is to discriminate various types of client proteins to adjust the molecular chaperone for proper substrate activation (Hawle et al., 2006). The C-terminal dimerization domain strengthens the weak association between the two N-terminal domains of the Hsp90 dimer (Terasawa et al., 2005). The C-terminal domain of eukaryotic Hsp90 has a conserved pentapeptide (MEEVD) implicated in binding to the tetratricopeptide repeat (TPR) domain of cochaperones, such as Hop (Hsp organizing protein) and Sti1 (stress-inducible protein 1, yeast homologue of Hop) (Pearl et al., 2008; Terasawa et al., 2005).

The “open” state of the Hsp90 dimer, with its two N-termini separated, can capture client proteins (Richter et al., 2008). ATP binding triggers the closure of the ATP pocket “lid” and brings the N-termini close to each other, resulting in the formation of a compacted, ring-shaped Hsp90 dimer (Richter et al., 2008; Wandinger et al., 2008). These conformational alterations lead to a “closed” state to “clamp” client proteins inside (Terasawa et al., 2005). The ATPase activity of Hsp90 itself drives the chaperone cycle (Kamal et al., 2004).

The Hsp90 multi-chaperone system has been extensively studied in the maturation of steroid receptor on yeast Hsp90 (Scheibel and Buchner, 1998). The chaperone cycle begins with a newly synthesized or misfolded steroid receptor binding to Hsp70/Hsp40 complex, associated with the “open” state Hsp90 via the bridging cochaperone Hop that interacts simultaneously with Hsp90 and Hsp70 (Pearl et al., 2008). Hop not only binds to the C-terminal MEEVD motif of Hsp90, but also connect with the N-terminal region of Hsp90, preventing the Hsp90 N-terminal domain association. Hop therefore inhibits the ATPase activity and promotes client transfer from Hsp70 to Hsp90 (Prodromou et al., 1999; Richter et al., 2003; Terasawa et al., 2005). Upon ATP binding to Hsp90, Hop is replaced by p23 and immunophilins, converting the intermediate chaperone complex into the mature complex (Neckers, 2003). Another cochaperone, Aha1 (activatior of Hsp90 ATPase), associates with the middle domain of Hsp90, facilitating conformational adjustments to favor ATP binding (Meyer et al., 2004). Both Aha1 and immunophilins stimulate the ATPase activity of Hsp90 (McLaughlin et al., 2002; Meyer et al., 2004; Panaretou et al., 2002). Upon ATP hydrolysis, the correctly-folded client protein is released from Hsp90 (Terasawa et al., 2005). Recent studies have shown that the mechanistic basis of the Hsp90 chaperone cycle are conserved in yeast and human, although a slower turnover rate was observed with human Hsp90 (Richter et al., 2008).

1.3. Hsp90, cdc37 and protein kinase clients

Protein kinases are the largest class of Hsp90 clients, with a similar chaperone cycle (Caplan et al., 2007; Pearl, 2005; Terasawa et al., 2005). However, minor differences are noticed with the cochaperones involved (Caplan et al., 2007). Cdc37 was originally discovered in yeast as an essential cell cycle protein (Reed, 1980), and later it was proved to be a kinase-specific cochaperone of Hsp90 (Pearl, 2005). The Hsp70/Hsp40 complex first prepares a newly synthesized or misfolded protein kinase for interaction with the N-terminal domain of Cdc37, followed by recruitment of Hsp90 to the complex with the help of Hop (Arlander et al., 2006; Mandal et al., 2007). The C-terminal side chain of Cdc37 associates with the “lid” of Hsp90, which closes the N-terminal ATP binding pocket (Roe et al., 2004). Crystallographic studies revealed that the insertion of Cdc37 C-terminus to the Hsp90 N-terminal ATP pocket inhibits the ATPase activity of Hsp90 and prevents its N-terminal dimerization (Siligardi et al., 2002). This holds Hsp90 in an “open” conformation in the intermediate complex, allowing later client loading (Roe et al., 2004). Although the release of Cdc37 C-terminus from Hsp90

N-terminal clamp is required for the transition of the "open" to the "closed", mature conformation, Cdc37 could stay in the complex by interacting with the client protein (Roe et al., 2004). Other cochaperones, such as p23 and Aha1, may be required as well (Wandinger et al., 2008). More details of kinase maturation in the complex remain to be understood.

2. Hsp90 inhibitors targeting the ATP Binding Site

2.1. Natural compounds and their derivatives

Benzoquinone ansamycins, represented by geldanamycin (Figure 1) (Workman et al., 2007), were the first class of natural Hsp90 inhibitors to be discovered and substantially studied. Geldanamycin, a natural occurring antibiotic, was originally isolated from Streptomyces hygroscopicus as early as 1970s (Messaoudi et al., 2008). Structural and biochemical studies demonstrated that GA is a competitive inhibitor of ATP binding to Hsp90 (Roe et al., 1999). Binding of GA in the N-terminal ATP pocket restrains Hsp90 in its ADP-bound conformation and prevents the subsequent "clamping" of Hsp90 around a client protein (Blagg and Kerr, 2006;Neckers, 2006), resulting in ubiquitination and proteasomal degradation of the client (Mimnaugh et al., 1996). This N-terminal ATP pocket has distinctive characteristics in comparison with most other nucleotide-binding proteins (Prodromou et al., 1997), which explains the selectivity of GA.

Although GA exhibited potent anti-cancer activities in preclinical in vivo studies, it was determined to have little clinical potential mostly due to the high hepatotoxicity observed in animal models (Neckers et al., 1999). As a result, this has encouraged the search for GA derivatives that maintain similar anti-cancer activities but with better toxicological properties. 17-AAG (17-allylamino-17-desmethoxygeldanamycin; tanespimycin, KOS-953) (Fig.1) (Solit et al., 2007; Workman et al., 2007), and more recently 17-DMAG (17-dimethylaminoethylamino-17-demethoxygeldanamycin) (Fig.1) (Smith et al., 2005; Workman et al., 2007) and IPI-504 (17-allylamino-17-demethoxygeldanamycin hydroquinone hydrochloride) (Fig.1) (Sydor et al., 2006; Workman et al., 2007) were therefore synthesized for further evaluation (Pacey et al., 2006).

A considerable amount of understanding has been obtained from the clinical experience of 17-AAG (Workman et al., 2007). 17-AAG entered Phase I trials in 1999 (Banerji et al., 2005; Pacey et al., 2006) and several intravenous formulations have completed Phase I testing (Heath et al., 2005; Ronnen et al., 2006). Early signs of therapeutic activity have been seen in melanoma, breast cancer, prostate cancer, and multiple myeloma (da Rocha Dias et al., 2005; Grbovic et al., 2006; Modi et al., 2007; Pacey et al., 2006; Solit and Rosen, 2006). Phase II clinical trials for 17-AAG are currently ongoing (Heath et al., 2005; Ronnen et al., 2006), which mainly focus on tumor types hallmarked by specific Hsp90 chaperoning targets, such as leukemia expressing Bcr-Abl and Her-2 positive breast cancer (Dai and Whitesell, 2005; Pacey et al., 2006). 17-AAG was recently administered in combination with trastuzumab in patients whose disease progressed following trastuzumab treatment; this trial has demonstrated promising anti-tumor activity and acceptable toxicity (Modi et al., 2007). However, several drawbacks of 17-AAG, including low water-solubility, instability in solution, and low oral bioavailability (McCollum et al., 2008; Messaoudi et al., 2008) may become the obstacle to further clinical application.

In addition to 17-AAG, other GA derivatives have been developed for clinical use as well. Currently 17-DMAG, a more water-soluble analogue of 17-AAG, has entered Phase I and Phase II clinical testing, and displayed higher oral bioavailability, lower toxicity, and increased stability compared with 17-AAG (Hollingshead et al., 2005; Ronnen et al., 2006). Another water soluble hydroquinone hydrochloride analogue of 17-AAG is IPI-504 (Didelot et al., 2007). IPI-504 is in Phase I and Phase II clinical trials to evaluate its potential for treating

cancer that has become resistant to therapy with tyrosine kinase inhibitors, such as Philadelphia chromosome-positive chronic myelogenous leukemia (CML) (Peng et al., 2007).

Radicicol (Fig. 1) (Workman et al., 2007) is a macrocyclic natural antibiotic initially isolated from the fungus Monocillium nordinii and Monosporium bonorden (Delmotte and Delmotte-Plaque, 1953). Like GA, radicicol was also shown to compete with nucleotide for N-terminal ATP pocket of Hsp90 (Roe et al., 1999). Similarly, this interaction between radicicol and Hsp90 constrains the chaperone in its ADP-bound conformation, leading to destabilization of Hsp90 client proteins (Mimnaugh et al., 1996). Radicicol displayed anti-cancer activity *in vitro* but not *in vivo*, which was explained by its chemical and metabolic instability (Pearl et al., 2008;Soga et al., 2003). Hence, synthetic efforts have been directed to generate radicicol derivatives with improved stability and *in vivo* efficacy (Proisy et al., 2006). Several oxime derivatives and cycloproparadicicol have been developed and shown to possess anti-tumor activity in preclinical animal models, as well as tolerable toxicity (Shiotsu et al., 2000;Soga et al., 2003;Yamamoto et al., 2003).

Novobiocin (Fig. 1) (Workman et al., 2007), a coumarin antibiotic isolated from Streptomyces species, was found to bind to Hsp90 at a newly suggested C-terminal ATP binding site with relatively weak activity (Marcu et al., 2000a). Inhibition of Hsp90 by novobiocin induced similar cellular responses as N-terminal inhibitors, i.e., destabilization of a range of Hsp90 client proteins such as Her-2, Raf-1 and p53 mutant via the ubiquitin-proteasome pathway (Allan et al., 2006;Marcu et al., 2000b;McConkey and Zhu, 2008). An allosteric regulation between the C-terminal and N-terminal domains of Hsp90 has been suggested, such that the interaction of ligands with one site might be affected by occupancy of the other site (Garnier et al., 2002;Marcu et al., 2000a;Marcu et al., 2000b). Two related coumarin antibiotics, chlorobiocin and coumermycin A1, also bind to the C-terminus of Hsp90 and have improved activity compared with novobiocin (Burlison and Blagg, 2006;Marcu et al., 2000b). Although a series of novobiocin analogues have been synthesized and screened for inhibitory activity against cancer cell proliferation (Burlison et al., 2006;Le Bras et al., 2007), currently available crystal structures have not yet confirmed the presence of such a second ATP binding site (Ali et al., 2006;Dollins et al., 2007;Shiau et al., 2006).

New natural product scaffolds are being discovered and tested. A recent example is the isoflavone derrubone (Fig. 1) from the Indian tree Derris robusta (Hadden et al., 2007). Derrubone was demonstrated to disrupt the interaction of Hsp90 and Cdc37 with heme-regulated eIF2a kinase (HRI), a Hsp90 client kinase, and exhibit antiproliferation activity in human breast cancer cell lines (Hadden et al., 2007). A green tea polyphenol catechin, epigallocatechin 3-gallate (EGCG) (Fig.1), was shown to inhibit the transcriptional activity of aryl hydrocarbon receptor (AhR) through a mechanism involving direct binding of EGCG to the C-terminus of Hsp90 (Palermo et al., 2005). It remains unclear whether EGCG could inhibit Hsp90 function through this direct binding. These findings may provide new natural product scaffolds to facilitate the development of novel Hsp90 inhibitors.

2.2. Potential resistance to ansamycins

Thus, encouraging clinical responses have confirmed the potential of targeting Hsp90. However, binding of these ansamycin drugs not only prevents ATP binding but also induces a stress response through the release, activation, nuclear localization and trimerization of heat shock factor-1 (HSF-1) (Kaur and Ralhan, 2000), a transcription factor that binds heat shock elements (HSE) to increase the mRNA and protein levels of Hsp70 (Whitesell et al., 2003). This stress-responsive up-regulation of Hsp70 is believed to reduce the Hsp90-targeted drug efficacy by inhibiting apoptosis signaling (Kaur and Ralhan, 2000; Schmitt et al., 2006). Furthermore, these ansamycins are P-glycoprotein (Pgp) substrates (Tsuruo et al., 2003). Interestingly, a very recent study suggested that HSF-1-mediated stress induction, such as

accumulation of Hsp70, may play a more important role in resistance to 17-AAG than drug efflux by Pgp, since depletion of Hsp70, but not P-gp inhibition increased the sensitivity of 17-AAG resistant cells to 17-AAG (McCollum et al., 2008b). These findings encourage the investigation of combining Hsp90 inhibitors, particularly of the ansamycin class, with abrogation of Hsp70 induction to enhance the clinical efficacy of Hsp90-targeted therapy (McCollum et al., 2008).

2.3. Synthetic small molecules and peptide derivatives

The search for natural Hsp90 inhibitors has been accompanied by a search for synthetic small molecule inhibitors that potentially possess more specific targeting and better pharmacological profiles. The availability of crystal structures of the Hsp90 N-domain and the development of structure-based design and high-throughput screening (HTS) assays have prompted successful exploitation of a range of new synthetic scaffolds that have Hsp90 inhibitory capacity (Huth et al., 2007; Messaoudi et al., 2008; Park et al., 2007).

The first group of these was empirically designed based on the purine scaffold (Figure 2) (Chiosis, 2006;Solit and Chiosis, 2008), which mimics the unique shape adopted by natural nucleotide ligand inside of the N-terminal pocket of Hsp90 (Chiosis, 2006;Chiosis et al., 2001). The favorable interaction between PU3 and the ATP pocket of Hsp90 was visualized in crystal structure, and consistently the similar biological effects of PU3 with geldanamycin were observed (Wright et al., 2004). PU3 thus became a starting point for the expansion and the improvement of clinically applicable purine-scaffold Hsp90 inhibitors, where efforts are directed toward either position C8 or C9 of purine (Chiosis, 2006). These synthetic derivatives could be roughly categorized to 8-benzyl (He et al., 2006;Vilenchik et al., 2004), 8-phenylsylfanyl (He et al., 2006;Llauger et al., 2005), 8-(7′-substituted benzothiazolothio) (Zhang et al., 2006), and 9-benzyl purine derivatives (Kasibhatla et al., 2007). Major improvements of purine-scaffold Hsp90 inhibitors include insensitivity to multi-drug resistance (Rodina et al., 2007), favorable water solubility, oral bioavailability, and metabolic stability (Biamonte et al., 2006;He et al., 2006;Kasibhatla et al., 2007;Zhang et al., 2006). For instance, PU-H71 and PU-DZ8 are currently under advanced preclinical investigation (Chiosis and Tao, 2006). CNF-2024, an orally available 9-benzyl purine derivative with low nanomolar potency, has entered Phase I clinical trials, tested in chronic lymphocytic leukemia, advanced solid tumors, lymphomas, and more recently in advanced breast cancer (Chiosis, 2006).

Pyrazole (Fig.2) (McDonald et al., 2006;Solit and Chiosis, 2008) is another important class of synthetic small molecules that has been identified (Cheung et al., 2005;Sharp et al., 2007a). A molecule of 3, 4-diaryl pyrazole (CCT018159) binds deeply into the N-terminal ATP pocket of Hsp90, as shown in crystallographic studies (Cheung et al., 2005). Similar with GA, a decrease in Hsp90 client protein levels (Raf-1 and Cdk4) and an increase in Hsp70 expression were observed in CCT018159-treated cancer cells (Cheung et al., 2005). Structure-based design generated more potent pyrazole amide CCT0129397 and isoxazole CCT0130024 (Sharp et al., 2007b); and an optimized analogue NVP-AUY922 has just entered clinical trials (Brough et al., 2008).

Finally, a novel family of short peptide derivatives composed of the three core amino acids, Phe-D-Trp-Leu, at the center of the molecule was recently reported to inhibit ATP binding to Hsp90 (Orosz et al., 2006). They killed various cancer cell lines, but not normal cells *in vitro* and did not show toxicity *in vivo* (Orosz et al., 2006). A few of these peptide derivatives were reported to cause a marked inhibition of multidrug resistance in human *MDR1* gene-transfected mouse lymphoma cells (Molnar et al., 2007).

3. Hsp90 inhibitors targeting co-chaperone/Hsp90 interactions

Hsp90 requires a series of co-chaperones to assemble a super-chaperone complex for its function. These co-chaperones bind and leave the complex at various stages to regulate the chaperoning process (Neckers, 2003). Arresting the chaperone cycle at these stages by targeting different cochaperone/Hsp90 interactions is likely to achieve similar consequences with the direct inhibition of Hsp90 (Gray et al., 2008; Zhang et al., 2008). Blockade of ATP binding seems to be the most direct and the simplest way to manipulate Hsp90, but its intrinsic non-selectivity for the Hsp90 clientele may limit its further application. Therefore, a potentially more specific approach is to develop drugs that block the interaction between Hsp90 and cochaperones (Pearl et al., 2008). Availability of crystal structures of co-chaperone/Hsp90 interactions plays a critical role in this process.

3.1. Targeting the cdc37/Hsp90 interaction

Cdc37 has a specialized and indispensable role in the maturation of a kinase sub-population of clients, including receptor tyrosine kinases such as epidermal growth factor receptor (EGFR), non-receptor tyrosine kinases Src and lymphocyte-specific protein tyrosine kinase (Lck), and intracellular serine/threonine kinases such as Raf-1and Cdk4 (Gray et al., 2008). Cdc37 acts as an adaptor, loading these kinases onto the Hsp90 complex, thereby facilitating their maturation (Pearl, 2005; Silverstein et al., 1998; Smith et al., 2008; Vaughan et al., 2006). Depletion of Cdc37 using RNAi in human colon cancer cells diminished the association of kinase clients with Hsp90 and decreased the levels of these clients including Her-2, Raf-1, Cdk4, and Akt, leading to reduced cell proliferation (Smith et al., 2008). Another noteworthy observation that silencing of Cdc37 did not induce Hsp70 up-regulation suggests that targeting Cdc37 might be advantageous compared with some Hsp90 inhibitors that induce Hsp70 accumulation (Smith et al., 2008). In addition, a recent study demonstrated that more extensive and sustained depletion of kinase clients and potentiated cell death could be achieved through combining Cdc37 silencing with the Hsp90 inhibitor 17-AAG (Smith et al., 2008).

A potential alternative strategy to direct Hsp90 inhibition was therefore brought up. The inhibition of Cdc37/Hsp90 interaction would probably represent an approach that may offer greater specificity and an improved side-effect profile (Gray et al., 2008; Gray et al., 2007). Indeed, our group has recently reported that celastrol (Figure 3A), a quinone methide triterpene from Tripterygium wilfordii Hook F, exhibits anti-pancreatic cancer activity both in vitro and in vivo through the disruption of Cdc37/Hsp90 association and the subsequent degradation of Hsp90 client proteins (Zhang et al., 2008). Celastrol was identified to block Cdc37 interaction with Hsp90 by molecular modeling (Zhang et al., 2008), taking advantage of the available crystal structure in which Cdc37 associates with the N-terminal domain of Hsp90 by inserting its C-terminal side chain into the mouth of the ATP binding pocket (Figure 3B) (Prodromou et al., 2000; Roe et al., 2004).

3.2. Targeting the Hsp70/Hsp90 interaction

Assembly of Hsp70/Hsp90 complex is achieved by associations of their C-terminal tails with two independent TPR domains of Hop, TPR1 and TPR2A, respectively (Yi and Regan, 2008). These interactions, especially the one between TPR2A of Hop and the C-terminal MEEVD motif of Hsp90, are essential for function of this multi-molecular complex (Chen and Smith, 1998; Scheufler et al., 2000). Recently, a designed TPR module, CTPR390+, which binds to the Hsp90 C-terminus with higher affinity and more specificity than TPR2A, was shown to be capable of competing with endogenous TPR2A for Hsp90 binding and thereby to prevent the formation of Hsp70/Hsp90 complex, triggering Her-2 degradation with consequent inhibition of breast cancer cell proliferation (Cortajarena et al., 2008). Furthermore, a high-throughput AlphaScreen assay was developed to identify novel small molecules that hinder

the Hsp90/TPR2A interaction and the identified compounds were shown to inhibit the growth of breast cancer cells, which was correlated with a reduced level of Her-2 (Yi and Regan, 2008). Remarkably, inhibition of Hsp90 through this way did not induce Hsp70 expression (Cortajarena et al., 2008; Yi and Regan, 2008). However, it is evident that these novel compounds will have modest selectivity for tumor versus non-tumor cells.

3.3. Targeting the Hop/Hsp90 interaction

In addition, recent work revealed that whilst the primary binding site of Hop is the C-terminal MEEVD peptide of Hsp90, binding also occurs at additional sites in the C-terminal and middle domains of Hsp90 (Onuoha et al., 2008). The feasibility of targeting Hop/Hsp90 interaction still needs to be addressed.

3.4. Targeting the Aha1/Hsp90 interaction

According to crystallogical studies, the N-terminal domain of Aha1 interacts with the middle segment of Hsp90 and triggers the ATPase activity of Hsp90 (Meyer et al., 2004). Similar to Cdc37, Aha1 knockdown was recently reported to decrease the activation status of Hsp90 clients and a synergistic interaction was observed between Aha1 depletion and 17-AAG in inhibition of cancer cell growth, which indicates that modulation of Aha1 might be another potential therapeutic strategy (Holmes et al., 2008).

4. Hsp90 inhibitors targeting client/Hsp90 associations

Inhibition of client/Hsp90 interactions offers the ultimate selectivity, but little is known about the molecular basis for these interactions (Pearl et al., 2008). The key to targeting the client/Hsp90 interaction is the ability to study the structure and biochemistry of the molecular complexes. An Hsp90/Cdc37/Cdk4 complex has been purified and its three dimensional structure has been determined by electron microscopy (Vaughan et al., 2006), providing the first structural view of the interaction between a client protein and Hsp90 (Ali et al., 2006; Pearl et al., 2008; Vaughan et al., 2006). However, most of the details of the client/chaperone interactions are still unclear, and therefore, the strategy of targeting these associations is more challenging.

Shepherdin, a peptidomimetic, was designed to specifically block the interaction between Hsp90 and the anti-apoptotic client survivin (Plescia et al., 2005). However, shepherdin can interact with the ATP pocket of Hsp90 as well, and affects a range of Hsp90 clients in addition to survivin (Pearl et al., 2008). Although shepherdin did exhibit anti-leukemia activity in animal models (Gyurkocza et al., 2006), its apparent interaction with the ATP pocket of Hsp90 and the effect on a range of Hsp90 clients suggests that it may have a different mode of action.

5. Post-translational modifications of Hsp90

Post-translational modifications, such as hyperphorphorylation, S-nitrosylation and reversible hyperacetylation, have been thought to be involved in regulating chaperone function of Hsp90 through affecting co-chaperone association and/or ATP binding (Neckers, 2007). The post-translational modifications of Hsp90 may open up a wide range of opportunities to indirectly interfere with its activity (Wandinger et al., 2006).

5.1. Hsp90 hyperacetylation

Several studies have correlated histone deacetylases (HDACs) with Hsp90 chaperone function. HDACs, in concert with acetyltransferases (HATs), control reversible acetylation of lysine residues on Hsp90 (Minucci and Pelicci, 2006), which regulates Hsp90 activity. Lysine K294 in the middle domain of Hsp90 has been recently identified to be an important acetylation site

(Kovacs et al., 2005; Scroggins et al., 2007). Identification of other acetylation sites and understanding of their functional significance will require more effort (Scroggins et al., 2007). A very recent work identified p300 as one of the HATs involved in acetylating Hsp90 (Yang et al., 2008). Hyperacetylation of Hsp90 has been reported after treatment with a variety of pan-HDAC inhibitors, such as LAQ824 and LBH589 (Bali et al., 2005; Kovacs et al., 2005). HDAC6, among the 18 HDAC family members identified so far, is unique in that it deacetylates *non*-histone proteins, such as Hsp90 and α-tubulin (Bali et al., 2005). Specific knockdown of HDAC6 using RNAi enhanced the degree of Hsp90 acetylation (Rao et al., 2008) and hyperacetylation of Hsp90 by either pan-HDAC inhibitors or HDAC6 knockdown was associated with a reduced binding of ATP and/or co-chaperones to Hsp90, thus promoting the degradation of Hsp90 client proteins (Rao et al., 2008). Also, HDAC1 may contribute to deacetylation of Hsp90 (Nishioka et al., 2008). MS-275, a novel synthetic selective HDAC1 (and not HDAC6) inhibitor (Minucci and Pelicci, 2006) induces apoptosis of acute myeloid leukemia (AML) cells expressing mutant fms-like tyrosine kinase 3 (FLT3) (Nishioka et al., 2008). MS-275, by inducing hyperacetylation of Hsp90 in these leukemia cells, inhibits the direct interaction between Hsp90 and FLT3, followed by its proteasome-dependent degradation (Nishioka et al., 2008). Overall, the hyperacetylation of Hsp90 was shown to inhibit binding of ATP, co-chaperone p23 and client proteins to Hsp90, directing the latter to polyubiquitylation and proteasomal degradation (Bali et al., 2005). Therefore, *inhibition* of *either* one of the two HDAC family members would probably be associated with hyperacetylation of Hsp90 and consequent impairment of Hsp90 chaperoning function. In addition, siRNA-mediated depletion of HDAC6 improved the affinity of 17-AAG for Hsp90 through enhancing the degree of Hsp90 acetylation (Rao et al., 2008). Moreover, co-treatment with Hsp90 inhibitors and HDAC inhibitors may produce synergistic effect, as suggested by the work on LBH589 and 17-AAG in CML and AML models (George et al., 2005).

5.2. Hsp90 thiol oxidation

A new type of inhibitor, tubocapsenolide A (TA) (Chen et al., 2008) was recently isolated from Tubocapsicum anomalum and shown to have potent anti-cancer activity in various human cancer cell lines (Hsieh et al., 2007). It was suggested that TA rapidly and selectively induced thiol oxidation of Hsp90 and Hsp70 respectively, thereby inhibiting the chaperone activity of the Hsp90/Hsp70 complex; indeed the TA-induced effects could be prevented by N-acetylcysteine, a thiol anti-oxidant. The TA activity was at least in part attributed to proteasomal degradation of Hsp90 clients such as Cdk4, cyclin D1, Raf-1, Akt and mutant p53 (Chen et al., 2008). This finding may open up another window for the targeting of Hsp90.

5.3. Hsp90 phosphorylation

It is known that phosphorylation negatively regulates Hsp90 chaperoning function (Yang et al., 2008) and disruption of a phosphatase responsible for Hsp90 phosphorylation resulted in inhibition of Hsp90 function (Wandinger et al., 2006). However, the role of site-specific phosphorylation in modulating Hsp90 function has not yet been clarified (Yang et al., 2008).

6. Hsp90 isoform function and drug selectivity

The two major isoforms of Hsp90 in humans, Hsp90α and Hsp90β, encoded by two distinct genes, share approximately 81% sequence homology (Eustace et al., 2004; Passarino et al., 2003). And both contain the same three highly conserved domains (Pearl and Prodromou, 2006; Prodromou and Pearl, 2003; Soti et al., 2002). Still, it has become increasingly clear that Hsp90α and Hsp90β play different roles (Pearl et al., 2008). While Hsp90β is constitutively expressed at high abundance in most tissues, Hsp90α is typically inducible in response to various cellular stress conditions (Sreedhar et al., 2004).

Although the unique functions and underlying molecular basis of the isoforms remain to be elucidated, the distinct ability to activate some client proteins and the *in vivo* isoform selectivity of different inhibitors have been described (Chan et al., 2008; Millson et al., 2007). Activation of certain Hsp90 clients, such as v-Src, was more efficient with Hsp90α, rather than Hsp90β (Millson et al., 2007). Geldanamycin and radicicol were reported to interact with Hsp90α and Hsp90β with similar efficiency, whereas some of the purine-scaffold drugs, such as PU-H71, were more effective against Hsp90α (Chan et al., 2008). It is still unclear whether the isoform specificity of any Hsp90 inhibitor would be more beneficial in a particular cancer type (Chiosis, 2006).

Another cytoplasmic variant in mammals, Hsp90N, completely lacks the N-terminal ATPase domain and appears to be membrane associated (Grammatikakis et al., 2002). Higher eukaryotes possess a distinct endoplasmic-reticulum isoform, Grp94 (Ni and Lee, 2007) and a mitochondrial isoform, Trap1 (Felts et al., 2000). Neither clientele nor the biological role of these isoforms is currently known.

7. Cell surface Hsp90 and tumor metastasis

Over the past years, most attention has been given to the study of the *intra*cellular Hsp90. However, a pool of Hsp90α has been described to be loosely attached to the cell membrane and facing the *extra*cellular space (Thomaidou and Patsavoudi, 1993), which was associated with tumor cell invasion (Eustace et al., 2004; Sidera et al., 2008; Tsutsumi et al., 2008). A range of conditions, such as serum starvation, hypoxia, high concentration of glucose, as well as oxidative stress have been shown to trigger the *extra*cellular localization of Hsp90α (Eustace and Jay, 2004; Li et al., 2007; Liao et al., 2000). It remains unknown how Hsp90α reaches the cell membrane and/or the extracellular environment (Sidera and Patsavoudi, 2008). One possible explanation is that Hsp90α might be secreted via an exosome pathway, as it was reported to be present in exosomes (Clayton et al., 2005). Although Hsp70, p23, Cdc37 and Hip are present in the extracellular space of tumors as well, their function there has not been explicated (Eustace and Jay, 2004; Shin et al., 2003). In addition, whether ATP binding and hydrolysis are required for an extracellular chaperone function of Hsp90α remains to be determined.

Cell surface Hsp90α was described to serve as a molecular chaperone of matrix metalloprotease 2 (MMP-2), an extracellular enzyme essential for cell invasion, to assist the maturation of this enzyme (Eustace et al., 2004; Passarino et al., 2003). Very recently, cell surface Hsp90α was also shown to specifically interact with the extracellular domain of Her-2 *in vitro* and *in vivo* (Citri et al., 2004; Sidera et al., 2008; Xu et al., 2001). This surface interaction is necessary for Her-2 activation and heregulin-induced heterodimerization with ErbB-3, which in turn mediates signal transduction pathways via MAPK and PI3K-Akt, leading to cytoskeletal actin re-arrangement essential for cell motility (Sidera et al., 2008; Sidera and Patsavoudi, 2008).

Inhibition of cell surface Hsp90α with antibodies or cell-impermeable inhibitors could block cell motility and invasion *in vitro* and metastasis *in vivo* (Tsutsumi and Neckers, 2007). A recent report identified a potent cell-impermeable Hsp90 inhibitor, DMAG-N-oxide, which is a polar derivative of 17-DMAG and currently in clinical trials (Tsutsumi et al., 2008). Although DMAG-N-oxide lacks the growth inhibitory ability of cell-permeable Hsp90 inhibitors, it displays significant anti-invasion activity *in vitro* and anti-metastasis activity *in vivo* (Tsutsumi et al., 2008). Moreover, it was described that the selective blockade of cell surface Hsp90α with a monoclonal antibody, mAb 4C5, could lead to specific disruption of the extracellular Hsp90α/Her-2 interaction, in turn preventing Her-2/ErbB-3 heterodimerization, actin reorganization, and consequent cell invasion (Sidera et al., 2008). Interestingly, disruption of the extracellular Hsp90α/Her-2 interaction did not lead to a reduced level of Her-2 protein on

the cellular membrane; instead, it significantly reduced the phosphorylation of Her-2, suggesting that this extracellular interaction is different from the previously characterized intracellular interaction (Sidera and Patsavoudi, 2008). These findings suggest that blockade of cell surface Hsp90α may have clinical benefit in limiting cancer cell invasion and metastasis (Li et al., 2007; Sidera et al., 2004; Stellas et al., 2007).

Recently K69 was identified as one of the acetylated lysine residues on cell surface Hsp90α hyperacetylated by pan-HDAC inhibitor LBH589; and hyperacetylation actually stimulates not only the extracellular localization of Hsp90α, but also promotes its association with MMP-2, resulting in an increased tumor cell invasion (Yang et al., 2008). This study further demonstrated that exposure of breast cancer cells to a novel antibody against the acetyl-K69 Hsp90α could markedly inhibit *in vitro* invasion compared with the inhibitory effect of an anti-Hsp90α antibody that non-specifically recognizes both acetylated and unacetylated Hsp90α (Yang et al., 2008).

Taken together, these intriguing findings raise the possibility that cell surface Hsp90α plays an important role in modulating cancer cell invasion and metastasis that is *in*dependent of the intracellular Hsp90 function, providing a novel extracellular drug target for metastatic cancer therapy (Sidera and Patsavoudi, 2008; Tsutsumi et al., 2008).

8. Hsp90 inhibition and cancer stem cells

It has become increasingly evident that cancer probably is initiated from and maintained by a small sub-population of undifferentiated, tumorigenic cells called cancer stem cells (CSCs) (Gonzalez-Sarmiento and Perez-Losada, 2008). Production of the main mass of the tumor may be attributed to this minor population of CSCs through a particular process of continuous self-renewal and differentiation (Gonzalez-Sarmiento and Perez-Losada, 2008). Thus, CSCs have come into sight as a potential target of cancer therapy (Hassane et al., 2008; Rottenberg and Jonkers, 2008).

Based on an *in silico* screen of public gene expression data, celastrol, a novel Hsp90 inhibitor reported by us (Zhang et al., 2008), was recently discovered to eradicate acute myelogenous leukemia stem cells through simultaneous inhibition of NF-κB-mediated survival signals and induction of oxidative stress (Hassane et al., 2008). A number of studies have documented a correlation between Hsp90 and NF-κB. Cdc37 and Hsp90 together are required for constitutive and inducible activation and nuclear localization of NF-κB (Broemer et al., 2004; Chen et al., 2002) and the expression of the Hsp90α gene was regulated by NF-κB (Ammirante et al., 2008). Therefore, a regulation loop is likely to exist between Hsp90 and NF-κB. Moreover, overexpression of Her-2, an Hsp90 client protein, was shown to increase the breast cancer stem cell population both *in vitro* and *in vivo* (Korkaya et al., 2008). The Wnt/β-catenin pathway is one of the pathways with a critical role in CSCs (Malanchi et al., 2008; Reya and Clevers, 2005) and β-Catenin has been suggested to be regulated by IKK that has been shown to be controlled by Hsp90, connecting the Wnt pathway to Hsp90 (Lamberti et al., 2001).

The putative spheroid-forming breast CSCs usually display resistance to chemotherapeutic drugs (Wright et al., 2008). Surprisingly, a synergistic effect was observed when breast CSCs were exposed to the Hsp90 inhibitor 17-DMAG simultaneously or after drugs such as doxorubicin, cisplatin or etoposide (Wright et al., 2008). Also, the Hsp90 inhibitor IPI-504 was shown to induce a dramatic suppression of leukemia stem cells in mice with Bcl-Abl-T315I-induced CML; in a sharp contrast, analysis of bone marrow from wild-type mice treated with IPI-504 showed that this drug did not affect normal hematopoietic stem cells (HSCs) (Peng et al., 2007). Finally, a growth inhibitory effect and degradation of Hsp90 client proteins including EGFR, Akt and MAPK by 17-AAG in a glioma stem cells *in vitro* was reported as well as reduction of the tumor-initiating capability of these cells in an orthotopic glioma model

(Sauvageot et al., 2008). These intriguing findings may implicate an association between Hsp90 and CSCs properties through one or more complicated pathway(s), which merit further investigation.

9. Conclusions and future perspectives

The most attractive advantage of targeting Hsp90 is the combined impact on many oncogenic pathways involved in multiple steps of carcinogenesis and cancer progression, as Hsp90 inhibition eventually leads to the ubiquitin-proteasome degradation of a large population of oncogenic client proteins (Workman, 2004). This review not only provides an up-to-date overview of mechanistic studies and the clinical prospect of currently available Hsp90 inhibitors, but also wishes to enhance the perspective both for designing and discovering novel inhibitors as well as to provide insight into less-understood and under-represented potential of Hsp90 in cancer therapy (illustrated in Figure 4).

Most studies have focused on the discovery or synthesis of Hsp90 inhibitors specifically acting against the ATP binding site at the N-terminus of Hsp90. Further evaluation of these Hsp90 inhibitors and development of new inhibitors belonging to this class remains of great interest. However, there are considerable possibilities for inhibition of the super-chaperone system in other ways. Targeting co-chaperone/Hsp90 interactions should receive more attention, as this approach is likely to improve the *specificity* of Hsp90 inhibition compared with direct inhibition of ATP binding. Another appealing advance is derived from the interference with post-translational modifications of Hsp90, where a majority of the efforts have been put into the hyperacetylation of Hsp90 by HDAC inhibitors. Disruption of client/Hsp90 interactions was suggested to provide the highest selectivity (Pearl et al., 2008). While the basis for antagonizing client/Hsp90 associations is the structural and biochemical understanding of the interactions, the reality is that even nowadays little is known about these interactions (Pearl et al., 2008). Besides, the initially-raised benefit of Hsp90 targeting is the simultaneous impact on *multiple* oncogenic pathways, which could be eliminated by only focusing on a specific client/Hsp90 interaction. Therefore, the strategy of targeting these client/Hsp90 associations is not only highly challenging, but also need to be further validated. A few studies have been performed to compare the properties and functions of distinct Hsp90 isoforms, but most details remain to be understood. The different functions of Hsp90 isoforms and the isoform selectivity of Hsp90 inhibitors will require further investigation. The inhibition of cancer metastasis by cell-impermeable Hsp90 inhibitors is an interesting area for research as well as specific targeting of cancer stem cells. Since the importance of CSCs in resistance to chemotherapy has been recognized, the evaluation the effect of Hsp90 inhibitors on CSCs properties would provide useful insight and clinical perspective for the future.

Since dose-limiting toxicity is likely to be an important issue when Hsp90 inhibitors are used as single agents, an alternative approach for the application of Hsp90 inhibition is to combine Hsp90 inhibitors with other therapeutic agents to enhance the efficacy with lowered dose-limiting toxicity. This perspective is substantiated by a large number of preclinical and clinical evaluations of Hsp90 inhibitors that have shown promising results in combination with chemotherapeutic drugs or other agents (Solit and Chiosis, 2008). 17-AAG and 17-DMAG are being investigated in preclinical, Phase I and II trials for breast cancers in association with trastuzumab, a Her-2 monoclonal antibody (Didelot et al., 2007). For example, a Phase I dose-escalation study has indicated that co-administration of trastuzumab and 17-AAG is well tolerated and exhibit anti-tumor activity in patients with trastuzumab-refractory Her-2 positive breast cancer (Modi et al., 2007). A recent Phase I study with 17-AAG plus paclitaxel was conducted in patients with advanced solid malignancies to determine the recommended Phase II dose of these two drugs (Ramalingam et al., 2008). Another Phase I dose-escalation trial using 17-AAG and irinotecan suggested that 17-AAG potentiated the anti-proliferative activity

of irinotecan possibly by depleting checkpoint kinase (Chk1) (Tse et al., 2008). Co-administration of 17-AAG and proteasome inhibitors such as bortezomib has also been reported to produce synergistic anti-cancer effects, which might result from the concomitant increase in protein misfolding and impairment of proteasome-dependent clearance (Whitesell and Lindquist, 2005). IPI-504 was demonstrated to be more effective when combined with imatinib than when used alone in prolonging survival of mice with Bcr-Abl positive leukemia cells (Peng et al., 2007). A recent report indicated cisplatin-mediated abrogation of the heat shock response through inhibition of HSF-1 activity that may, at least in part, contribute to the synergistic effect of GA and cisplatin (McCollum et al., 2008a). An interesting observation by our group is that an Hsp90 inhibitor and glycolysis inhibitor synergistically inhibited tumor growth in a pancreatic tumor model, possibly by preferentially targeting hypoxic tumor cells (Cao et al., 2008).

In summary, the targeting of Hsp90 for anti-cancer therapeutics has a potentially bright future. Further progress in the development of Hsp90 inhibitors and a deeper understanding of the Hsp90 characteristics further strengthen its promise in cancer therapy.

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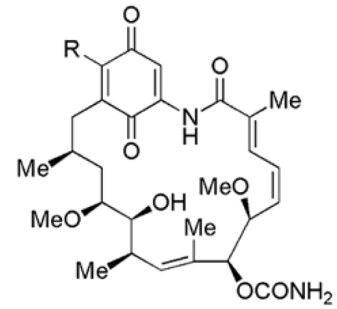
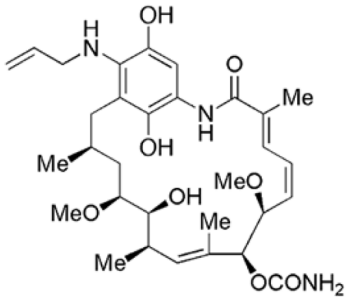


Geldanamycin R = OCH_3
17-AAG R = $NHCH_2CH{=}CH_2$
17-DMAG R = $NHCH_2CH_2N(CH_3)_2$



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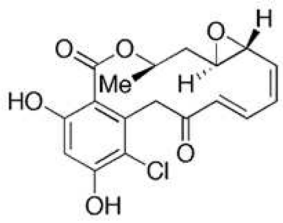

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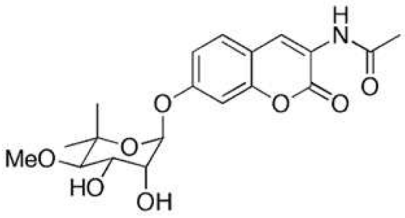

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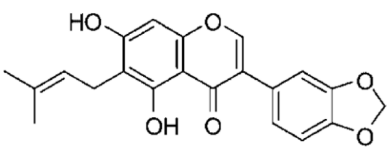
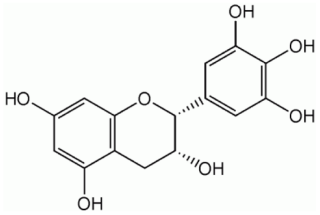


Derrubone



Epigallocatechin 3-gallate

Figure 1.
Chemical structures of the natural product Hsp90 inhibitors.

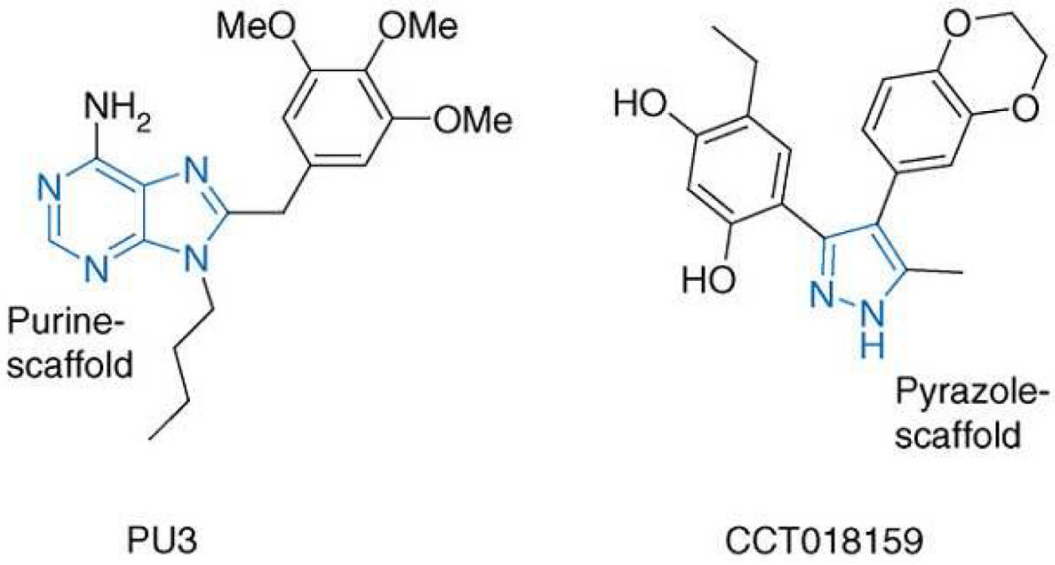


Figure 2.
Chemical structures of synthetic small molecule Hsp90 inhibitors. The molecular scaffolds are depicted in blue (Chiosis, 2006; McDonald et al., 2006; Solit and Chiosis, 2008).

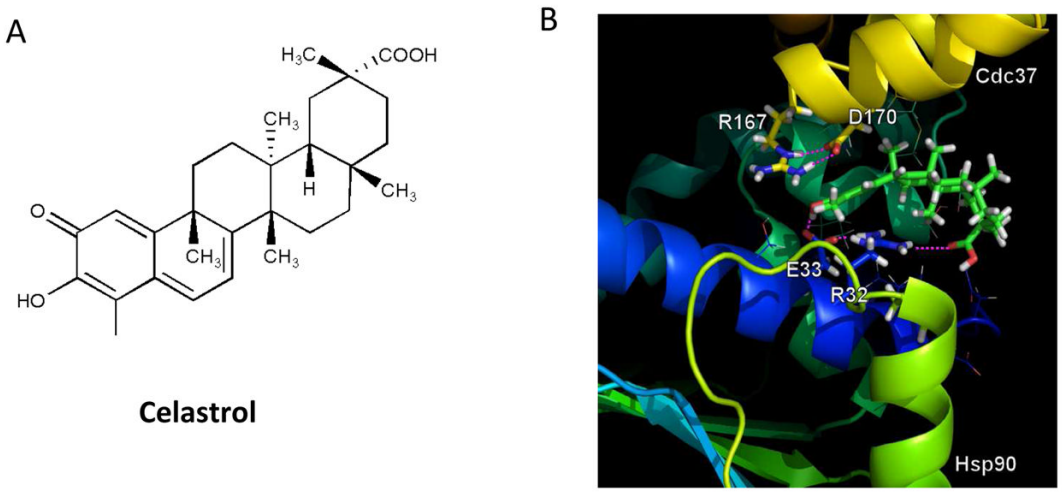


Figure 3.
(A) Chemical structure of celastrol. (B) Molecular docking of celastrol with Hsp90-Cdc37 complex. Ribbon view of the Hsp90-Cdc37-celastrol binding pocket.

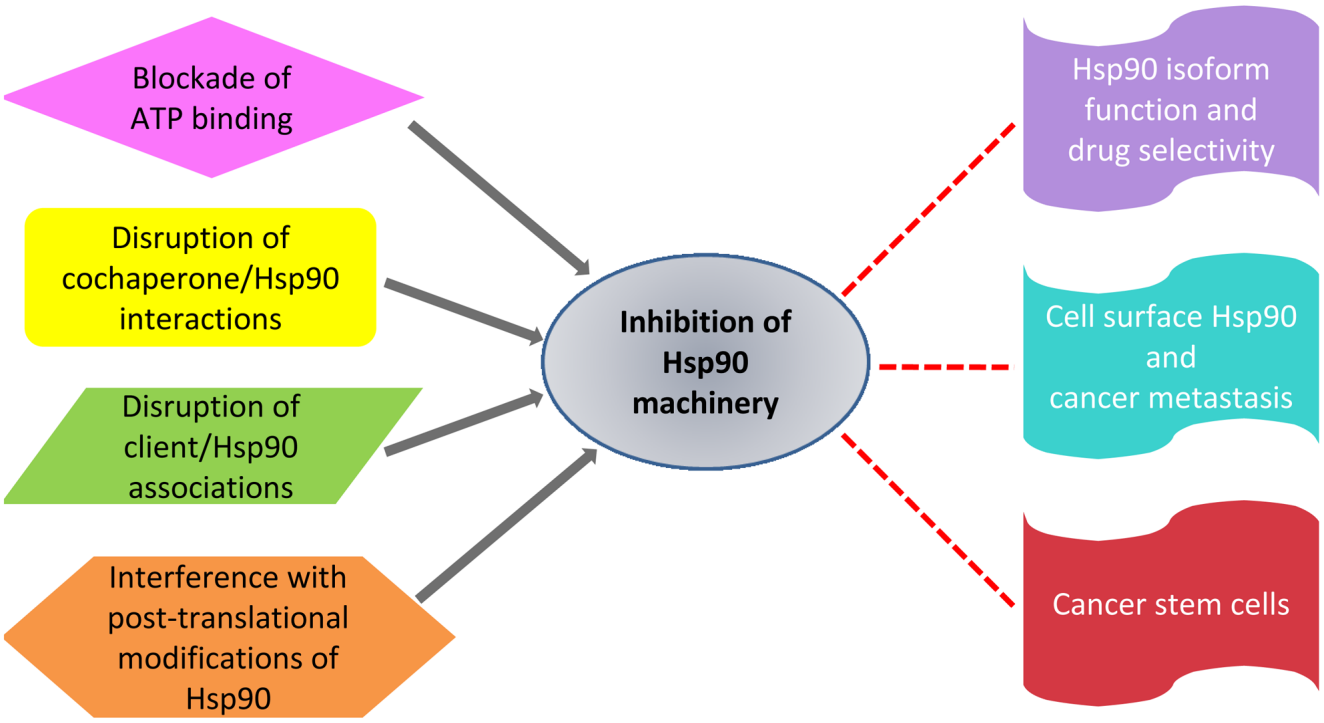


Figure 4.
Schematic illustration for designing and discovering Hsp90 inhibitor, as well as more potential of Hsp90 inhibition.