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Hsp90 Inhibitors Disrupt Mitochondrial Homeostasis in Cancer Cells

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Hsp90 is a cytosolic molecular chaperone whose paralog in mitochondria, TRAP1, protects cells from oxidative stress. The recent study in *Cell* by Kang et al. [1] now identifies the molecular components of the proapoptotic network regulated by TRAP1, that includes Hsp90. Targeting Hsp90/TRAP1 inhibitors to mitochondria induces rapid tumor cell-specific apoptosis.

The family of mammalian heat shock protein (Hsp) 90 molecular chaperones includes the cytosolic Hsp90α (stress-inducible) and β (constitutive) isoforms, the endoplasmic reticulum (ER)-restricted glucose-regulated protein (Grp) 94, and the mitochondrially localized paralog TRAP1. Among the family, the function(s) of cytosolic Hsp90 have been most intensively investigated. These include regulating the stability and activity of between one and two hundred cytosolic and nuclear ''client'' proteins among which are numerous protein kinases, transcription factors, and other proteins that serve as nodal points which integrate cellular responses to multiple extracellular signals. Thus, cytosolic Hsp90 function is a key component underlying maintenance of cellular homeostasis in the face of environmental fluctuation. This is particularly important to cancer cells, which frequently face both noxious extracellular (e.g., hypoxia) and intracellular (e.g., protein misfolding) environments.

Hsp90 is a conformationally flexible protein that associates with a distinct set of cochaperones depending on ATP or ADP occupancy of an amino-terminal binding pocket. Nucleotide exchange and ATP hydrolysis by Hsp90 itself, with the assistance of cochaperones, drive the Hsp90 chaperone machine to bind, chaperone, and release client proteins. Nucleotide-dependent cycling of the cytosolic Hsp90 chaperone machine is critical to its function. Pharmacologic inhibitors of Hsp90's nucleotide binding prevent chaperone cycling and generally promote the proteasome-mediated degradation of client proteins [2]. Because of the diverse functions of its numerous client proteins, Hsp90 inhibition is predicted to impact all of the six hallmarks of cancer, as defined by Hanahan and Weinberg [3], and indeed these drugs have demonstrated anticancer activity in both diverse animal xenograft models and in several human clinical trials [4].

Much less is known about the function(s) of Grp94 and TRAP1. Unlike cytosolic Hsp90, neither the ER nor the mitochondrial paralog is known to interact with cochaperones nor have any of their ''client'' proteins been convincingly identified. Like Hsp90, both proteins utilize their N-terminal domains to bind and hydrolyze ATP and both proteins interact with Hsp90 inhibitors [5–7]. Although Grp94 and TRAP1 both bind N-terminal small molecule inhibitors with an affinity similar to that of cytosolic Hsp90, no significant sequellae of drug binding to these proteins have been identified. Recently, several reports have implicated TRAP1 in protecting cells from mitochondria-mediated apoptosis induced by oxidative stress [8–10]. Inactivation or depletion of TRAP1 may play a role in neurodegenerative diseases of the central nervous system [9].

In general, small molecule inhibitors of Hsp90 induce growth arrest. In certain tumor-specific instances, induction of apoptosis has been observed [11]. In contrast, Altieri and colleagues previously reported that a peptide-based Hsp90 inhibitor, shepherdin, caused rapid and extensive mitochondria-dependent apoptosis in all tumor types examined [12]. The study by Kang et al., published in the October 19, 2007, issue of *Cell*, serves to extend this initial observation and to offer an explanation for the apparent discrepancy between the cellular response to small molecule and peptide-based Hsp90 inhibitors [1]. Kang et al. also propose that mitochondrial homeostasis is differentially regulated in tumor versus normal cells, in part due to elevated expression in mitochondria not only of TRAP1 but also of Hsp90 itself.

Unlike the small molecule Hsp90 inhibitors, the peptide-based inhibitor shepherdin by itself is unable to cross the plasma membrane. In order to circumvent this problem, Altieri and colleagues included the helix III homeodomain cell-penetrating sequence from *Antennapedia* in the shepherdin peptide. The current study by Kang et al. reports that the *Antennapedia* sequence not only facilitates intracellular uptake of shepherdin but unexpectedly serves to target the peptide to mitochondria, where its accumulation results in membrane depolarization and cytochrome *c* release. In contrast, while small molecule Hsp90 inhibitors readily cross the plasma membrane, they remain primarily cytosolic and neither localize to mitochondria nor promote mitochondrial membrane depolarization. This differential intracellular localization appears to be key to the proapoptotic effects of shepherdin, since removing the *Antennapedia* sequence from shepherdin abrogates its ability to affect the membrane

permeability of isolated mitochondria. Further, coupling the prototypical Hsp90 inhibitor geldanamycin to the *Antennapedia* sequence allows the drug to efficiently penetrate the mitochondrial membrane and to promote depolarization and cytochrome *c* release similar to shepherdin. Thus, the discrepant cellular activity of shepherdin and the small molecule Hsp90 inhibitors appears due to their differential ability to penetrate into mitochondria.

In their original study, Altieri and colleagues demonstrated that the apoptosis-inducing effects of shepherdin were limited to transformed cells and that the viability of normal cells was unaffected by this Hsp90 inhibitor [12]. In the current article in *Cell* [1], Kang et al. provide one rationale for this observation. They report that mitochondria isolated from a panel of tumor cell lines express markedly elevated levels of TRAP1 and, more surprisingly, of Hsp90 itself. Examination of mitochondria isolated from a number of nontransformed fibroblast cell lines and from a panel of normal tissues reveals much reduced expression of TRAP1 and little to no detectable Hsp90. Interestingly, the sole exceptions are mitochondria isolated from mouse brain and testis, in which Hsp90 expression is readily observed. Is there a common factor that may link these observations? Because of their dependence on glycolysis and frequent exposure to fluctuating oxygen levels, tumor cells exist in a general state of oxidative stress. Because they are both subject to high energy demands, testis and brain rely heavily on aerobic glycolysis to generate large amounts of ATP, and they are also prone to oxidative stress [13, 14]. Overexpression of TRAP1 and Hsp90 may serve to buffer mitochondrial (and in the case of Hsp90 also cytosolic) proteins from direct damage caused by exposure to prolonged oxidative stress. Indeed, it is possible that appearance of Hsp90 in mitochondria of tumor cells is merely a matter of degree—because environmentally stressed tumor cells contain significantly more of the chaperone than do nontransformed cells, a proportionally greater amount of Hsp90 may be detectable in tumor mitochondria.

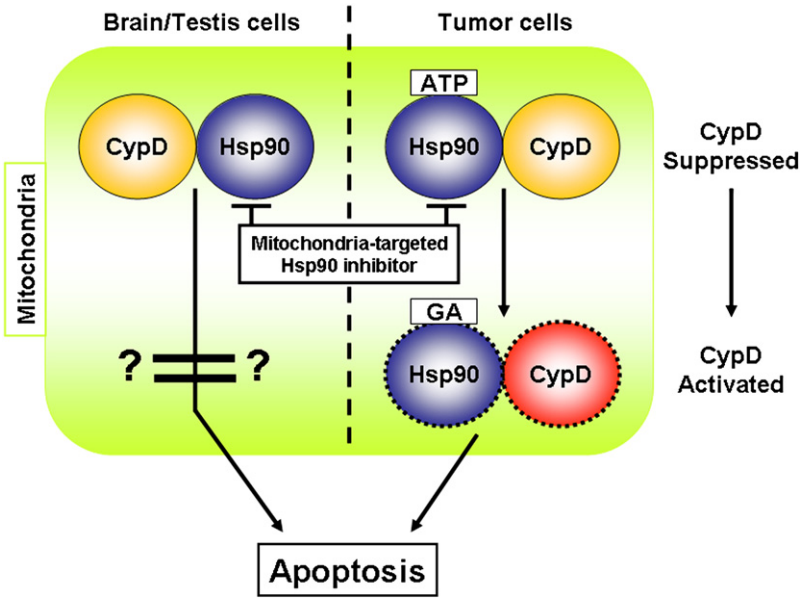

Figure 1. Hsp90 Inhibitors Trigger Mitochondrial Membrane Depolarization-Dependent Apoptosis in Tumor Cells

Based on the recent paper by Kang et al. [1], Hsp90 is overexpressed in mitochondria of tumor cells (as well as brain and testis) and associates spontaneously with the immunophilin Cyclophilin D (CypD). In the presence of Hsp90 inhibitor, which displaces ATP from Hsp90, CypD (still bound to Hsp90) becomes active and promotes mitochondrial membrane depolarization. This causes cytochrome *c* to be released into the cytosol, triggering the intrinsic apoptotic pathway [15]. Surprisingly, even though CypD is also highly expressed in neuronal mitochondria, as is Hsp90, apoptosis induced by Hsp90 inhibitors appears to be tumor-specific.

Why does the mitochondrial targeting of Hsp90 inhibitors promote apoptosis? Kang et al. implicate the mitochondrially localized immunophilin Cyclophilin D (CypD) in this phenomenon (see Figure 1). They show that CypD, a mediator of oxidative stress-induced cell death [15], associates with both Hsp90 and TRAP1 (although apparently in separate complexes) and that the CypD inhibitor Cyclosporin A (CsA) abrogates Hsp90 inhibitor-induced membrane depolarization and cytochrome *c* release. CsA prevents association of both Hsp90 and TRAP1 with CypD, suggesting that this association may be necessary for CypD activity. In support of this hypothesis, Hsp90 inhibitors do not dissociate CypD from either Hsp90 or TRAP1. How can we reconcile the observation that both Hsp90 and TRAP1 spontaneously associate with CypD, yet CypD activity is only apparent following Hsp90 inhibition? One possibility is that the nucleotide state of mitochondrial Hsp90 (and TRAP1) may be a determining factor. When associated with the ATP-bound conformation of either chaperone, the activity of CypD is suppressed. However when the ATP concentration falls to dangerously low levels or when the Hsp90/TRAP1 nucleotide binding site is occupied by an inhibitor, conformation of the chaperone/CypD complex alters to permit CypD activity. CypD may also require continued association with Hsp90/TRAP1 to deliver it to an appropriate location or protein to be modified, since Hsp90 inhibitor treatment does not dissociate CypD from Hsp90/TRAP1. Since CsA inhibits CypD-mediated membrane depolarization, it is likely that this process requires the immunophilin's peptidylprolyl isomerase activity (inhibited by CsA), which may also be necessary for association of the immunophilin with Hsp90/TRAP1. A contribution of oxidative stress to elevated chaperone expression cannot be overlooked. Certainly, some of the increased Hsp90/TRAP1 in tumor cell mitochondria may be protecting key mitochondrial proteins from oxidative damage. Is association of either Hsp90 or TRAP1 with CypD affected by the necessity

to chaperone oxidatively damaged mitochondrial proteins? If such a mitochondrial ATP- or protein damage-sensing "suicide" mechanism exists, can it be exploited to preferentially kill cancer cells, as suggested by Kang et al.?

A growing body of evidence, including the Kang et al. study, proposes a role for TRAP1 in cellular protection from oxidative stress-induced apoptosis [1, 8–10]. The identification of CypD as a TRAP1-interacting protein provides an attractive mechanism to explain these observations. As all provocative studies should, the paper by Kang et al. raises many additional questions for future exploration. Does localization of Hsp90 to mitochondria depend on oxidative stress and does it contribute to the maintenance of mitochondrial homeostasis under extreme conditions? Perhaps this possibility might be explored by determining the effect of reducing agents or free radical scavengers on Hsp90 localization (and TRAP1 expression). Why are both Hsp90 and TRAP1 necessary to promote mitochondrial homeostasis in tumor cells if they both serve to regulate the same apoptosis transducer (CypD)? How is Hsp90 targeted to mitochondria of tumor cells? Is it merely a correlative result of cellular overexpression of the chaperone or is a unique process (e.g., posttranslational modification of Hsp90) responsible? Isolation and characterization of the mitochondrial pool of Hsp90 in comparison with the cytosolic pool might address this question. Is mitochondrial homeostasis differentially susceptible to chaperone interference in certain tumor types? Small molecule Hsp90 inhibitors cause marked cytochrome *c*-dependent apoptosis in small cell lung cancer and in other neuroendocrine tumors in which the retinoblastoma protein is defective [11]. Do these drugs penetrate the mitochondria in these tumors? Interestingly, cytosolic Hsp90 negatively regulates Apaf-1 oligomerization (relieved by Hsp90 inhibition), which is mediated by cytochrome *c* released from mitochondria and is required for activation of procaspase-9 [16]. Thus, in sensitive cells Hsp90 inhibitors may impact the intrinsic apoptotic pathway at multiple points.

Lastly, it is very important to understand why neither shepherdin nor *Antennapedia*-coupled geldanamycin seem to cause membrane depolarization and cytochrome *c* release in brain mitochondrial preparations (as described in the Kang et al. study). Mitochondrial CypD is found in most if not all normal tissues, and in fact the immunophilin is abundantly expressed in neuronal mitochondria [17]. Indeed, inhibition of neuronal CypD has been suggested as an approach to prevent dopaminergic neurodegeneration associated with Parkinson disease (PD) [18]. Since TRAP1 inactivation has recently been suggested to be a determinant of PD pathogenesis [9], it will be essential to thoroughly eliminate the possibility of inadvertent activation of neuronal CypD when considering the future therapeutic benefit of targeting Hsp90 inhibitors to mitochondria. To this end, it is very important to understand why tumor cell CypD appears to be preferentially sensitive to activation by Hsp90 inhibitors directed to this subcellular compartment.

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