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Invited Mini Review

TRAP1 regulation of mitochondrial life or death decision in cancer cells and mitochondria-targeted TRAP1 inhibitors

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Hsp90 is one of the most conserved molecular chaperones ubiquitously expressed in normal cells and over-expressed in cancer cells. A pool of Hsp90 was found in cancer mitochondria and the expression of the mitochondrial Hsp90 homolog, TRAP1, was also elevated in many cancers. The mitochondrial pool of chaperones plays important roles in regulating mitochondrial integrity, protecting against oxidative stress, and inhibiting cell death. Pharmacological inactivation of the chaperones induced mitochondrial dysfunction and concomitant cell death selectively in cancer cells, suggesting they can be target proteins for the development of cancer therapeutics. Several drug candidates targeting TRAP1 and Hsp90 in the mitochondria have been developed and have shown strong cytotoxic activity in many cancers, but not in normal cells *in vitro* and *in vivo*. In this review, recent developments in the study of mitochondrial chaperones and the mitochondria-targeted chaperone inhibitors are discussed [BMB reports 2012; 45(1): 1-6]

INTRODUCTION

After exposure to proteotoxic insults such as heat and oxidative stresses, cells elevate the expression of a set of proteins, collectively called heat shock proteins (Hsp), as a protective mechanism (1). The inducible heat shock proteins and their cognate constitutively expressed members are recognized as molecular chaperones (2). The most fundamental functions of these chaperones are to fold and maintain the proper conformation of other proteins, named clients, and to guard them from misfolding and aggregation, consequently regulating the cellular function of clients (3). The 90-kDa heat shock protein, Hsp90, is one of the most conserved heat shock proteins, found in bacteria and all eukaryotes, and is ubiquitously expressed in cells (4, 5). Hsp90 is an essential component of the protective heat shock responses, and participates in stabilizing and regulating many client proteins involved in cellular signaling networks (6).

Cancer cells reside in harmful and stressful environments, wherein they express higher levels of Hsp90 and other chaperone molecules than normal cells, in order to sustain viability (3). Hsp90 is critical for tumor cells to tolerate the stressful environments which induce protein unfolding, and to maintain the function of accumulated mutant proteins produced during tumorigenesis (6, 7). Many client proteins stabilized by Hsp90 in cancer cells are associated with, and regulate, the proteins in tumorigenic signaling pathways related to cell proliferation, evasion of apoptosis, gene replication, angiogenesis, and metastasis (8). Due to this nodal regulatory function of Hsp90 in malignant transformation, Hsp90 has become a target protein for development of anticancer therapeutics (9). Various classes of Hsp90 inhibitors have been developed, and some of them are already in clinical trials (10).

The Hsp90 family in mammalian cells is composed of 4 major homologs: Hsp90α (inducible form) and Hsp90β (constitutive form) are cytosolic isoforms; the 94kDa glucose-regulated protein (GRP94) is localized to the endoplasmic reticulum, and TRAP1 resides in the mitochondrial matrix (6, 11). In this review, recent progress concerning mitochondrial Hsp90 studies associated with cytoprotective functions in cancer cells, and the development of mitochondria-targeted inhibitors will be discussed.

TRAP1: HOMOLOGOUS TO Hsp90 BUT FUNCTIONALLY DIFFERENT FROM Hsp90

The tumor necrosis factor (TNF) receptor-associated protein 1 (TRAP1), alternatively known as heat shock protein 75 (Hsp75), was initially identified as an Hsp90 homolog interacting with the TNF receptor (12) and the retinoblastoma protein (Rb), irrespective of mitochondrial location (13). However, subsequent careful analyses of the TRAP1 cDNA sequence and subcellular localization using biochemical and microscopic techniques have proven the existence of a mitochondrial targeting sequence at its N-terminal end and mitochondrial accumulation of the protein (14).

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TRAP1 is a mitochondrial homolog of Hsp90

Hsp90 has 3 domains which are resistant to proteolysis; the N-terminal domain, the middle segment, and the C-terminal domain, or briefly, the N, M, and C domains (4). TRAP1 and Hsp90 share conserved domain architectures with high amino acid sequence similarity, molecular chaperone functions, and homo-dimeric quaternary structures (11-13, 15). Apart from the structural similarity between them, TRAP1 has the additional mitochondrial targeting sequence at its N-terminus, which is cleaved off after mitochondrial translocation, and lacks the highly charged flexible region between the N and M domains of Hsp90 (11-13).

The N domain contains an ATP binding site and is the most conserved region between Hsp90 and TRAP1 (12, 13). Both chaperones have ATPase activities and are inhibitable by a prototype Hsp90 inhibitor, geldanamycin, at a comparable working concentration (14). This indicates that Hsp90-targeted drugs designed to occupy the ATP pocket in the Hsp90 N domain can also inhibit the TRAP1 chaperone function *in vitro*. The common Hsp90 inhibitors, however, cannot compromise the function of TRAP1 *in vivo* due to its mitochondrial location (16, 17).

TRAP1 and Hsp90 in mitochondria have disparate properties compared to cytosolic Hsp90

Each organelle contains its own unique set of proteins and specialized distribution of biomolecules. Thus, though they are homologous to each other, TRAP1 in the mitochondrial microenvironment could have different cellular functions from cytosolic Hsp90, due to the potential differential distribution of interactors, such as clients and regulators of the chaperone located inside and outside of the mitochondria. TRAP1 does not interact with the co-chaperones or the client proteins of Hsp90 *in vitro*, which further implies the regulation and function of TRAP1 in mitochondria are different from those of Hsp90 (14). A pool of Hsp90 is also found inside mitochondria in various cancer cells and some normal mouse tissues (16), and is likely to be modulated differentially from cytosolic Hsp90 for the same reason. The mitochondrial Hsp90 does not form protein complexes with TRAP1, even though it is compartmentalized in the same place; the mitochondrial matrix (16). Considering no unique functions have been assigned to either of them at this point, TRAP1 and Hsp90 seem to work independently and be functionally redundant in mitochondria.

THE FUNCTION OF TRAP1 AND Hsp90 IN MITOCHONDRIA

TRAP1 is synthesized in the cytoplasm, translocated into the mitochondria, and maturated by cleaving off the N-terminal mitochondrial targeting sequence (18). A pool of mitochondrial TRAP1 seems to be exported from the mitochondria to interact with extramitochondrial proteins such as a TNF receptor (12), retinoblastoma protein (13), and tumor suppressor EXT proteins (19). Even with a mitochondrial targeting sequence at their N-terminus, the extramitochondrial location of other mitochondrial chaperones, such as Hsp60 and mortalin, is often found in many cancer cells (20). Whereas its functional roles in maintaining mitochondrial integrity in stressful environments and inhibiting cell death have been well documented, the exact export mechanism and cytoplasmic functions of TRAP1 are not fully understood.

TRAP1 protects mitochondria from oxidative stresses

Excessive production of oxygen-containing reactive free radicals induces oxidative stress and can be the cause of mitochondrial dysfunction and cell death related to various human diseases, including neurodegeneration, heart attack, stroke, and cancers (21, 22). Much literature has demonstrated that TRAP1 plays an important role in inhibiting cell death caused by a reactive oxygen species (ROS) (23, 24). Silencing TRAP1 through siRNA increases ROS accumulation, whereas TRAP1 over-expression decreases ROS production (25, 26). Granzyme M, a serine protease capable of inducing apoptosis, can cleave TRAP1 to compromise the ATPase activity and abolish its antagonistic functions against ROSs, resulting in ROS accumulation and cell death (25). Thus, as a molecular chaperone, TRAP1 prevents damaged proteins from unfolding and refolds denatured proteins (27), regulates ROS metabolism to antagonize ROS production, and thereby maintains the integrity of the mitochondria under oxidative stress.

TRAP1 modulates the permeability transition pore to inhibit cell death

ROS production can trigger cell death by opening the permeability transition (PT) pore in the mitochondrial inner membrane (28). The pore opening results in the loss of mitochondrial inner membrane potential, mitochondrial swelling, and a concomitant rupture of the outer membrane, which results in necrotic or apoptotic cell death (29). Initially, the molecular identity of the PT pore had been considered to consist of the protein complex of the voltage-dependent anion channel (VDAC) and the adenine nucleotide translator (ANT), which are located in the mitochondrial outer and inner membranes, respectively (30). Later, it was clearly shown that the mitochondrial PT pore was still operational, even after the genetic deletion of VDAC or ANT genes, suggesting they are not the sole PT pore components (31, 32). In contrast to the ambiguous molecular identity of membranous PT pore components, a series of knockout mouse studies showed that a mitochondrial matrix protein, cyclophilin D (Cyp-D), plays an important role in the formation of the pore in the mitochondrial inner membrane (33-35). Cyp-D is a peptidyl-prolyl cis-trans isomerase presumed to catalyze protein folding and is located in the mitochondrial matrix where it interacts with the PT pore components in the inner membrane (36). Once activated under stress conditions, such as elevated ROS/calcium and depleted ATP, Cyp-D switches the conformation of proteins constituting

the PT pore to generate non-selective pores through the membrane (29).

TRAP1 and the mitochondrial pool of Hsp90 directly interact with Cyp-D and modulate its function, i.e. inhibit the pore opening triggered by Cyp-D activation, which confers the various cell types resistance to a variety of stresses, and is therefore an important survival mechanism in cancer cells (16, 37) (Fig. 1). The ATPase activity of the chaperones is critically involved in the inhibition of Cyp-D function and concomitant PT pore formation. The protein-protein interaction between Hsp90/TRAP1 and Cyp-D, however, is not important because it was maintained even after treatment with inhibitors compromising the protective function of the mitochondrial Hsp90/TRAP1 (16). This indicates that the protein folding and refolding activities of the chaperones, not the protein interaction *per se*, seems to be critical in regulating Cyp-D functions involving the conformational switch of PT pore components.

Regulation of TRAP1 and mitochondrial Hsp90

Though the regulation of TRAP1 expression is not fully understood, several lines of evidence suggest a close relationship between TRAP1 expression and tumorigenesis. Expression analysis with oligonucleotide microarrays revealed that TRAP1 is one of the target genes elevated by the Myc oncogene (38). TRAP1 was over-expressed in primary human fibroblasts and mouse prostate by SV40-induced malignant transformation (39, 40). Various cancer cell lines, including mouse adenocarcinoma, and specimens from human cancer patients also consistently showed over-expression of TRAP1, while it remained undetectable in normal cells and tissues (16, 40).

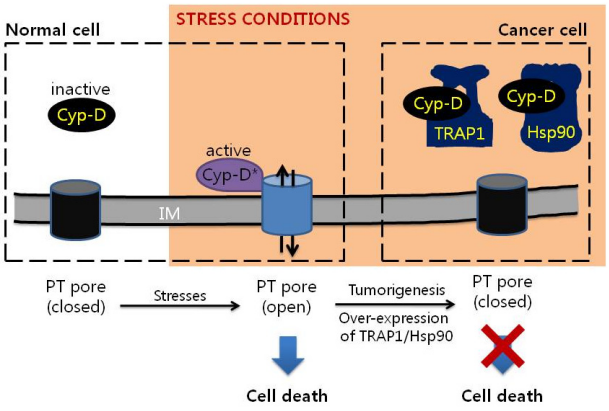

Fig. 1. Regulation of a PT pore in a mitochondrial inner membrane (IM). Under normal physiological conditions, the Cyp-D in a mitochondrial matrix is inactive (black) and the PT pore is closed (black). Under stress conditions, the Cyp-D is activated (purple) and interacts with the PT pore components in the IM, which triggers opening of the pore (blue) and results in the increase of non-selective permeability in the IM, swelling of the mitochondria, rupture of the mitochondrial outer membrane, and concomitant induction of cell death. In cancer cells, however, though they are constantly exposed to the same unfavorable conditions, the PT pores remain in a closed state due to the elevated expression of chaperones such as TRAP1 and Hsp90 in the mitochondrial matrix, which inhibits the pore-forming function of the Cyp-D through direct interactions.

The elevated expression of Hsp90 in the mitochondria of many cancer cells is readily observed (16). The transport mechanism of cytoplasmic Hsp90 into the mitochondria, however, is currently unclear. It is possible that machineries involved in the preprotein import are modified to enhance the mitochondrial transport of Hsp90. This may be associated with the functional modifications of unidentified cytosolic regulatory factors regulating protein trafficking during malignant transformation (41).

Post-translational modification, such as phosphorylation, is an important means to regulate the protein function *in vivo*. Phosphorylation of TRAP1 by PTEN induced putative kinase 1 (PINK1) is responsible for the protection of ROS mediated cell death (42). It is likely that a PINK1-TRAP1 pathway affects mitochondrial integrity by regulating the Cyp-D function, but the potential regulatory pathway has not yet been fully investigated. In addition to phosphorylation, considering the regulation of Hsp90 functions by other post-translational modifications such as acetylation and nitrosylation in cytoplasm (43), we cannot exclude the possibility that such modifications also occur and critically affect the function of mitochondrial chaperones under normal or pathological conditions.

INHIBITORS TARGETING MITOCHONDRIAL TRAP1 AND Hsp90 AS CANCER THERAPEUTICS

TRAP1 and mitochondrial Hsp90 critically inhibit cell death in cancer cells

Cancer cells are under stress conditions due to the insufficient supply of oxygen and an increased metabolic demand compared to normal cells, which, without protective mechanisms, could easily trigger opening of the PT pore (29, 44). TRAP1 over-expression can be one of the protective mechanisms adopted during tumor progression and is closely related to the multi-drug resistance commonly found in cancer cells (24, 45). Loss of the chaperone functions directly induce mitochondrial membrane permeabilization (MMP), followed by immediate cell death in many cancer cells, but not in normal cells. This strongly argues that the chaperone networks operate in a cancer-specific manner and can be the Achilles heel of many cancer cells (37).

Cytosolic Hsp90 has the role of chaperoning labile mutated proteins in cancer cells to allow them to be functional, otherwise they are aggregated and inactivated (3). Similarly, ablation of the chaperone function of TRAP1 and mitochondrial Hsp90 by pharmacological reagents or genetic methods increased aggregation of many proteins in cancer mitochondria and triggered unfolded protein responses (UPR). This further affected cytosolic survival pathways including NF-κB signaling and increased the sensitivity to anticancer therapeutics (27, 46, 47),

Therefore, in addition to the intra-mitochondrial triggering of cell death programs, targeted inhibition of TRAP1 and Hsp90 in mitochondria results in the perturbation of global survival pathways outside the mitochondria in cancer cells, further strengthening the rationale of targeting mitochondrial Hsp90 and TRAP1 for cancer therapies.

Gamitrinibs accumulate in mitochondria and inhibit TRAP1 and Hsp90

Widely used conventional Hsp90 inhibitors may be effective in inhibiting the function of mitochondrial TRAP1 and Hsp90, as long as they are delivered into the mitochondria. In practice, those inhibitors were unable to pass through the mitochondrial membranes, which suggests they neither affect the function of the chaperones therein, nor induce direct organelle dysfunction, *i.e.* MMP (37). To make them reach the target proteins and directly induce MMP, it is required to use a drug delivery system targeted to the mitochondria. Gamitrinibs are the first mitochondria-targeted small molecules which inhibit TRAP1 and Hsp90 inside the mitochondria (17). Gamitrinibs directly induced MMP, resulting in loss of mitochondrial inner membrane potential and discharge of cytochrome c into the cytosol, followed by extensive cell death in many cancer cells (17, 48, 49).

Gamitrinibs are composed of 3 modules: 1) a mitochondrial targeting module, cyclic guanidinium or triphenylphosphonium, 2) the prototype Hsp90 inhibitor, geldanamycin, and 3) short linkers connecting between them (17). The design of Gamitrinibs was originated and developed from the peptide-based Hsp90 inhibitor, shepherdin, and the peptide-chemical hybrid compound, Ant-GA (Fig. 2). Both molecules use a cell penetrating peptide (CPP) sequence, helix III of the Antennapedia homeodomain protein, to pass through the plasma membrane and deliver the inhibitory sequence inside the cell, and unexpectedly, were delivered and accumulated inside the mitochondria (16, 50).

The major prominent difference between CPP-containing and mitochondria-targeted inhibitors is the effect on the Hsp90 in the cytoplasm. Gamitrinibs have no effect on cytosolic Hsp90, and thereby neither change client expression nor induce heat shock responses. Meanwhile, CPP-containing inhibitors compromise the function of cytoplasmic Hsp90, which, as a result, elevates the expression of Hsp70 as a heat shock response and degrades client proteins (17). Gamitrinibs show much improved anticancer activity compared to the CPP-containing shepherdin and Ant-GA when they are used to treat cancer cell types *in vitro* or mouse tumor xenografts *in vivo*, probably due to the effective accumulation of the drugs in the target organelle mitochondria (16, 17).

Hence, when considering broad and selective cytotoxic activities in various cancer cells *in vitro* and *in vivo*, targeting the mitochondrial chaperone is a feasible strategy to develop a novel class of anticancer drugs such as Gamitrinibs, with potent anticancer activity and a unique mechanism of action (37, 51).

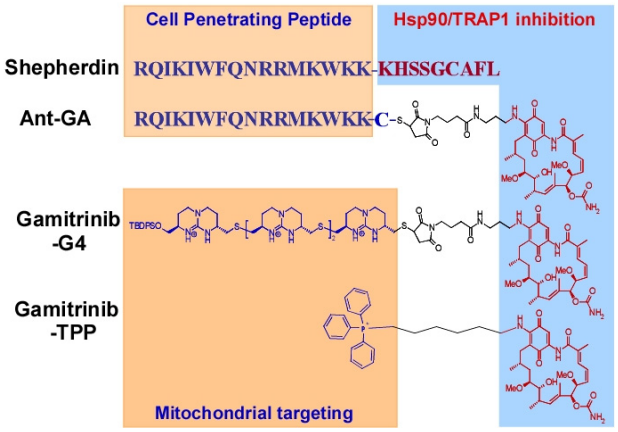

Fig. 2. Mitochondria-targeted Hsp90/TRAP1 inhibitors. Both shepherdin and Ant-GA have an amino acid sequence of cell penetrating peptide, RQIKIWFQNRRMKWKK. Shepherdin contains an amino acid sequence from survivin, KHSSGCAFL, which is involved in the interaction with Hsp90, and Ant-GA has a prototype Hsp90 inhibitor, geldanamycin, to inhibit Hsp90 and TRAP1. Gamitrinib-G4 and TPP adopted a modular structure: 1) mitochondrial targeting module, tetracyclicguanidinium or triphenylphosphonium, 2) Hsp90/TRAP1 inhibition module, geldanamycin, and 3) linkers connecting both of them.

CONCLUSIONS

A growing body of evidence now supports the idea that TRAP1 is associated with important human diseases including cancer and neurodegeneration (37, 42). However, the molecular regulatory mechanisms and signaling pathways affecting the chaperone functions are largely unknown at this point. Considering the dynamic chaperone complexes in the regulation of Hsp90 in cytoplasm, there may be many unidentified post-translational modifications and protein interaction networks involving regulatory proteins or co-chaperones which modulate mitochondrial chaperones. Understanding the precise mechanisms of mitochondrial chaperones can shed light not only on the molecular mechanisms of mitochondrial transformation caused by disease onset and progression, but also the therapeutic value of mitochondria-targeted inhibitors in treating human diseases. Furthermore, considering the mitochondria-targeted delivery and direct induction of MMP, Gamitrinibs could sensitize the mitochondria to a variety of cell death stimuli using an entirely different mechanism from other cancer drugs. Thus, a combination treatment of Gamitrinibs with other anticancer drugs currently in the clinic should be considered to formulate better therapeutics to fight cancer.

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