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Extracellular Hsp90 (eHsp90) as the Actual Target in Clinical Trials: Intentionally or Unintentionally

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Abstract

Despite extensive investigative studies and clinical trials over the past two decades, we still do not understand why cancer cells are more sensitive to the cellular toxicity of Hsp90 inhibitors than normal cells. We still do not understand why only some cancer cells are sensitive to the Hsp90 inhibitors. Based on studies of the past few years, we argue that the selected sensitivity of cancer cells to Hsp90 inhibitors, such as 17-*N*-allylamino-17-demethoxygeldanamycin, is due to inhibition of the extracellular Hsp90 (eHsp90) rather than intracellular Hsp90 by these inhibitors. Because not all tumor cells utilize eHsp90 for motility, invasion and metastasis, only the group of “eHsp90-dependent” cancer cells is sensitive to Hsp90 inhibitors. If these notions prove to be true, pharmaceutical agents that selectively target eHsp90 should be more effective on tumor cells and less toxic on normal cells than current inhibitors that nondiscriminatively target both extracellular and intracellular Hsp90.

1. INTRODUCTION

The 90-kDa heat shock protein (Hsp90) was initially reported half a century ago as an intracellular protein whose cellular level increases in response to heat (Ritossa, 1996). Since then, Hsp90 has been found to be present in most cells and has been characterized as an intracellular chaperone protein that assists the conformational activation of a long list of client proteins under both physiological and stress conditions (Young et al., 2001; Whitesell and Lindquist, 2005). Since Hsp90 does not exhibit the conventional traits of an oncogene, for a long time it was not considered as a tumor-specific target for therapeutics. This status of Hsp90, however, started to change in the early 1990s. During this period, anticancer drugs targeting a single oncogene faced the common problem of cancer plasticity and drug resistance. Therefore, alternative strategies for cancer therapeutics had been sought. It was noticed that cancer drug resistance is due to either additional mutations in the same target gene, such as the case of imatinib that targets BCR-ABL’s tyrosine kinase, or activation of

independent pathway(s) in the same cells (Neckers and Neckers, 2002; Workman, 2004; Workman et al., 2007). Therefore, it became desirable to search for a single drug target with combined effects, i.e. inhibition of this target would simultaneously shut down multiple cellular signaling pathways related to the established hallmarks of cancer (Hanahan and Weinberg, 2011). Concurrently, the Hsp90 field was rapidly expanding the list of its “client” proteins, many of which play a critical role in the survival, migration, proliferation, differentiation and apoptosis of the cells, such as ErbB2, MET, RAF, AKT, BCR-ABL, CDK4, and HIF-1 α (Isaacs et al., 2003; Whitesell and Lindquist, 2005; Powers and Workman, 2006). Neckers et al. provided the first evidence that Hsp90 played a role in maintaining the oncogenic function of an oncogene. They analyzed Hsp90 in v-src-induced cell transformation and found that treatment with inhibitors such as geldanamycin (GA) and radicicol (RD) interfered with the formation of v-src and Hsp90 heteroprotein complex and reverted v-src-induced cell transformation (Whitesell et al., 1994). In line with subsequent studies from many other laboratories, a consensus arose that Hsp90 acts as a “nodal protein” in multimolecular complex formation required for oncogene-mediated transformation (McClellan et al., 2007). This finding laid the foundation for the concept that inhibiting the ATPase of Hsp90 will lead to simultaneous collapse of multiple signaling pathways in cancer cells. This new strategy was thought to be able to better deal with plasticity and drug resistance of the cancer cells (Workman, 2004).

Many cancer cell lines were reported to express 2- to 10-fold higher levels of Hsp90 than their normal counterparts, providing an additional support for targeting Hsp90 in cancers (Isaacs et al., 2003; Banerji, 2009). Even in some cancer cells that do not have an elevated level of Hsp90, Hsp90 appeared to be more active than in normal cells. Kamal et al. reported that Hsp90-client complexes in tumor cells have 100-fold higher affinity for 17-*N*-allylamino-17-demethoxygeldanamycin (17-AAG), an analog of GA, and ATP than normal cells. Results of their competition-binding assays showed that 17-AAG inhibited biotinylated GA binding to Hsp90 in BT474 breast cancer cells with an IC₅₀ (half maximum inhibitory concentration) of 6 nM, in comparison to binding to Hsp90 from normal dermal fibroblast (NDF, IC₅₀ = 400 nM) and primary human renal epithelial cells (RPTEC, IC₅₀ = 350 nM). Furthermore, they showed that tumor-cell-derived Hsp90 bound ATP with higher affinity (IC₅₀ = 100 μ M) than Hsp90 from normal cells (IC₅₀ = 1000 μ M in NDF and IC₅₀ = 2900 μ M in RPTEC). Finally, coimmunoprecipitation experiments revealed that Hsp90 from tumor cells form more complexes than Hsp90 from normal cells (Kamal et al., 2003).

Pharmacokinetic and pharmacodynamic studies of 17-DMAG, a water-soluble form of 17-AAG, revealed that 17-DMAG (dimethylaminoethylamino-17-demethoxygeldanamycin-N-oxide) was retained longer in MDA-MB-213 human breast cancer xenografts than in normal tissues (Eiseman et al., 2005). Similarly, two other groups observed that although Hsp90 is ubiquitously expressed in most cell types, 17-AAG preferentially accumulates in human tumor xenografts (Egorin et al., 1998; Chung et al., 2003). Moreover, Vilenchik et al. showed that PU24FCI, another GA-derived inhibitor, accumulated in tumors, but not in normal tissues, and exerted a strong antitumor activity (Vilenchik et al., 2004). Taken together, these studies suggested that multiple Hsp90-client protein complexes are found in tumor cells that exhibit higher biochemical activity and increased binding affinity to 17-

AAG. These results were interpreted as the reason behind tumor cells being more sensitive to the ansamycin inhibitors.

Despite these encouraging preclinical results, the effects of the GA-derived anti-Hsp90 inhibitors in anticancer clinical trials have not been as strong as they were predicted. The stability and solubility of the ansamycin inhibitors in patients are believed to be among those intrinsic problems. Perhaps more importantly, there is still a deficiency in understanding the biology of Hsp90, including differences between Hsp90 α and Hsp90 β isoforms and the recent discovery of extracellular Hsp90 (eHsp90) in the pathogenesis of cancer (Ferrarini et al., 1992; Eustace and Jay, 2004; Tsutsumi and Neckers, 2007; Schmitt et al., 2007; Gopal et al., 2011; Li et al., 2012a,b). These studies raised a provocative and previously unrecognized possibility that the GA inhibitors in clinical trials simultaneously targeted both intracellular and eHsp90 proteins. Furthermore, the anticancer effect of these GA inhibitors might be in fact due to their inhibition of the eHsp90, instead of the intracellular Hsp90 chaperone. In this review, we provide our analyses of the studies that challenge the notion that Hsp90 proteins are chaperones anywhere and under all circumstances. We suggest that intracellularly and extracellularly localized Hsp90 molecules carry evolutionarily distinct functions.

2. HSP90 α VERSUS HSP90 β

Vertebrates have two cytosolic Hsp90 genes (Hsp90 α and Hsp90 β), which share 86% amino acid (aa) identity and are ubiquitously expressed in all nucleated cells. In addition, another cytosolic N-terminal ATPase-free Hsp90, Hsp90N, and two organelle-residing isoforms, Grp94 and TRAP1, are grouped into the Hsp90 family (Sreedhar et al., 2004). However, Zurawska et al. subsequently reported that Hsp90N was either a cDNA artifact or a chromosomal translocation product only from that particular cell line (Zurawska, 2008). Here we discuss studies that directly addressed differences between Hsp90 α and Hsp90 β at developmental and cellular levels.

2.1. Roles in Mouse Development

Voss et al. reported that Hsp90 β -knockout mice showed a primary defect in the allantoic membrane, which results in embryonic lethality (Voss et al., 2000). In addition to lack of continued studies following the initial report, concerns with the study by Voss et al. include lack of validation that Hsp90 β was truly nullified in the knockout mice and verification for unaffected presence of Hsp90 α at mRNA or protein levels. Nonetheless, this finding suggests that Hsp90 β is essential for life and implies one of the following two possibilities: (1) the role of Hsp90 β is distinct and it cannot be replaced by Hsp90 α or (2) Hsp90 β and Hsp90 α together make up a threshold of the activity that carries out the same functions. Therefore, reduction in either Hsp90 β or Hsp90 α level would show defects. However, the phenotype of Hsp90 α -knockout mice did not support the “threshold” possibility that presence of both Hsp90 β and Hsp90 α is necessary to avoid lethality.

Three articles on Hsp90 α -knockout mice have recently appeared. Picard's group first reported generation of the knockout mice carrying a gene trap insertion in intron 10 of the Hsp90 α gene. This insertion could potentially produce a truncated Hsp90 α protein lacking

the C-terminal 36 aas, but for unknown reasons it did not occur and the mice had an Hsp90 α knockout-like environment. The lack of expression of truncated Hsp90 α appeared not due to a compromised stability of the protein (such as due to failed dimerization), because Li's group detected little difference in expression between a mutant Hsp90 α with deletion of the 36 aas and the wild-type Hsp90 α in human keratinocytes (Cheng et al., unpublished). Surprisingly, except for the lack of sperms in the male mice due to an apparently higher rate of apoptosis of the spermatocytes in the testes, these Hsp90 α -knockout mice developed normally with only a slightly increased level of Hsp90 β (Grad et al., 2010). The role of Hsp90 α in spermatogenesis even in the adult mice was confirmed by an independent study (Kajiwara et al., 2012). This finding suggests that, in contrast to Hsp90 β , Hsp90 α is not essential for life and, perhaps more interestingly, its tissue-specific role could not be replaced by the presence of Hsp90 β .

During the same period of time as Picard's report, Imai et al. generated conditional Hsp90 α -knockout mice by floxing the exons 9 and 10 in Hsp90 α gene. Again, the mice showed a normal phenotype (Imai et al., 2011). In addition, there were several additional interesting observations from this study: (1) Hsp90 β takes up at least 50% of the total Hsp90 in the cells (assuming that the pan anti-Hsp90 antibody used recognized Hsp90 α and Hsp90 β with similar affinities), (2) Hsp90 α is responsible for cytosolic translocation of extracellular antigen across the endosomal membrane into the cytosol, and (3) eHsp90 α is not involved in this process. However, their inhibitor "pre-treatment" strategy would have left the secreted Hsp90 untouched and functional during the entire period of the experiments. Using the same mice, a group found a defect in major histocompatibility complex (MHC) class II antigen presentation (Li et al., 2012a). Recent studies have demonstrated that eHsp90 α plays a critical role in skin wound healing and tumor progression (Li et al., 2012a,b). Therefore, it would be interesting to see if these Hsp90 α -knockout mice have defects in wound healing and show less support to growth of those tumors that use eHsp90 for invasion and metastasis (see more in later sections).

2.2. Functions at Cellular Levels

Saribek et al. reported that Hsp90 β mediates the signaling of prolactin to trigger apoptosis (Saribek et al., 2006). Because prolactin has more than 300 biological effects reported, it is not clear if this was a primary or secondary effect. Further, GA, which was used in this study to distinguish Hsp90 α from Hsp90 β , is not a specific inhibitor for the ATPase of Hsp90 α . Kunisawa and Shastri showed that Hsp90 α , but not Hsp90 β , was required to interact with the C-terminally extended proteolytic intermediates, an early stage in antigen processing, even with partial (approximately 50%) knockdown of the Hsp90 α proteins (Kunisawa and Shastri, 2006). The reverse was true for CpG-B oligodeoxynucleotide's (ODN's) antiapoptotic signaling in macrophages and dendritic cells. Similarly, using RNA interference (RNAi) technology, Kuo et al. reported that Hsp90 β , but not Hsp90 α , is involved in the CpG-B ODN signaling. However, no experiments were included for targeting Hsp90 α as a control (Kuo et al., 2007). Didelot et al. provided strong evidence that chaperone function of Hsp90 toward cellular inhibitor of apoptosis protein-1 (c-IAP1) was the specific action of Hsp90 β , as depletion of Hsp90 α did not affect the c-IAP1 content and did not inhibit cell differentiation (Didelot et al., 2008). Bouchier-Hayes et al. provided a

possible mechanism for how Hsp90 α or Hsp90 β is involved in antiapoptosis. Using RNAi approach, they showed that Hsp90 α is a key negative regulator of heat-shock-induced caspase-2 activation (Bouchier-Hayes et al., 2009). This study did not determine whether Hsp90 α acts alone or requires coparticipation of Hsp90 β , i.e. downregulation of Hsp90 β would not affect the status of caspase-2. Metchat et al. reported that Hsp90 α is involved in the differentiation process of oocyte meiosis. They showed that knocking out heat shock factor 1 (HSF-1) caused Hsp90 α depletion, similar to the treatment with 17-AAG (Metchat et al., 2009). However, HSF-1 has many downstream target genes and 17-AAG could not distinguish between Hsp90 α and Hsp90 β . Houlihan et al. also reported that downregulation of either Hsp90 α or Hsp90 β alone was sufficient to cause decreased MHC class II presentation of both endogenous and exogenous antigens (Houlihan et al., 2009). Chatterjee et al. recently showed that Hsp90 β plays a more important role than Hsp90 α in the control of multiple myeloma cell survival (Chatterjee et al., 2007). It has been previously shown that both intracellular Hsp90 and eHsp90 play a role in antigen presentation (Basu et al., 2000; Binder et al., 2004; Imai et al., 2011).

2.3. Discovery of eHsp90 Provides a New Take-home Message

Besides the intracellular Hsp90 α and Hsp90 β chaperone proteins, identification of eHsp90 α , but less of eHsp90 β , from conditioned media of a wide variety of normal or tumor cell lines has added another layer of complexity to Hsp90 biology. If one extrapolates from these new findings within the context of Hsp90 α and Hsp90 β chaperones, it suggests that (1) Hsp90 α and Hsp90 β represent two pools of Hsp90 with fundamentally distinct roles; (2) intracellular Hsp90 β , but not Hsp90 α , is the effective target for inhibitors, such as 17-AAG; and (3) Hsp90 α is mainly used for cells' responses to micro- or macro-environmental stress via the action of eHsp90 α .

3. eHSP90, BUT NOT INTRACELLULAR HSP90 CHAPERONE: DIFFERENCE IN PHYSIOLOGY AND PATHOPHYSIOLOGY

eHsp90 has been reported to participate in various physiological and pathophysiological processes, such as wound healing (Li et al., 2007), angiogenesis (Song et al., 2010), cell rearrangements in cranial mesenchyme during neurulation (Sarkar and Zohn, 2012), activation of monocytes, macrophages and dendritic cells (Cecchini et al., 2011) as well as tumor invasion and metastasis (Eustace et al., 2004; Tsutsumi et al., 2008). Since this chapter focuses on cancer, an ideal anticancer agent would be one that targets a cellular element that is vital for the cancer but not for normal cells. eHsp90 may be just such a long-sought-after anticancer target. Here we emphasize the lines of evidence supporting the hypothesis that eHsp90 is a more druggable therapeutic target than the intracellular Hsp90.

3.1. Normal Cells Do not Secrete Hsp90 unless under an Environmental Stress

Technically speaking, there is no such thing as "physiological conditions in vitro". The currently used tissue or cell culture conditions are not the same as those in the bodies of animals or humans. Even obtaining cell or tissue samples from an experimental animal or a human patient would introduce a level of stress such as ischemia, hypoxia, oxidation, and fluid loss. The degree of stress on the obtained tissues or cells would depend on the length of

time needed going from the in vivo condition to the in vitro ones, such as formalin treatment or ultra low temperature freezing. These are important concerns for immunochemistry studies of animal and human tissue specimens, since varying degrees of stress could cause variations in gene and protein expression in the tissues after their isolation from the hosts. For example, we know from cell culture experiments that Hsp90 accounts for 1-2% of the total proteins in a cell, but it is not clear if the stress from the in vitro conditions has caused any significant changes in Hsp90 from its real setting in the hosts. That is to say whether or not the cell really contains 1-2% Hsp90 proteins of its total protein pool when it is in animals or humans remains unconfirmed. Having been mindful of these often overlooked concerns, the reality remains that cultured cells from healthy tissue or donors are widely regarded as “normal cells” (versus tumor cells) and their culture conditions are referred as “physiological conditions”. We continue taking these inaccurate statements as necessary assumptions for this chapter. Several laboratories showed that noncancerous cells under optimized culture conditions do not secrete Hsp90 until an acute environmental change occurs (Li et al., 2012b). The reported environmental changes that trigger normal cells to secrete Hsp90 include reactive oxygen species (Liao et al., 2000), heat (Hightower and Guidon, 1989; Clayton et al., 2005), γ -irradiation (Yu et al., 2006), hypoxia (Li et al., 2007; Woodley et al., 2009), injury-released growth factors (Cheng et al., 2008), serum starvation (Chen et al., 2010) and virus infection (Hung et al., 2011). A schematic illustration of how normal cells secrete Hsp90 is shown in Fig. 5.1, which emphasizes that normal cells secrete Hsp90 only under stress. In some of the above studies, the observed basal levels of secreted Hsp90 detected from the conditioned media of normal cells could be explained by the imperfect in vitro environment or stress of the cell culture conditions.

3.2. Tumor Cells Constitutively Secrete Hsp90

Constitutive secretion of Hsp90 has been reported in a variety of tumor cell lines, including SH-76 hybridoma cells (Kuroita et al., 1992); HT-1080 fibrosarcoma cells and MDA-MB-231 breast cancer cells (Eustace et al., 2004; Wang et al., 2009; McCready et al., 2010); MCF-7 breast cancer cells (Wang et al., 2009); HCT-8 colorectal cancer cells (Chen et al., 2010); T24 bladder cancer cells; B16 melanoma cells and PC3 prostate cancer cells (Tsutsumi et al., 2008); SKBR3, MDA-MB-453, MDA-MB 435 and MDA-MB-468 breast cancers; CaoV-3 ovarian cancer and HepG2 hepatoma (Wang et al., 2010; McCready et al., 2010; Sahu et al., 2012); A172 glioblastoma and SUM159 breast cancer (McCready et al., 2010) and MG63 osteocarcinoma (Hsp90 β) (Suzuki and Kulkarni, 2010). Figure 5.1 also illustrates how tumor cells secrete Hsp90 and emphasizes that constitutive Hsp90 secretion is linked to abnormalities in tumor suppressor genes and proto-oncogenes, as discussed in the following sections.

3.3. Upstream Regulators of Hsp90 Secretion in Normal and Tumor Cells

There have been a few reported upstream regulators of Hsp90 secretion, including p53, HIF-1 α and Hectd1 ubiquitin ligase. First, Levine's laboratory used non-small-cell lung cancer cell lines, H460 (wild type p53) and H1299 (p53-null), and the MEF (mouse embryonic fibroblast) cells with or without endogenous p53 expression, to investigate the role of p53 in the control of exosome formation and secretion in response to DNA damage. They made the following interesting observations: (1) p53 is involved in exosome

formation; (2) Hsp90 β , but not Hsp90 α (since the anti-Hsp90 β antibody used by the authors was raised against a peptide derived from the C-terminus of Hsp90 β that has less than 40% identity with the same region in Hsp90 α), was detected in the conditioned medium and isolated exosomes from irradiated cells after they were dissolved in sodium dodecyl sulfate sample buffer; (3) whether Hsp90 α was also present in the conditioned media was not tested; and (4) downregulation of p53 in H460 cells by RNAi or reintroduction of wild-type p53 gene into the p53-null H1299 cells blocked or rescued exosome secretion and detection of Hsp90 β from the conditioned media of the cells, respectively (Yu et al., 2006). It is unlikely, however, that p53 has a direct regulatory effect on Hsp90 β secretion via exosomes, due to the length of radiation treatment (16-24 h) in order to detect secreted Hsp90 β . Finally, whether eHsp90 β plays a role in response to γ -radiation-caused DNA damage and p53-mediated DNA repair processes remains unknown.

Second, Li's group reported that HIF-1 α mediates hypoxia-triggered Hsp90 α secretion in primary human dermal fibroblasts and keratinocytes (Li et al., 2007; Woodley et al., 2009). These studies showed that a dominant negative mutant of HIF-1 α (DN-HIF-1 α) blocks Hsp90 α secretion, whereas a constitutively active mutant of HIF-1 α (CA-HIF-1 α) makes the cells to secrete Hsp90 α even under normoxia. The same mechanism appears to take place in tumor cells. Depletion of HIF-1 α or HIF-1 β from the metastatic breast cancer cell line, MDA-MB-23, by RNAi completely blocked the constitutive secretion of Hsp90 α by these cells. Furthermore, this inhibition could be rescued by exogenously reintroducing the CA-HIF-1 α , but not DN-HIF-1 α , gene into the endogenous HIF-1 α -downregulated cells (Sahu et al., 2012). As previously described, since approximately 50% of all invasive human tumors express higher levels of HIF-1 α (Semenza, 2007; Semenza, 2012b), eHsp90 could be used as new diagnostic and/or therapeutic target for the "HIF-1 α -positive" tumors.

Third, Sarkar and Zohn reported that Hectd1 ubiquitin ligase negatively regulated the intracellular localization and secretion of Hsp90 in control of the cranial mesenchyme during neurulation. In Hectd1 mutant cranial mesenchyme cells, both enhanced secretion of Hsp90 and emigration of cells from cranial mesenchyme explants were highly dependent on eHsp90 secreted from the mutant cells. Since Hectd1 ubiquitinates the lysine-63 (K-63) in Hsp90 α , it would be interesting to find out whether K-63 ubiquitination plays a role in Hsp90 α secretion (Sarkar and Zohn, 2012).

Finally, it needs to be pointed out that Hsp90 released by necrotic cells has been shown to participate in binding and presenting antigens to antigen-presenting cells (Basu et al., 2000; Binder et al., 2005), but Hsp90 released by necrosis of a cell is different from the Hsp90 actively secreted by living cells in response to environmental stress signals or by tumor cells driven by internal oncogenic signals (Li et al., 2012b). Thus, eHsp90 released by necrotic cells is not discussed in this chapter.

3.4. Regulating Elements within Hsp90 for Secretion

Several laboratories identified aa motifs within Hsp90 α that influence Hsp90 secretion. Cheng et al. demonstrated that the ATPase activity of Hsp90 α is not required for Hsp90 α membrane translocation and secretion. They fused green fluorescent protein (GFP) gene with the cDNAs of Hsp90 α -wt, Hsp90 α -E47A, Hsp90 α -E47D and Hsp90 α -D93N, which

encode proteins containing 100, 50 or 0% of the ATPase activity, respectively. They infected primary human keratinocytes with lentivirus carrying each of the fusion genes and studied their membrane translocation and secretion in response to environmental stress signals with anti-GFP antibodies. They reported that the mutations in the ATP-binding and ATPase site of Hsp90 α decreased the efficiency of membrane translocation in response to transforming growth factor- α (TGF α), but did not affect TGF α -stimulated secretion of the GFP-Hsp90 α fusion proteins by human keratinocytes. Functionally, these mutations showed little effect on the promotility activity of the recombinant proteins of these genes in vitro (Cheng et al., 2008).

Tsutsumi et al. reported that a conserved hydrophobic motif at the boundary between the N-terminal domain, specifically at the Ile218 and Leu220 residues, and the charged linker (starting at glu236) of the human Hsp90 α is required for its chaperone function and secretion (Tsutsumi et al., 2009). Moreover, they showed that mutations at the Ile218 and leu220 motif altered the accessibility of the charged linker by the monoclonal antibody K41233 (that recognizes an epitope between aa 236 and aa 270). This indicates that the mutations caused a conformational change in the charged linker. It should be pointed out that the epitope of the K41233 antibody is located within the reported F-5 fragment, which retains the full promotility activity of the full-length Hsp90 α (Cheng et al., 2011). Therefore, the Ile218 and Leu220 mutations could affect the conformation and function of F-5 as an extracellular promotility factor. However, it is unclear whether Ile218 and Leu220 are directly involved in the regulation of Hsp90 α secretion or these aa substitutions caused a conformational change in the F-5 domain of Hsp90 that interfered with the secretion process.

Wang et al. reported that the secreted Hsp90 α is a truncated protein missing at least the last four aas, EEVD. In addition, these authors showed that both the C-terminal EEVD aas and phosphorylation on threonine-90 in Hsp90 α regulate Hsp90 α secretion (Wang et al., 2009). However, for unknown reasons, the truncation event appeared only to occur with the exogenously expressed epitope-tagged Hsp90 α . The molecular weight of the endogenous Hsp90 α , inside or outside of the cells, remained the same. Nonetheless, this finding was interesting in reference to an observation made by Grad et al., as previously mentioned. These authors showed that the Hsp90 α mutant gene lacking the C-terminal 36 aas was unable to be expressed in mouse tissues during development (Grad et al., 2010). According to Wang et al., this particular Hsp90 α mutant gene product is stable in cells, but cannot be secreted. Reason for this discrepancy remains unknown.

3.5. Exosome Pathway for Hsp90 Secretion

Hsp90 is secreted from cells via the exosome protein trafficking pathway (Yang and Robbins, 2011; Li et al., 2012b). Evidence to support this mechanism came from studies with chemical inhibitors, proteomic analysis and electron microscopic (EM) visualization of Hsp90-containing exosomes. First, brefeldin A (BFA) and dimethyl amiloride (DMA) are two chemical inhibitors that selectively block the classical endoplasmic reticulum (ER)/Golgi protein transport pathway and the exosome-mediated protein secretion pathway, respectively (Lancaster and Febbraro, 2005; Savina et al., 2003). Several groups reported that

DMA, but not BFA, inhibited the membrane translocation and secretion of Hsp90 α , Hsp90 β and/or Hsp70 in various cell types (Li et al., 2012b). Second, Clayton et al. used proteomic methods to analyze the peptide contents of B-cell-secreted exosomes under either physiological temperature (37 °C) or heat shock (42 °C for 3 h). Heat shock increased the presence of Hsp90 α in secreted exosomes isolated by ultracentrifugation (Clayton et al., 2005). Third, Yu et al. provided EM evidence that Hsp90 β is located in exosomes outside the cells in response to γ -irradiation (Yu et al., 2006).

A major difference between the exosome pathway and the classical ER/Golgi peptide transport pathway is that the former does not require a signal sequence at the amino terminus of the protein, while the latter pathway does. Hsp90 lacks any signal sequence and, therefore, cannot be secreted via the classical peptide trafficking pathway. Exosomes or “intraluminal vesicles” are non-plasma-membrane-derived vesicles that are 30-90 nm in diameter and contained within the multivesicular bodies (MVBs). Among other functions, such as fusion with the lysosomes, MVBs can also fuse with the plasma membrane to release their cargo proteins, such as Hsp90, into the extracellular space via the following steps: (1) sorting into smaller vesicles, (2) fusion with the cell’s surface membrane and (3) release of the small vesicles to extracellular space (Février and Raposo, 2004; Stoorvogel et al., 2002). Many other proteins that do not have any signal sequence have been found in the cell cytosol or endosomal compartments, but never in the ER, Golgi apparatus, mitochondria, or nucleus (Théry et al., 2002). What is not clear is whether eHsp90 stays inside exosomes all the time or is “spilled” out to the environment after the exosomes get to the cell surface or outside the cells.

4. eHsp90 AS AN UNCONVENTIONAL PROMOTILITY FACTOR

The main function of eHsp90 is to promote cell motility, first demonstrated by using anti-Hsp90 neutralizing antibodies (Sidera et al., 2004) and recombinant human Hsp90 α proteins (Li et al., 2007). Only one earlier report by Kuroita et al. showed that purified Hsp90 α from conditioned media of human hybridoma SH-76 cells had a growth-stimulating activity at the concentration of 0.1 μ M (Kuroita et al., 1992). Four lines of evidence support the notion that eHsp90 represents a naturally occurring promotility factor that is abundantly stored in all cells and has unique properties that enable it to deal with a variety of environmental insults, in comparison to conventionally recognized pro-motility factors, such as growth factors.

Cheng et al. recently showed that a 115-aa peptide, called F-5 (aa 236 to aa 350), retains the full promotility activity of the full-length Hsp90 α (Cheng et al., 2011). This region appears to locate at a loop at the surface of Hsp90 α protein (Csermely et al., 1998). More intriguingly, this region appears to be one of the two most immunogenic epitopes in human Hsp90 α . Udonon et al. (Riken Institute, Japan) injected full-length human recombinant Hsp90 α protein into mice and selected for productive monoclonal-antibody-producing clones. They obtained and made commercial availability of several clones that produce high titers of antibodies against human Hsp90 α (Cosmo Bio Co., Ltd, Japan). Interestingly, the majority of these antibodies recognize either the C-terminal or the F-5 region. Two clones produce antibodies that recognize aa 247-aa 257 and aa 263-aa 270, both of which are present in the heart of the F-5 fragment. The significance of such a correlation remains to be

seen. Two other monoclonal antibodies recognize the extreme C-terminal tail of Hsp90 α , between aa residues 604-732 and 702-716.

4.1. Hsp90 Proteins are Stockpiled in all Cells

When cells are under stress, such as heat shock, they reduce their overall protein synthesis rate and yet selectively upregulate the expression of some of the heat shock proteins, at least for a certain period of time following the heat insult (Horwich et al., 1990; Young et al., 2001). These seemingly contradictory events were interpreted to help the folding and stability of (existing) proteins. As a matter of fact, it does not make a perfect sense since each cell already contains Hsp90 proteins at up to 100-fold higher concentration than any of its client proteins (Li et al., 2012b). The preexisting client proteins were already bound to their Hsp90 chaperones prior to the insult. Hence, one needs to consider other possible reasons why cells maintain such a high concentration of Hsp90. First, how much Hsp90 exactly does a cell have? By using a textbook protein quantitation methodology, Sahu et al. re-examined four types of normal cells against four lines of tumor cells. They found that (1) Hsp90 α accounts for 2-3% of the total cellular proteins in the four types of normal cells tested and the amount could go up to 7% of the total cellular proteins in certain tumor cell lines and (2) Hsp90 α is not elevated in all cancer cells tested. A \$64,000 question is whether there are two distinct pools of Hsp90 in each cell, a “pool of chaperone Hsp90” and a “pool of promotility eHsp90”. These two pools of Hsp90 could have been designed (by Mother Nature) to serve the common purpose—to deal with environmental stresses and to protect the cell from both inside and outside. Csermely et al. questioned “Why do we need constitutively so much Hsp90 α ?” They reasoned “the 1-2% of the total cellular protein seems to behave like a ‘fireman’ of the cell, sitting quietly and doing nothing most of the time – a luxury that is seldom tolerated by evolution”. They went on to speculate that the major cellular function of Hsp90 may not be its well-recognized function as an intracellular chaperone, but rather another unrecognized cellular role that would require such a large and steady-state amount of Hsp90 α (Csermely et al., 1998). Since the mid-1980s, there have been a number of studies suggesting that Hsp90 α may also have an extracellular function. For instance, Hsp90 α was repeatedly found on the surface of cancer cells and has been shown to be a tumor-specific antigen. It is of a great interest to investigate the “two pool” hypothesis.

4.2. Fast Release and Only Need to be Local

It is critical for a cell to rapidly respond to environmental stress and to repair any tissue damages. In comparison to some stress-responding factors such as conventional growth factors, using eHsp90 for the job may have several advantages. The most obvious one is the fact that Hsp90 proteins are “pre-made” in the cell and ready to go out prior to any environmental signals. Therefore, the time for eHsp90 to respond to environmental stress signals is significantly shorter than the time for release of a growth factor under similar conditions. Taking insulin production and release as an example, insulin is exclusively produced by pancreatic beta cells located in clusters known as the islets of Langerhans in the pancreas. In response to the signal of a rising blood glucose level, the insulin gene is first transcribed into an mRNA transcript. The mRNA is then translated into an inactive protein called preproinsulin. Preproinsulin contains an amino-terminal signal sequence that is

required for the precursor hormone to pass through the membrane of the ER for posttranslational processing. This posttranslational processing clips away those portions not needed for the bioactive insulin. Then, three critical disulfide bonds are formed within the proinsulin, prior to further specific peptidase cleavages in the proinsulin. These modifications and processing finally result in the mature and active insulin. Insulin is then pack-aged and stored in secretory granules in the cytoplasm of the beta cells, until its release is triggered. Therefore, in comparison, eHsp90 takes less time to respond to environmental signals and to get out of its producing cells since eHsp90 is prestored in all cells. Second, eHsp90 does not have to travel a long distance, like insulin, to build up a threshold working concentration. Instead, it works in a local microenvironment, such as in a wounded tissue or a tumor microenvironment and can quickly reach its threshold working concentration.

4.3. Requirement for Transmembrane Signaling by eHsp90

The mechanism of action of eHsp90 to promote cell motility has two distinct, but not necessarily mutually exclusive, theories: (1) eHsp90 acts as an accessory protein to bind and activate other cell surface or secreted proteins such as HER2, matrix metalloproteinases (MMPs), ECMs (extracellular matrices) and cochaperones (Eustace et al., 2004; Stellas et al., 2010; Sims et al., 2011) or (2) eHsp90 acts as a bona fide extracellular signaling peptide, like insulin, that binds to a cell surface receptor and triggers a cross-the-membrane signaling. The best-characterized receptor for eHsp90 is LRP-1 (low-density lipoprotein (LDL) receptor-related protein-1, also called α 2-macroglobulin receptor, CD91 or TGF β R-V) (Li et al., 2012b).

The first reported extracellular target for eHsp90 α is MMP2 (Eustace et al., 2004). Eustace et al. showed that eHsp90 α binds and somehow mediates MMP2 activation. Inhibition of eHsp90 α decreased both MMP2 activity and invasiveness of the tumor cells. This study did not, however, address whether extracellular MMP2 was directly involved in eHsp90 α -induced tumor cell invasion. Song et al. reported that MMP2 is required for eHsp90 signaling to promote endothelial cell migration. However, their study did not distinguish intracellular versus extracellular MMP2, making it difficult to assess the potential importance of eHsp90-MMP2 interaction in the control of cell migration (Song et al., 2010). Stellas et al. reported that a monoclonal antibody (mAb) against Hsp90 α , 4C5, prevented Hsp90 binding to and activating MMP2 in vitro and inhibited MDA-MB-453 cell tumor growth in vivo (Stellas et al., 2010). These experiments emphasized the importance of eHsp90 α , but not MMP2. For instance, their experiments did not rule out the possibility that 4C5 also interfered with interactions of eHsp90 with other extracellular targets. Similar arguments go to the membrane-impermeable inhibitors of Hsp90 (Eustace et al., 2004) and the study showing that inhibition of Hsp70 cochaperone reduced Hsp90-mediated activation of MMP2 (Sims et al., 2011). It is important to know if specific inhibition of the extracellular MMP2 affects eHSP90 α -driven cell migration and tumor invasion.

LRP-1 has been shown in vitro and in vivo to play an essential role in mediating eHsp90 signaling, including activation of the Akt pathway, stimulation of cell migration, promotion of wound healing and tumor formation. The general function of LRP-1 is a “protector” of tissue damage, which can be taken advantage of by tumors (Lillis et al., 2008). LRP-1

belongs to a family of seven members related to the LDL receptor. Deletion of the LRP-1 gene leads to embryonic lethality in mice (Herz and Strickland, 2001). LRP-1 is widely expressed in various types of normal and cancer cells and has been reported to bind a wide variety of extracellular ligands, including lipoproteins, proteases and their inhibitors, ECMs and growth factors. LRP-1 expression is altered in certain cancer cells and this alteration influences the invasiveness of the cancer cells (Lillis et al., 2008). Structurally, LRP-1 consists of a 515-kDa extracellular subunit and a membrane-anchoring 85-kDa subunit, which are formed from proteolytic products of a common 600-kDa precursor (Strickland et al., 1990). Cheng et al. provided direct evidence that the LRP-1 receptor mediates eHsp90-stimulated human skin cell migration in vitro and wound healing in vivo. Their study showed that neutralizing antibodies against LRP-1's ligand binding domain blocked recombinant Hsp90-induced cell migration. Lentiviral-vector-mediated short hairpin RNA expression and downregulation of LRP-1 abolished normal cell migration and cancer cell migration and invasion in response to recombinant Hsp90 α . Reintroduction of LRP-1 (minireceptor) rescued the response (Cheng et al., 2008). Blocking the signaling of LRP-1 by RAP (receptor-associate protein) dramatically delayed wound healing in mice (Cheng et al., 2011). Breast cancer cell migration and invasion in vitro and tumor formation in vivo were greatly reduced by downregulation of LRP-1 in these cells (Sahu et al., 2012). Recently, Li's group showed that eHsp90 α promotes skin cell migration and accelerates wound closure by engaging the NPVY motif in the cytoplasmic tail of the LRP-1 receptor to activate downstream Akt kinases, providing direct evidence for cross-membrane signaling by eHsp90 (F. Tsen, C-F. Cheng, K. O'Brien, M. Chen, N. Hay, B. Stiles, D. T. Woodley and W Li, unpublished).

4.4. What is Unique about eHsp90?

First, a comparison was made between eHsp90 and growth factors on their actions in wound healing. The conventional wisdom is that growth factors are the major driving force leading to wound closure (Singer and Clark, 1999; Martin, P., 1997). However, after two decades of studies and clinical trials with individual or combination of growth factors, only PDGF-BB (becaplermin gel/RegranexTM) received the US Food and Drug Administration (FDA) approval for treatment of human foot diabetic ulcers. Subsequent multiple-center, double-blind, randomized and placebo-controlled clinical trials showed that Regranex had a modest efficacy, albeit its high cost and "black-box" warning for higher risk of causing cancer in patients (Nagai and Embil, 2002; Mandracchia et al., 2001). While such overall disappointing outcomes for growth factors in wound healing were unexpected, the reason long remained unclear until recently.

Badyopahdhay et al. switched their studies of human skin cell migration from using fetal bovine serum (FBS) to human serum. They argued that human cells are never in contact with FBS in reality. Results of their new experimental design led to the discovery that TGF β 3 present in human serum selectively blocked growth-factor-stimulated migration of the dermal cells (dermal fibroblasts and dermal microvascular endothelial cells) and proliferation of both epidermal and dermal cells. In contrast, this important effect was not detected in FBS (Bandyopadhyay et al., 2006). An interpretation of this finding is that the conventional growth factors present in human serum, which represents the main soluble

environment in the wound, are in fact unable to promote cell migration and growth, as they were thought, due to the copresence of TGF β 3. This previously unrecognized “defect” of growth factors (i.e. overridden by TGF β) might be the main reason for their low efficacy in promoting wound healing in humans in the clinical trials.

In contrast to conventional growth factors, Cheng et al. reported that eHsp90 α is able to override the inhibitory effect of TGF β and promotes migration of all three human skin cell types in vitro, even in the presence of TGF β 3 (Cheng et al., 2008, 2011). This unique property in eHsp90 α was better demonstrated in vivo. They showed that topical application of recombinant Hsp90 α enhanced skin wound healing in nude mice and db/db diabetic mice three times stronger than FDA-approved Regranex (PDGF-BB) (Cheng et al., 2011).

Second, expression of the receptor for Hsp90 α signaling, LRP-1, is detected in all the three types of human skin cells (Cheng et al., 2008; Woodley et al., 2009). In comparison, growth factor receptors often have a limited cell type distribution. For example, the receptors for PDGF-BB and vascular endothelial growth factor A, both regarded as key factors for wound healing, were only detectable in dermal fibroblasts and the microvascular endothelial cells, respectively (Cheng et al., 2010). These findings again question how effective a growth factor treatment, such as Regranex, can be, if it only acts one of the three cell types involved in a biological process.

Last, eHsp90 has no reported functions in development (Li et al., 2012b). In normal tissues, the function of eHsp90 is “to repair”. eHsp90 does not, otherwise, exist if tissue repair is not needed. This unique property of eHsp90 differs from the intracellular Hsp90 chaperone, which plays equally important roles in normal and cancer cells. Therefore, this feature grants eHsp90 the status of an ideal target for therapy of certain cancers that constitutively secrete Hsp90. If all these hold up, targeting eHsp90 would do little harm to normal cells and tissues. Hence, the tolerating concentrations for an anti-eHsp90 inhibitor for cancer patients could easily surpass the effective threshold concentrations that effectively damage cancer cells.

5. eHsp90 IN BLOOD CIRCULATION IN NORMAL AND CANCER PATIENTS

5.1. eHsp90 in Plasma Versus Serum

If eHsp90 plays a critical role in tissue wound repair and cancer progression, one would expect that eHsp90 is present in the plasma of normal and cancer patients. One would expect that the eHsp90 levels might be higher in cancer patients. Recent studies suggested that this is the case. Wang et al. reported increased Hsp90 α in plasma of breast, lung, pancreas and liver cancer patients in the range of 0.05-0.6 μ g/mL and a correlation of elevated plasma levels of eHsp90 and tumor malignancy (Wang et al., 2009). Chen et al. examined the eHsp90 α levels in the serum of 172 colorectal cancer patients, in comparison to 10 normal human volunteers. They reported that the mean Hsp90 α level in sera of the cancer patients was 1 mg/mL, whereas that of healthy controls was approximately 0.2 mg/mL (Chen et al., 2010). Although the two studies show the same trend, the absolute values of serum Hsp90 differed in the range of 1000– fold. Such huge differences in the absolute values might be due to the fact that one study used serum and the other study used plasma in their analyses.

5.2. Technical Challenges in Preparing Plasma and Serum

First, the large difference in the amount of eHsp90 in plasma and serum was likely caused by the differences in the protein contents between plasma and serum. In unwounded tissues, the blood vessels are intact and the resident cells are nourished by a filtrate of plasma. However, when tissues are injured, the resident cells encounter an acute transition from an initial stage of plasma to a stage of serum for the first time, called blood coagulation. As the tissue injury heals and subsequent wound remodeling initiates, the resident cells experience a transition from serum back to plasma. In fact, the plasma to serum to plasma transition coincides with the classical phases of intact tissue to wounded tissue to repaired and remodeled tissue transition. Coagulation begins almost instantly after an injury to the blood vessel, leading to activation of the blood platelets. Activated platelets release the contents of stored granules into the blood plasma, a process called degranulation. Due to degranulation, the levels of many peptides and proteins, such as growth factors and cytokines, undergo dramatic changes. Therefore, it is the eHsp90 α in plasma, but not in serum, that should represent the eHsp90 in the patients' blood circulation. Second, even for studies of eHsp90 in human plasma (Wang et al., 2009), additional cautions need to be taken. For instance, due to technical limitations, it is extremely difficult to collect platelet-free plasma and to completely prevent degranulation of the contaminated platelets because platelets tend to resuspend during plasma preparation. Sun et al. recently reported dramatically different levels of serum Hsp90 from hepatocellular carcinoma patients with a range of 100–200 ng/mL (Sun et al., 2010). Third, the wide variations in age, gender, or racial background of the selected donors for the human subjects could also contribute to the variations. The much higher levels of eHsp90 α in serum, for instance, could be due to released Hsp90 α proteins from platelets' degranulation as well as due to secretion of Hsp90 α by other blood cells under the stress from the serum preparation procedures.

The complexity of plasma and serum preparations and its variability can often be overlooked. For instance, Fredly et al. recently reported that sera from patients with acute myeloid leukemia showed more than 10-fold increase in Hsp90, from 10–20 ng/mL (normal control) to 300 ng/mL (Fredly et al., 2012). In this study, the time from blood to serum preparation took an average of 2 h, which should be sufficient for changes in the contents that would be different from those of the patients' plasma. Moreover, Zagouri et al. could not find significant association between serum Hsp90 levels and the severity of the lesion in ductal and lobular breast tumors (Zagouri et al., 2011). For patients with other abnormalities, Lee et al. reported that plasma from acute respiratory distress syndrome group had higher levels of GRP94, Hsp90, Hsp60, Hsp47, GPx-3, and interleukin-8 (Lee et al., 2012). Musial et al. reported higher levels of Hsp90 α in children with chronic kidney disease undergoing dialysis (Musial et al., 2009a) and children undergoing chronic hemodialysis (Musial et al., 2009b). Finally, Hacker et al. reported that Hsp27, Hsp70 and Hsp90 α were significantly altered in patients with chronic obstructive pulmonary disease, a leading cause of death characterized by increased cellular stress and inflammation (Hacker et al., 2009). In summary, whether or not eHsp90 in blood circulation can be used as a diagnostic marker for diseases such as cancer remains unclear.

6. HSP90 VERSUS eHsp90: RELATED BUT NOT THE SAME MOLECULE ANY MORE

The central theme for anticancer drugs is to target a life-control molecule that shows differences between normal and tumor cells. The first small-molecule inhibitor of Hsp90 was identified almost 30 years ago. However, the clinical values of GA and RD were quickly put in question, because both inhibitors proved to be poorly soluble in water and too toxic even in animal studies (Neckers and Neckers, 2002). The modified versions have since been developed, synthesized and tested on patients. In 1999, the first Hsp90 inhibitor that entered clinical trials as a potential anticancer agent was 17-AAG, a derivative of GA as previously mentioned. Until recently, there have been more than a dozen anti-Hsp90 inhibitor clinical trials on various human cancers; however, these trials have either been terminated at the late phase or still are in their early phases. No Hsp90 inhibitor has yet made it through clinical trials and received a regulatory approval for treatments of human cancer. The overall outcomes of the cancer clinical trials targeting the Hsp90's ATPase activity have not been as promising as they were initially hoped to be (Drysedale et al., 2006; Miyata et al, 2013; Whitesell et al. 2012).

6.1. eHsp90 is not a Chaperone

Inside the cells, the N-terminal ATPase of Hsp90 is considered the heart of the protein's function that has reportedly hundreds of client proteins (Young et al., 2001). Inhibition of the ATPase activity has been shown to cause serious consequences to the client proteins, including misfolding, loss of function and degradation (Obermann et al., 1998; Whitesell et al., 1994). Since many of these clients proteins are involved in one or more of the fundamental cell events, including survival, growth, migration, differentiation, senescence, and apoptosis, the Hsp90's ATPase has become an attractive target for drugs that aim to manipulate all these processes at once and significantly reduce the chance of occurrence of drug resistance, which has been a major issue for anticancer drugs that target a single signaling pathway.

Does the N-terminal ATPase still play a similar role in eHsp90's function outside the cells? Results of initial studies suggested that it is the case. Two groups used cell-membrane-impermeable inhibitors of Hsp90 to specifically target eHsp90 and found that the N'-terminal ATPase of Hsp90 is also required for surface-bound or secreted Hsp90 α -mediated tumor cell migration and invasion. Eustace et al. found Hsp90 α (but not Hsp90 β) in the conditioned medium of HT-1080 fibrosarcoma cells. They used bead-linked 17-AAG to selectively target the secreted Hsp90 α and reported that the inhibitor decreased cancer cell invasion in vitro (Eustace et al., 2004). In another study, Tsutsumi et al. utilized DMAG-N-oxide, a cell-impermeable and water-soluble form of 17-AAG, to specifically block eHsp90. They showed that this inhibitor did not affect the stability of several known intracellular clients of Hsp90, such as Akt, Raf-1 and Hsp90, in comparison to its membrane-permeable counterpart 17-AAG that caused degradation of these signaling proteins. Under these conditions, the DMAG-N-oxide pretreatment inhibited motility and invasion of bladder cancer, breast cancer, prostate cancer, and melanoma cells in vitro and reduced lung colonization by melanoma cells in mice (Tsutsumi et al., 2008). Since these inhibitors target

the ATPase of eHsp90, results of these two studies suggest that the N'-terminal ATP-binding region and ATPase of Hsp90 α are also required for eHsp90 α function.

To directly address this important issue, Cheng et al. carried out gene mutagenesis studies. They obtained the cDNAs of human Hsp90 α -wt, Hsp90 α -E47A, Hsp90 α -E47D and Hsp90 α -D93N from Hartl's laboratory (Obermann et al., 1998), subcloned them into the pET15b system, and expressed these constructs in bacteria. Following FPLC (Fast protein liquid chromatography) purification, they showed that recombinant Hsp90 α -wt exhibited full ATPase activity, Hsp90 α -E47D mutant lost half of the ATPase activity, and Hsp90 α -E47A and Hsp90 α -D93N mutants lost the entire ATPase activity of Hsp90 α -wt, consistent with a previous report (Obermann et al., 1998). However, Cheng et al. found that all the ATPase-mutant proteins still retained the full promotility activity of the wild-type Hsp90 α (Cheng et al., 2008). Then, they used sequential deletion mutagenesis to have narrowed down the promotility activity to a 115-aa fragment between the middle domain and the linker region in Hsp90 α , called F-5. Furthermore, recombinant F-5 was fully functional in both in vitro migration assays and in vivo wound healing assays. In contrast, the entire N-terminal and C-terminal domains showed little migration-stimulating activity (Cheng et al., 2011). These results indicated that the N-terminal ATPase of Hsp90 is dispensable for eHsp90 functions. A schematic representation of the two independent, one intracellular and one extracellular, functional elements in Hsp90 α is shown in Fig. 5.2.

6.2. ATPase Inhibitors Should Not Affect the eHsp90 Function, but They Do

Cheng et al. then tested the effect of a nonpermeable GA (NPGA), similar to the DMAG-N-oxide used by Tsutsumi et al. (Tsutsumi et al., 2008; McCready et al., 2010; Gopal et al., 2011), on cell migration stimulated by the full length and F-5 fragment of Hsp90 α . Their results showed that NPGA inhibitor selectively inhibited cell migration stimulated by full-length Hsp90 α , but not by the F-5 fragment (F. Cheng and W. Li, unpublished). Using the same protein stocks from Cheng et al., Gopal et al. reported that NPGA inhibited cancer cell migration and invasion driven by full-length Hsp90 α , but not that driven by the F-5 fragment (Gopal et al., 2011). These results demonstrated that the N-terminal ATPase domain and the C-terminal dimer-forming and cofactor-binding domains are dispensable for eHsp90 α to promote cell motility. Based on these seemingly contradictory results, it is possible that the inhibitor's binding to the N-terminal ATPase domain of the full-length Hsp90 α caused a conformational change in the protein, so that the F-5 epitope within the LR and M domains in Hsp90 α becomes unavailable for binding to its cell surface receptor, LRP-1. Currently, there is little evidence for or against this conformational change hypothesis. However, such an "accident" by these GA inhibitors to block eHsp90 functions may have provided an important insight into the two critical questions: (1) why cancer cells are more sensitive to the inhibitors than normal cells and (2) why only some cancers, but not others, respond to the inhibitors.

7. eHsp90: ACTUAL TARGET FROM DAY ONE?

7.1. Status of the Latest Clinical Trials

The termination of a phase 3 17-AAG trial by Bristol-Myers Squibb (BMS) reflected the complexity of targeting the ATPase of Hsp90 in humans. BMS acquired the California-based Kosan Biosciences in 2008 and launched a phase 3 clinical trial of tanespimycin (17-AAG) (intravenous) on multiple myeloma in combination with Velcade (bortezomib). While results of the tests were described “very encouraging” by scientists involved in the trials, BMS permanently terminated the trial in 2010 without giving specific reasons. Infinity Pharmaceutical, Inc. launched a phase 2 clinical trial of IPI-504 (retaspimycin hydrochloride) (intravenous), a water-soluble version of 17-AAG, in combination with Trastuzumab on breast cancers in 2009. This trial was terminated in 2011 because while it had “modest clinical effect,” the data fell short on the prespecified efficacy criteria for continued trial expansion (Modi et al., 2011). Biogen Idec has recently completed a phase 2 clinical trial on CNF2024 (BIIB021) (oral) in gastrointestinal stromal tumors and breast cancer. Similarly, NCI (National Cancer Institute) has completed a phase 1 trial on SNX-5422 (mesylate) (oral) in the treatment of refractory solid tumors and lymphomas. No further information is yet available for these trials. There are two ongoing phase 2 clinical trials with STA-909 (infusion) in late-stage non-small-cell lung cancer (Synta Pharmaceuticals Corp.) and with AU922 in advanced gastric cancer (Novartis Pharmaceuticals).

Additional half a dozen small-molecule inhibitors targeting the Hsp90's ATPase with unreleased molecular structures have also entered phase 1 trials for the past 2-3 years. It is hard to predict the outcomes of these trials, based on the multiple failures of similar inhibitors in previous trials. RD inhibitors have never entered clinical trials, so is the case for inhibitors that target the C-terminal/middle domains, the client-binding domain, cochaperone-binding regions and membrane-impermeable inhibitors of Hsp90.

7.2. Only Those “Hsp90-Secreting” Tumors are More Sensitive to and “Hurt” by Inhibitors

Didier Picard (Geneva, Switzerland) recently reminded us of the two unresolved puzzles for Hsp90 inhibitors. He wrote “we have yet to understand why cancer cells are more sensitive to Hsp90 inhibitors (and) why the (Hsp90) inhibitors seem to work for some but not for other cancers” (Picard, 2012). These questions might have been critical ones, so that lack of answers to them may have been a significant factor for the overall disappointing outcomes of the clinical trials. This notion is based on recent new studies that have put eHsp90 on the radar of Hsp90 therapeutics. The most intriguing notion is that the effects of those Hsp90 inhibitors on various cancers may have had nothing to do with inhibition of the intracellular Hsp90 chaperone, which is also the reason for the cytotoxicity of the trials. Instead, the observed effects of Hsp90 inhibitors, such as 17-AAG, on certain cancers might have come from previously unrecognized inhibition of the eHsp90 secreted by the cancer cells. Assuming that this were the case, how one would explain the two questions raised by Picard? First, why are cancer cells more sensitive to Hsp90 inhibitors? A possible answer is that cancer cells constitutively secrete Hsp90, whereas normal cells do not (unless they are subjected to stress) (Li et al., 2012b). Therefore, inhibitors such as 17-AAG would first “hit” the extracellularly located eHsp90, before it penetrates into the cells and inhibits the

intracellular Hsp90. It is known that selective inhibition of eHsp90 significantly decreases cancer cell invasion in vitro and the ability to form tumors in mice (Eustace et al., 2004; Sidera et al, 2008; Tsutsumi et al., 2008; Wang et al., 2009; Song et al., 2010; Sahu et al., 2012). Second, why did Hsp90 inhibitors seem to work for some but not for other cancers? A possible answer is that not all cancers secrete Hsp90 and depend on eHsp90 for progression. 17-AAG would only show inhibitory effect on those “Hsp90-secreting” cancers at a concentration range that normal cells and tissues could tolerate. For instance, a well-characterized upstream regulator of Hsp90 secretion is HIF-1 α (Li et al., 2007; Woodley et al., 2009; Sahu et al., 2012). HIF-1 α remains undetectable in normal cells under physiological conditions (normoxia). In contrast, the constitutive presence of HIF-1 α was detected in approximately 50% of all invasive tumors in humans (Semenza, 2012a). Therefore, only those cancers that constitutively express HIF-1 α and, therefore, constitutively secrete Hsp90 α will be sensitive to 17-AAG, whereas other “HIF-1 α -negative” cancers will not.

7.3. eHsp90 Should be the Selected Target

If the above speculations were correct, membrane-impermeable inhibitors of Hsp90 would show stronger inhibition of tumor progression than the membrane-permeable ones, since these inhibitors do not have to bother the intracellular Hsp90 chaperone in normal cells. A schematic illustration of this idea is shown in Fig. 5.3. To do this experiment in an orthotopic tumor mouse model, for instance, one could inject cancer cells into the mice that have been systemically administered a membrane-impermeable inhibitor into the circulation and then assess primary tumor formation, invasion and tumor metastasis, in comparison to controls. Tsutsumi et al. tried to carry out similar experiments. Unfortunately, they reported that the NPGA inhibitors were structurally unstable in circulation (Tsutsumi et al., 2008). Another reported membrane-impermeable inhibitor is mAb, 4C5. Patsavoudi's group developed anti-Hsp90 mAb, 4C5, and showed that injection of 4C5 into mice that were preinjected with B16 mouse melanoma cells or mixing 4C5 with MDA-MB-45 human breast cancer cells prior to injecting them to mice decreased tumor formation of B16 cells and lung deposition of MDA-MB-453 cells, respectively (Stellas et al., 2007, 2010). Based on recent studies, there is a need to develop new cell-impermeable inhibitors that specifically target the F-5 region of eHsp90.

8. CONCLUSIONS AND PERSPECTIVES

For the past few years, we have learned a few things new from Hsp90. (1) Hsp90 α is not essential for development in mammals; (2) eHsp90 α is a novel promitility factor and critical for wound healing, tumor progression and possibly more; (3) reported effects of GA inhibitors in cancer clinical trials might come from inhibition of eHsp90, whereas their penetrations into normal cells limit the effectiveness; (4) The real purpose for Hsp90 α might be the source that supplies eHsp90 α that protects and repairs extracellular tissue damages, important tasks during adulthood and aging; and (5) eHsp90 α can also nonvoluntarily help “the wounds that do not heal”, i.e. tumor progression (Dvorack, 1986). A potential clinical contradiction is that we need more of the eHsp90 to repair injured tissues but we also have to kill the same eHsp90 in the environment that supports tumor progression. Taken together,

it may be time that we stop meddling with the business of intracellular Hsp90 in search for anticancer drugs, especially that of Hsp90 β . Instead, we should specifically target eHsp90 α in the tumor environment. Nonetheless, more studies are required for investigating whether or not a new era for the Hsp90 proteins has arrived.

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Figure 5.1. Hsp90α secretion occurs in normal cells only under stress, but constitutively in certain tumor cells

(Left part) In normal cells, secretion of Hsp90 does not occur unless cells are “hit” with environmental stress cues, as listed. Almost all types of stress have been shown to trigger Hsp90 secretion. The mechanisms by which the stress signals cause Hsp90 secretion remain to be further studied. The main function of the eHsp90 is to help tissue repair by promoting the cells at the edge of damaged tissue to migrate into the damaged area. (Right part) In tumors, constitutively activated oncogenes, such as HIF-1α, trigger Hsp90 secretion even in the absence of environmental stress cues. Tumor-secreted Hsp90α promotes both tumor and tumor stroma cell migration during invasion and metastasis. (For color version of this figure, the reader is referred to the online version of this book.)

Figure 5.2. Required elements in Hsp90α for intracellular and extracellular functions of Hsp90α
 (Lower part) The intracellular “chaperone” function of Hsp90 requires almost the entire molecule, especially the amino terminal (blue), the middle (red) and the carboxyl terminal (green) domains. It arguably regulates hundreds of signaling pathways and gene expression. (Upper part) The extracellular “promotility” function of Hsp90α depends on less than a 115-amino acid fragment (F-5) located at the boundary between the linker and the M domain. This region appears at the surface of Hsp90 protein and one of the two highly immunogenetic regions (with the C-terminal end). F-5 acts likely a Mother-Nature-designed “trooper” to take care of extracellular crisis. (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.)

Figure 5.3. A “trooper” that might have been the accrual target

We know now the following facts: (1) Hsp90α is unessential for development of life in mammals, (2) eHsp90α is critical for wound healing and tumor progression, (3) Hsp90β has no proven extracellular roles, (4) geldanamycin inhibitors have been inhibiting eHsp90 in clinical trials for over a decade without being noticed and (5) geldanamycin inhibitors’ penetration into normal cells limit their effectiveness on tumor cells. We propose that drugs that target the F-5 region of eHsp90α without penetrating cells are more effective and safer in the treatment of cancer patients. (For color version of this figure, the reader is referred to the online version of this book.)