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REVIEW

Organelle-specific Hsp90 inhibitors

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Abstract Heat shock protein 90 (Hsp90) is an ATP-dependent molecular chaperone that is involved in the folding, activation, and stabilization of numerous oncogenic proteins. It has become an attractive therapeutic target, especially for eradicating malignant cancers and overcoming chemotherapy resistance. The Hsp90 family in mammalian cells is composed of four major homologs: Hsp90α, Hsp90β, 94-kDa glucose-regulated protein (Grp94), and TNF receptor-associated protein 1 (Trap1). Hsp90α and Hsp90β are mainly localized in the cytoplasm, while Grp94 and Trap1 reside in the endoplasmic reticulum and the mitochondria, respectively. Additionally, some Hsp90 s are secreted from the cytoplasm, commonly called extracellular Hsp90. Interestingly, each Hsp90 isoform is localized in a particular organelle, possesses a unique biological function, and participates in various physiological and pathological processes. To inhibit the organelle-specific Hsp90 chaperone function, there have been significant efforts to accumulate Hsp90 inhibitors in particular cellular compartments. This review introduces current studies regarding the delivery of Hsp90 inhibitors to subcellular organelles, particularly to the extracellular matrix and the mitochondria, and discusses their biological insights and therapeutic implications.

Keywords Heat shock protein 90 · Extracellular matrix · Mitochondria · Organelle · Hsp90 inhibitors

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Introduction

In the post-genomic era, the identification and modulation of a novel molecular target provides the promise of great specificity coupled with a reduction in harmful side effects for the treatment of cancer (Aggarwal 2010). Despite enormous efforts taken to modulate a single molecular target to combat this devastating disease, cancer continues to be a global health problem and a leading cause of death worldwide, probably because cancer is heterogeneous and caused by multiple genetic abnormalities. Accordingly, the strategy of targeting a single genetic abnormality faces the potential peril of being unable to successfully control heterogeneous cancers (Petrelli and Giordano 2008; Boran and Iyengar 2010). Cancer cells survive against therapeutic toxins by mutating target genes and activating alternative pathways. In this regard, it is now recognized that cancers cannot be treated by targeting a single genetic abnormality.

Heat shock protein 90 (Hsp90) represents an attractive therapeutic target to interfere with multiple genetic abnormalities, and inhibition of Hsp90 function therefore provides a promising strategy to effectively eradicate malignant cancers and overcome unwanted drug resistance (Whitesell and Lindquist 2005; Mahalingam et al. 2009). Hsp90 is an ATP-dependent molecular chaperone that is responsible for the late-stage maturation, activation, and stabilization of many oncogenic proteins, including epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (Her2), mesenchymal-epithelial transition factor (Met), cyclin-dependent kinase-4 (Cdk4), protein kinase B (Akt/PKB), cellular rapidly accelerated fibrosarcoma (c-Raf), hypoxia-inducible factor 1α (Hif-1α), and matrix metalloproteinase-2 (MMP2) (Mahalingam et al. 2009). Hsp90 inhibitors are now actively being pursued by both the pharmaceutical industry and academia,

with several strategies being employed to provide clinically potent Hsp90 inhibitors. The most widely used strategy is to mimic Hsp90's endogenous ligand, ATP, which binds to the chaperone's N-terminal nucleotide-binding pocket. This strategy causes the potent and selective blockage of ATP binding to the N-terminal domain of Hsp90. To date, hundreds of inhibitors have been developed to target the N-terminal ATP-binding pocket of Hsp90, and this is considered the most effective way to inhibit Hsp90 chaperone function (Wright et al. 2004; Brough et al. 2008; Lee and Seo 2013; Seo 2013; Jeong et al. 2014; Kusuma et al. 2014; Seo and Park 2014; Sidera and Patsavoudi 2014). Currently, 13 Hsp90 inhibitors are undergoing clinical development, all of which are N-terminal inhibitor (Sidera and Patsavoudi 2014). Other strategies employed to inhibit Hsp90 chaperone function are to block the C-terminal nucleotide-binding domain (Marcu et al. 2000; Allan et al. 2006; Kusuma et al. 2014), to disrupt protein–protein interactions of Hsp90 (Yi and Regan 2008; Zhang et al. 2008; Pimienta et al. 2011; Li et al. 2012b; Seo 2015), and to inhibit Hsp90 isoforms residing in a specific subcellular organelle (Kang et al. 2007; Tsutsumi et al. 2008; McCready et al. 2014).

In the past few decades, technology to deliver pharmaceutical agents to particular cellular compartments has become the new frontier in drug delivery (Rajendran et al. 2012; Milane et al. 2015). Utilizing the technology of drug delivery, there have been significant efforts to deliver pan-Hsp90 inhibitors to subcellular organelles, particularly to the extracellular matrix and the mitochondria (Kang et al. 2007; Tsutsumi et al. 2008; McCready et al. 2014). This present review will introduce the technology used to deliver Hsp90 inhibitors to the extracellular matrix and the mitochondria, and discuss their biological insights and therapeutic implications.

The Hsp90 family and their subcellular localization

The Hsp90 family in mammalian cells consists of four major homologs (Fig. 1) (Sreedhar et al. 2004; Whitesell and Lindquist 2005; Taipale et al. 2010). Hsp90α (inducible form/major form) and Hsp90β (constitutive form/minor form) are mostly found in the cytoplasm and the nucleus. Hsp90α and Hsp90β share 86 % amino acid identity and are ubiquitously expressed in all nucleated cells. In addition to Hsp90α and Hsp90β, there are two organelle-residing isoforms, the 94-kDa glucose-regulated protein (Grp94) and tumor necrosis factor (TNF) receptor-associated protein 1 (Trap1). Grp94 is localized to the endoplasmic reticulum while Trap1 resides in the mitochondrial matrix and the inner membrane space. Additionally, there are cell-surface-bound

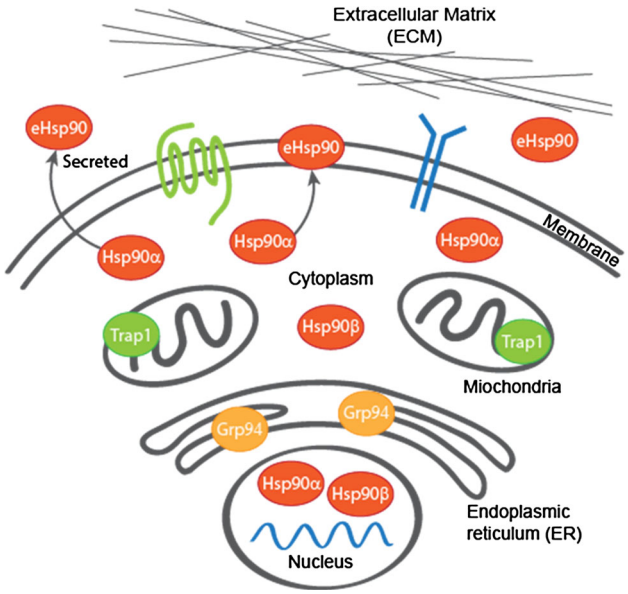

Fig. 1 The subcellular localization of the Hsp90 family. Hsp90α (inducible form) and Hsp90β (constitutive form) are cytosolic isoforms. GRP94 is localized in the endoplasmic reticulum, and Trap1 resides in the mitochondrial matrix. eHsp90 is secreted from cytoplasmic Hsp90α and resides outside the cells. *eHsp* extracellular heat shock protein, *Hsp* heat shock protein, *GRP* glucose-regulated protein, *Trap* tumor necrosis factor receptor-associated protein

or secreted Hsp90 s residing outside the cytoplasm, generally referred to as extracellular Hsp90 (eHsp90) (Tsutsumi and Neckers 2007; Li et al. 2012a). Technically, eHsp90 is not an isoform of the Hsp90 family but is actually an Hsp90 that is cell-surface-bound or secreted from the cytoplasm. Nevertheless, each individual isoform possesses a unique biological function, and participates in various physiological and pathological processes (Sreedhar et al. 2004).

Extracellular Hsp90

The vast majority of cancer-related deaths result from the formation of secondary metastases rather than the primary cancer (Chaffer and Weinberg 2011). To date, there are no anti-metastasis drugs and the discovery of such drugs will address an important unmet medical need in the treatment of metastatic cancers. Although cancer metastasis is a complex multi-step process, there are several key components of the metastatic process, including migration and invasion of cancer cells into the circulatory system, survival of cancer cells while circulating, and eventual colonization of distant secondary sites (Chaffer and Weinberg 2011). Accordingly, interfering with any of these steps will be beneficial in inhibiting the metastasis of cancer cells.

eHsp90 refers to Hsp90 that is cell-surface-bound, released and secreted from the cytoplasm (Li et al. 2012a).

It is now well documented that eHsp90 possesses functions that are distinct from those of intracellular Hsp90. eHsp90 expression correlates positively with metastatic potential and its inhibition has been reported to reduce cell migration and invasion both in vitro and in vivo (Tsutsumi and Neckers 2007; Tsutsumi et al. 2008). Of particular interest, patients with metastatic tumors exhibit higher serum levels of Hsp90 (Burgess et al. 2008; Wang et al. 2009). Therefore, eHsp90 clearly plays an important role in cancer cell metastasis distinct from Hsp90's intracellular chaperone function. The inhibition of eHsp90 is believed to be beneficial in reducing metastasis without serious toxicity, implying the clinical potential of inhibiting eHsp90 without affecting Hsp90's intracellular functions.

The first validation of eHsp90's role in the migration and invasion of cancer cells was obtained by the blockage of eHsp90 function with either anti-Hsp90 antibody or geldanamycin coupled to cell-impermeable agarose beads (Becker et al. 2004; Eustace et al. 2004). To date, the accumulated data has reinforced the pro-mobile and pro-invasive role of eHsp90 in cancer and revealed the repertoire of eHsp90 clients, including MMP2, MMP9, LDL receptor-like protein (LRP1), and EGFR2/Her2/ErbB2, as shown in Fig. 2 (Eustace et al. 2004; Sidera et al. 2008; Woodley et al. 2009; Stellas et al. 2010; Hance et al. 2012).

MMP2 and MMP9 are enzymes that are responsible for the proteolytic processing of extracellular matrix structural proteins, and the secretion and activation of MMP2 and MMP9 represent a critical proteolytic hub in cancer cell invasion and metastasis (Segarra et al. 2005; Karagiannis and Popel 2006). MMP2 was first identified to be regulated by eHsp90, illustrating the pro-invasive role of eHsp90, and eHsp90 was also later reported to regulate MMP9 activity in tumor invasion (Eustace et al. 2004; Stellas et al. 2010). Likewise, eHsp90 also interacts with a number of receptors, such as LRP1 and EGFR2/Her2/ErbB2, to regulate signaling pathways (Sidera et al. 2008; Woodley et al. 2009). LRP1 is a member of the LDL receptor family, and is associated with tumor growth and metastasis development. Interestingly, eHsp90 interacts with LRP1 to promote cell motility in a number of cancer cells. Coupled with LRP1, eHsp90 also interacts with EGFR2/Her2/ErbB2 to activate EGFR signal transduction. Direct interaction of eHsp90 with these receptors initiates signaling events via extracellular signal-regulated kinase (ERK) and nuclear factor kappa B (NF-κB) to induce the epithelial to mesenchymal transition (EMT) (Chen et al. 2010; Thuringer et al. 2011; Hance et al. 2012; Bohonowych et al. 2014). EMT activation collaborates with eHsp90-dependent signaling to further upregulate the expression of MMPs, EMT-activating transcription factor, and cytokines.

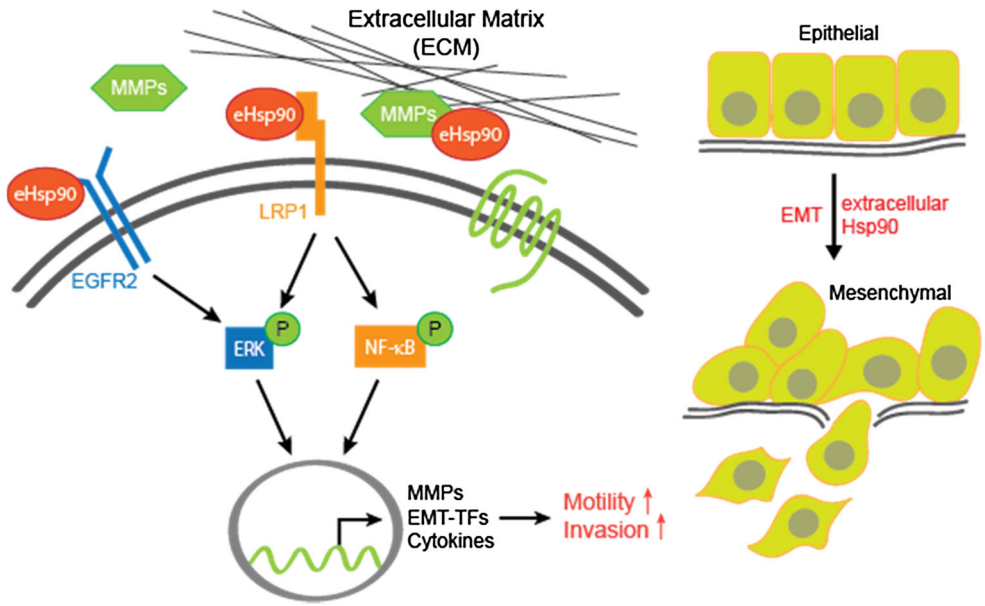

Fig. 2 Schematic representation of eHsp90 as a regulator of the EMT. eHsp90 interacts with a number of receptors (EGFR2 and LRP1) and initiates ERK- or NF-κB-mediated signaling pathways, which facilitate cell mobility and invasion. In addition, eHsp90 is associated with MMPs to promote migration of cancer cells. *eHsp* extracellular heat shock protein, *EMT* epithelial–mesenchymal transition, *EGFR* epidermal growth factor, *ERK* extracellular signal-regulated kinase, *Hsp* heat shock protein, *GRP* glucose-regulated protein, *LRP* low-density lipoprotein receptor-like protein, *MMP* matrix metalloproteinase, *NF-κB* nuclear factor kappa B, *P* phosphoylation, *TF* transcription factor, *Trap* tumor necrosis factor receptor-associated protein

Inhibitors selectively targeting extracellular Hsp90

In 2008, Tsutsumi et al. identified DMAG-N-oxide (**2**) as a cell-impermeable Hsp90 inhibitor for the first time by analyzing a number of geldanamycin-derived Hsp90 inhibitors for their membrane permeability and binding affinity to Hsp90 (Fig. 3) (Tsutsumi et al. 2008). The study indicated that impermeant analog DMAG-N-oxide (**2**) showed a similar Hsp90 binding affinity (K_d of 0.6 μM) to its parent cell-permeable inhibitor, 17-DMAG (**1**). The cell impermeability of DMAG-N-oxide (**2**) is caused by modifying the dimethyl amino group of 17-DMAG (**1**) to the polar N-oxide group shown in red (Fig. 3). With DMAG-N-oxide (**2**) confirmed as a cell-impermeant Hsp90 inhibitor, they examined the ability of DMAG-N-oxide (**2**) to affect the stability of Hsp90 clients, Akt and Raf-1. As expected, DMAG-N-oxide (**2**) did not affect the steady-state level of Akt and Raf-1 in T24 bladder carcinoma cells, while geldanamycin significantly decreased the expression levels of Atk and Raf-1 in a dose-dependent manner. Interestingly, DMAG-N-oxide (**2**) did not induce the heat shock response that is an abrogate biomarker of intracellular Hsp90 inhibition. Heat shock factor 1 (Hsf-1) is a transcription factor that is repressed by Hsp90, and Hsf-1 exists in an inactive form in a complex with Hsp90. When cells are exposed to Hsp90 inhibitors, Hsf-1 is dissociated from Hsp90 and induces a heat shock response. The finding that DMAG-N-oxide (**2**) is unable to induce a heat shock response is probably owing to its cell impermeability. To further investigate whether DMAG-N-oxide (**2**) possesses anti-metastatic activity, they performed Matrigel invasion, wound healing, and in vivo colonization assays. The study showed that DMAG-N-oxide (**2**) successfully acts as an antagonist of cancer cell mobility. DMAG-N-oxide (**2**) inhibited cell migration and invasion in vitro, and brief ex vivo exposure of cancer cells to DMAG-N-oxide (**2**) significantly reduced tumor colonization at distant sites in vivo.

Recently, McCready et al. introduced another impermeant Hsp90 inhibitor, STA-12-7191 (**4**), which is a biotinylated analog of ganetespib (**3**) (Fig. 4) (McCready et al. 2014). Ganetespib (**3**) is a non-geldanamycin-derived Hsp90 inhibitor developed by Synta Pharmaceuticals that is currently in a phase III clinical trial (Proia and Bates 2014). Ganetespib is known to bind to the ATP-binding pocket in the N-terminal domain of Hsp90α with a K_d value of 110 nM (Shimamura et al. 2012). To accumulate the Hsp90 inhibitor outside the cells, they attached the polar biotin moiety to ganetespib (**3**) to generate an impermeant Hsp90 inhibitor, STA-12-7191 (**4**). They measured the binding affinity of STA-12-7191 to Hsp90α using a fluorescence polarization assay by competing with FITC-labeled geldanamycin. The assay displayed an EC_{50} value of 62 nM, suggesting that STA-12-7191 was bound tightly to the ATP-binding pocket in the N-terminal domain of Hsp90α, comparable to its parent inhibitor, ganetespib (Table 1). As expected, STA-12-7191 (**4**) was 100-fold less effective at Her2 degradation, which is considered as an intracellular function of Hsp90. Consequently, the difference in the EC_{50} values between the Hsp90α fluorescence polarization assay

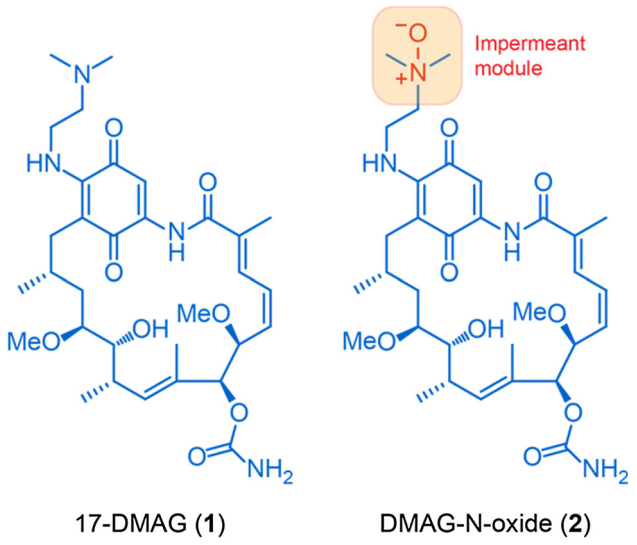

Fig. 3 Structures of Hsp90 inhibitor (DMAG, **1**) and its impermeant analog (DMAG-N-oxide, **2**). The Hsp90-inhibiting module and the impermeant module are depicted in *blue* and *red*, respectively. *17-DMAG* 17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin, *Hsp* heat shock protein

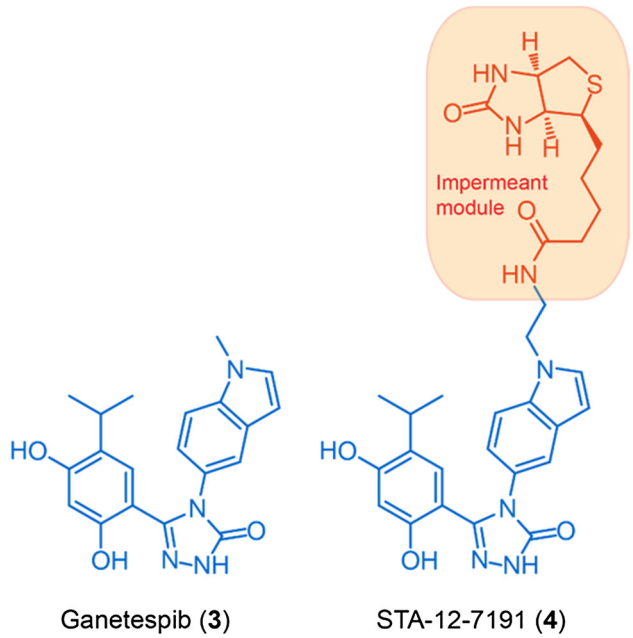

Fig. 4 Structures of a Hsp90 inhibitor (ganetespib, **3**) and its impermeant analog (STA-12-7191, **4**). The Hsp90-inhibiting module and the impermeant module are depicted in blue and red, respectively. *Hsp* heat shock protein

Table 1 EC_{50} for fluorescence polarization (FP) assay and Her2 degradation

Compound	EC_{50} (FP) (nM)	EC_{50} (Her2) (nM)
Ganetespib (**3**)	110	29
STA-12-7191 (**4**)	62	2557

and the cell-based Her2 degradation assay proved that STA-12-7191 (**4**) could not penetrate the cell membrane. Comparative evaluation of cell viability with STA-12-7191 (**4**) and ganetespib (**3**) showed that STA-12-7191 (**4**) had a 6-fold higher LD_{50} compared with that of ganetespib (**3**) in HEK293T cells (306 nM and 54 nM, respectively) owing to its reduced ability to penetrate into the cytoplasm. They further evaluated the anti-metastatic activity of STA-12-7191 (**4**) against highly invasive cancer cell lines MDA-MB-231 and A172 GBM, which were reported to highly express eHsp90 (McCready et al. 2010). The study demonstrated that STA-12-7191 (**4**) efficiently hampered cancer cell migration by inhibiting eHsp90.

Recently, a number of patents have been published that report antibody-based therapeutic applications for the treatment of metastatic cancers. They claim that cell-impermeable anti-Hsp90 antibodies inhibit eHsp90 functions and can be applied for the treatment of a range of cancers (Sidera and Patsavoudi 2014).

Mitochondrial chaperone Trap1

Trap1 is a molecular chaperone with prevalent mitochondrial localization (Felts et al. 2000). Trap1 and Hsp90 share high amino acid sequence identity, molecular chaperone functions, and quaternary structure similarity (Song et al. 1995; Chen et al. 2005; Leskovar et al. 2008). Both Trap1 and Hsp90 have an ATP-binding site in the N-terminal domain, and the process of ATP binding and hydrolysis is required for Hsp90 and Trap1 chaperone function. Trap1 is synthesized in the cytoplasm, transferred to the mitochondria, and maturated by cleaving off the mitochondrial targeting sequence at the N-terminus (Song et al. 1995; Chen et al. 1996, 2005; Schleiff and Becker 2011). Trap1 possesses anti-apoptotic and antioxidant properties. Several studies have demonstrated that Trap1 plays a critical role in protecting mitochondrial integrity from reactive oxygen species (ROS)-mediated lethal stress (Masuda et al. 2004; Montesano Gesualdi et al. 2007). ROS production induces oxidative stress, promotes mitochondrial dysfunction and cell death, and is associated with diverse human diseases, such as heart attack, stroke, cancer, and neurodegenerative diseases (Patten et al. 2010; Sotgia et al. 2011). Trap1 regulates ROS metabolism and chaperones denatured proteins in mitochondria. Moreover, Trap1 is upregulated in several human malignant tumors, such as prostate, colorectal, nasopharyngeal, and ovarian carcinomas (Fang et al. 2008; Costantino et al. 2009; Landriscina et al. 2010; Leav et al. 2010). Nonetheless, the physiological function and precise mechanism of Trap1 are still open to exploration. Collectively, the anti-apoptotic function and the overexpression of Trap1 in human malignancies strongly suggest that Trap1 could serve as a potential target in the war on cancer.

Inhibitors selectively targeting mitochondrial Hsp90 and Trap1

In 2007, Kang et al. used a novel approach to deliver a small-molecule Hsp90 inhibitor to mitochondria and discovered two mitochondrial Hsp90 or Trap1-specific inhibitors, gamitrinib-$G_{1\text{-}4}$ (**5**) and gamitrinib-TPP (**6**), as shown in Fig. 5 (Kang et al. 2007). They attached a mitochondria-accumulating moiety, one to four repeat units of cyclic guanidinium or triphenylphosphonium to the prototype Hsp90 inhibitor, geldanamycin, to synthesize gamitrinib-$G_{1\text{-}4}$ (**5**) and gamitrinib-TPP (**6**). Interestingly, they demonstrated that conventional Hsp90 inhibitors did not directly affect the function of mitochondrial Hsp90 and Trap1 nor induce mitochondrial dysfunction, probably owing to the incapability of penetrating the mitochondrial membrane, but their mitochondria-targeted inhibitors directly inhibited mitochondrial Hsp90 and Trap1 function. Therefore, to direct Hsp90 inhibitors to the mitochondrial matrix and induce mitochondrial membrane permeability, it is indispensable to tag a mitochondria-accumulating moiety onto the conventional Hsp90 inhibitors.

The pioneering work done by Kang et al. demonstrated that Trap1 plays a critical role in regulating the opening of the mitochondrial transition pore induced by excessive ROS production (Kang et al. 2007, 2009; Kang and Altieri 2009). CypD is a peptidyl-prolyl *cis, trans* isomerase that is considered to be involved in the mitochondrial permeability transition. It is reported that elevated ROS generation and ATP depletion activate CypD to switch the permeability pore open, release cytochrome c, and ultimately lead to cell death (Baines et al. 2005; Basso et al. 2005; Nakagawa et al. 2005). The study demonstrated that mitochondrial Hsp90 and Trap1 directly interact with CypD, and hamper the pore opening triggered by CypD activation, which is considered as an essential survival mechanism of various cancer cells (Fig. 6). MTT assay demonstrated that Trap1-specific inhibitor, gamitrinib-G_4, showed good anti-proliferative activity ($IC_{50} = 4.0$ μM) against squamous cell carcinoma, A431. As a result, inhibition of mitochondrial Hsp90 and Trap1 by the

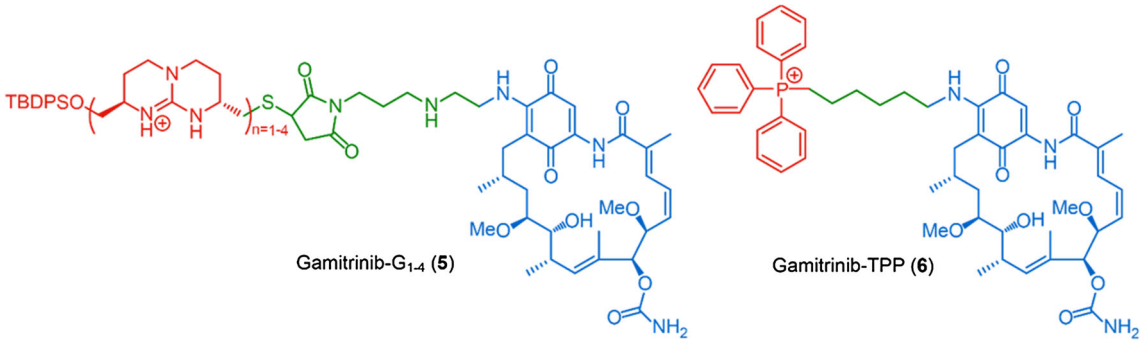

Fig. 5 Structures of mitochondria-targeted Hsp90 (Trap1) inhibitors. The mitochondria-targeting module, the linker, and the Hsp90-inhibiting module (geldanamycin) are illustrated in *red*, *green*, and *blue*, respectively. *Hsp* heat shock protein, *Trap* tumor necrosis factor receptor-associated protein

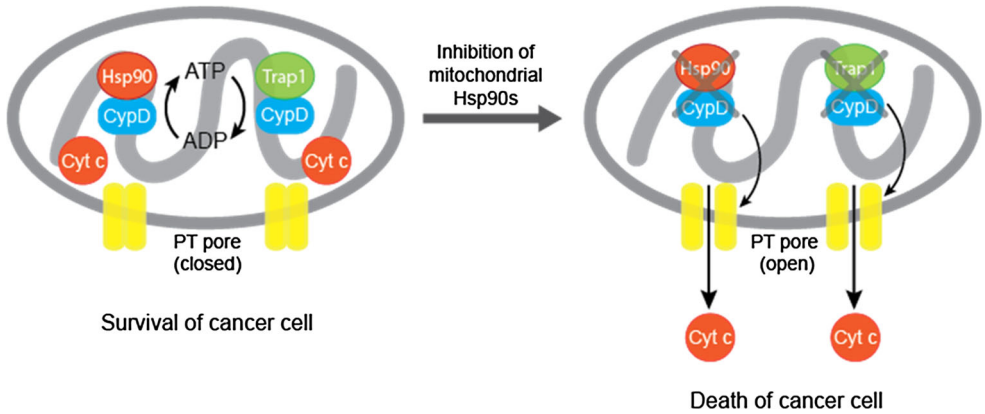

Fig. 6 Mitochondrial Hsp90 and Trap1 regulate cancer cell survival. Mitochondrial Hsp90 and Trap1 form a complex with CypD and antagonize its function. Inhibition of mitochondrial Hsp90 and Trap1 promote the activation of CypD, lead to mitochondrial PT pore opening, and release Cyt c, thus inducing cancer cell death. *ADP* adenosine diphosphate, *ATP* adenosine triphosphate, *CypD* cyclophilin D *Cyt c* cytochrome c, *Hsp* heat shock protein, *PT* permeability transition, *Trap* tumor necrosis factor receptor-associated protein

mitochondria-specific Hsp90 inhibitors gamitrinib-G_{1-4} (**5**) or gamitrinib-TPP (**6**) promoted the activation of CypD, mitochondrial permeability transition pore opening, released cytochrome c, and induced cancer cell death.

Concluding remarks

Hsp90 is responsible for diverse cellular functions and pathophysiological processes in various cancer cells, and Hsp90 has therefore become an active molecular target for the treatment of cancers. Despite the enormous amount of studies that have been conducted to discover Hsp90 inhibitors for clinical application, there are no FDA-approved Hsp90 inhibitors available today. Organelle-specific inhibition of Hsp90 is an interesting area, not only for scientific research but also for therapeutic applications. Extracellular and mitochondrial Hsp90 inhibitors efficiently impair Hsp90 functions in an organelle-specific way, and thereby minimize lethal damage to normal cells. Accordingly, this alternative strategy provides a potential breakthrough in overcoming the long-standing challenge of conventional pan-Hsp90 inhibitors of toxicity versus efficacy and has afforded an opportunity for the development of Hsp90 inhibitors with clinical applications. In this regard, the technology to deliver Hsp90 inhibitors to particular cellular compartments provides an exciting novel weapon to selectively deal with subcellular localized Hsp90 isoforms. The technology has not only provided meaningful therapeutic outcomes for the treatment of cancer but has also provided biological tools to reveal the physiological functions of individual Hsp90 isoforms. In this review, we provided an overview of current efforts to discover organelle-specific Hsp90 inhibitors, specifically against eHsp90 and Trap1, and we discussed their biological insights and therapeutic implications.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest with any person or any organization.

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