Stuart K. Calderwood
Michael Y. Sherman
Daniel R. Ciocca
Editors

Heat Shock Proteins Volume 2

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Heat Shock Proteins in Cancer

Springer

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HEAT SHOCK PROTEINS

Volume 2

Series Editors:

A. A. A. Asea
Effie and Wofford Cain Centennial Endowed Chair in Clinical Pathology,
Chief, Division of Investigative Pathology, Scott & White Memorial Hospital and Clinic
and The Texas A&M University System Health Science Center College of Medicine

S. K. Calderwood
Division of Molecular and Cellular Radiation Oncology,
Beth Israel Deaconess Medical Center and Harvard Medical School

Heat Shock Proteins in Cancer

Edited by

Stuart K. Calderwood
Harvard Medical School, Boston, U.S.A.

Michael Y. Sherman
Boston University School of Medicine, Boston, U.S.A.

and

Daniel R. Ciocca
National Research Council CONICET, Mendoza, Argentina

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LIST OF CONTRIBUTORS

Andre-Patrick Arrigo Stress, Chaperons and Cell Death Laboratory, CNRS UMR 5534, Centre de Génétique Moléculaire et Cellulaire, Université Claude Bernard, LYON 1, 16 rue Dubois, 69622 Villeurbanne Cedex, France, e-mail: arrigo@univ-lyon1.fr

Alexzander Asea Division of Investigative Pathology, Scott & White Memorial Clinic and Hospital and the Texas A&M University System Health Science Center College of Medicine, 1901 South 1st Street, Temple, Texas 76504, USA

María A. Bausero Cell Biology and Flow Cytometry Unit, Institut Pasteur de Montevideo, Calle Mataojo 2020 CP 11400 Montevideo, Uruguay

Ajit Bharti Boston University Medical Center, 650, Albany Street, Boston, MA 02118

Marco Biamonte Biogen Idec, 5200 Research Place, San Diego, CA 92122, USA

Marcus F. Boehm Biogen Idec, 5200 Research Place, San Diego, CA 92122, USA

Cristina Bonorino Faculdade de Biociências e Instituto de Pesquisas Biomédicas, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil

M. Brunet INSERM UMR-866, Faculty of Medicine and Pharmacy, 7 Boulevard Jeanne d'Arc, 21039 Dijon, France

Vishwanie S. Budhram-Mahadeo Medical Molecular Biology Unit, Institute of Child Health, University College London, 30 Guilford Street, London, WC1N 1EH

Francis J. Burrows Biogen Idec, 5200 Research Place, San Diego, CA 92122, USA

Stuart K. Calderwood Division of Molecular and Cellular Radiation Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215; Department of Medicine, Boston University School of Medicine, MA 02118, USA, e-mail: scalderw@bidmc.harvard.edu

Avrom J. Caplan Department of Pharmacology and Biological Chemistry, Mount Sinai School of Medicine, New York, NY 10029, USA

Daniel R. Ciocca Oncology Laboratory, Institute of Experimental Medicine and Biology of Cuyo (CRICYT-CONICET), and Argentine Foundation for

Cancer Research (FAIC), Mendoza, C. C. 855, 5500 Mendoza, Argentina, e-mail: dciocca@lab.cricyt.edu.ar

F. Dario Cuello-Carrión Oncology Laboratory, Institute of Experimental Medicine and Biology of Cuyo (CRICYT-CONICET), and Argentine Foundation for Cancer Research (FAIC), Mendoza, C. C. 855, 5500 Mendoza, Argentina

Custer C. Deocaris Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki, Japan

C. Didelot INSERM UMR-866, Faculty of Medicine and Pharmacy, 7 Boulevard Jeanne d'Arc, 21039 Dijon, France

Mariel A. Fanelli Oncology Laboratory, Institute of Experimental Medicine and Biology of Cuyo (CRICYT-CONICET), and Argentine Foundation for Cancer Research (FAIC), Mendoza, C. C. 855, 5500 Mendoza, Argentina

Vladimir L. Gabai Boston University Medical School, Boston, MA 02118, USA

C. Garrido INSERM UMR-866, Faculty of Medicine and Pharmacy, 7 Boulevard Jeanne d'Arc, 21039 Dijon, France

Jianlin Gong Department of Medicine, Boston University School of Medicine, Boston, MA 02118, USA

Phillip J. Gray Jnr Division of Molecular and Cellular Radiation Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215

Richard J. Heads Cardiovascular Division, King's College London School of Medicine, Department of Cardiology, The Rayne Institute, St Thomas's Hospital, Lambeth Palace Road, London SE1 7EH, UK

J.L. Holmes Signal Transduction and Molecular Pharmacology Team, Cancer Research UK Centre for Cancer Therapeutics, The Institute of Cancer Research, Haddow Laboratories, Sutton, Surrey, SM2 5NG, UK

Adeela Kamal Biogen Idec, 5200 Research Place, San Diego, CA 92122, USA

Srinivas Kasibhatla Biogen Idec, 5200 Research Place, San Diego, CA 92122, USA

Sunil C. Kaul National Institute of Advanced Industrial Science & Technology (AIST), Central 4, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan

Md Abdul Khaleque Division of Molecular and Cellular Radiation Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215

Goro Kutomi Department of Pathology, Sapporo Medical University, School of Medicine, South 1 West 16, Cho-ku, Sapporo 060-8556, Japan

Stan Lepchammer Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA

Karen Lundgren Biogen Idec, 5200 Research Place, San Diego, CA 92122, USA

Robert Matts Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK 74078, Oklahoma, USA

Catherine A. McLellan Whitehead Institute for Biomedical Research Cambridge, MA 02142, USA

Yoshihiro Morishima Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI 48109-0630, USA

Len Neckers Urologic Oncology Branch, National Cancer Institute, Bethesda, MD 20892, USA

Cornelia O'Callaghan-Sunol Boston University Medical School, Boston, MA 02118, USA

Yoichi Osawa Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI 48109-0630, USA

Jun Oura Department of Pathology, Sapporo Medical University, School of Medicine, South 1 West 16, Cho-ku, Sapporo 060-8556, Japan

William B. Pratt Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI 48109-0630, USA

A.L. Rérole INSERM UMR-866, Faculty of Medicine and Pharmacy, 7 Boulevard Jeanne d'Arc, 21039 Dijon, France

Noriyuki Sato Department of Pathology, Sapporo Medical University, School of Medicine, South 1 West 16, Cho-ku, Sapporo 060-8556, Japan

S.Y. Sharp Signal Transduction and Molecular Pharmacology Team, Cancer Research UK Centre for Cancer Therapeutics, The Institute of Cancer Research, Haddow Laboratories, Sutton, Surrey, SM2 5NG, UK

Michael Y. Sherman Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118, USA

Ana Paula Souza Faculdade de Biociências e Instituto de Pesquisas Biomédicas, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil

S. Subramaniam INSERM UMR-866, Faculty of Medicine and Pharmacy, 7 Boulevard Jeanne d'Arc, 21039 Dijon, France

Yasuaki Tamura Department of Pathology, Sapporo Medical University, School of Medicine, South 1 West 16, Cho-ku, Sapporo 060-8556, Japan

Jimmy R. Theriault Department of Radiation Oncology, Beth Israel Deaconess Medical Center, Boston, MA 02215

A. de Thonel INSERM UMR-866, Faculty of Medicine and Pharmacy, 7 Boulevard Jeanne d'Arc, 21039 Dijon, France

Toshihiko Torigoe Department of Pathology, Sapporo Medical University, School of Medicine, South 1 West 16, Cho-ku, Sapporo 060-8556, Japan

Renu Wadhwa National Institute of Advanced Industrial Science & Technology (AIST), Central 4, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan

Luke Whitesell Whitehead Institute for Biomedical Research Cambridge, MA 02142, USA

P. Workman Signal Transduction and Molecular Pharmacology Team, Cancer Research UK Centre for Cancer Therapeutics, The Institute of Cancer Research, Haddow Laboratories, Sutton, Surrey, SM2 5NG, UK

Julia Yaglom Department of Biochemistry, Boston University Medical School, Boston, MA 02118, USA

Nava Zaarur Boston University Schools of Medicine, Boston, MA 02118, USA

Hong Zhang Biogen Idec, 5200 Research Place, San Diego, CA 92122, USA

Lin Zhang Biogen Idec, 5200 Research Place, San Diego, CA 92122, USA

CHAPTER 1

THE HSP90 CHAPERONE MACHINERY ACTS AT PROTEIN FOLDING CLEFTS TO REGULATE BOTH SIGNALING PROTEIN FUNCTION AND PROTEIN QUALITY CONTROL

WILLIAM B. PRATT, YOSHIHIRO MORISHIMA AND YOICHI OSAWA*

Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI 48109-0630, USA

Abstract: The Hsp90 chaperone machine functions as a 'cradle-to-grave' system for regulating the function, trafficking and turnover of a variety of key 'client' proteins that are involved in the genesis and progression of cancer, such as p53, RAF, ERBB2, AKT, and CDK4. The multiple effects of Hsp90 on signaling proteins issue from the focal interaction of the multichaperone machinery with protein folding clefts in natively folded proteins. Clefts in the client proteins that are stringently regulated by Hsp90 are inherently 'metastable' in that they open more readily. These metastable clefts are usually (or always) clefts that must open to bind ligands, such as steroids, heme, or ATP in the protein interior. The Hsp90 chaperone machine acts both to promote cleft opening and to limit further cleft opening by forming stable Hsp90 heterocomplexes with the client protein. By promoting cleft opening, the Hsp90 machinery regulates protein function. By forming stable client protein•Hsp90 heterocomplexes, the machinery performs its protein quality control function in preventing further cleft unfolding that triggers chaperone-dependent ubiquitination and proteasomal degradation. Natural mutations occurring in a variety of proteins associated with human malignancies affect the stability of protein folding clefts to convert them from weak to stringent regulation by Hsp90 and *vice versa*. Here, we use two well studied Hsp90 client proteins, the glucocorticoid receptor and nNOS, to illustrate how Hsp90 interacts with protein folding clefts to regulate protein activity and turnover

Keywords: Hsp90, Hsp70, glucocorticoid receptor, nNOS, ERBB2, p53

*Corresponding author: Department of Pharmacology, University of Michigan Medical School, 1301 MSRB III, Ann Arbor, MI 48109-0630, USA. Tel: 734 936 5797, Fax: 734 763 4450, e-mail: osawa@umich.edu

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INTRODUCTION

In the two decades since Hsp90 was shown to regulate the function of steroid receptors (reviewed in Pratt and Toft, 1997), more than 200 signaling proteins have been found to be regulated by hsp90 (reviewed Pratt and Toft, 2003). In as much as many of these Hsp90 'client proteins' are critical components of regulatory pathways involved in cancer cell growth, inhibitors of Hsp90 function, such as geldanamycin and its analogs, have emerged as a promising new class of anticancer drugs (reviewed in Sharp and Workman, 2006). The recognition that geldanamycin inhibits Hsp90 (Whitesell et al., 1994) was a turning point leading to our understanding that this chaperone plays a role in multiple cellular processes, including protein trafficking, protein complex assembly, protein function, and protein quality control. It has been difficult to place these varied effects within the context of a single mechanism for Hsp90 interaction with its varied protein substrates. Thus, investigators have used somewhat vague terms like Hsp90 'chaperoning' of protein folding or Hsp90-dependent protein 'maturation' to account for its actions.

In this chapter, we provide a basic conceptual framework for understanding how Hsp90 and Hsp70 function both as regulatory proteins and as molecular chaperones in protein quality control. To do this, it is first necessary to realize that these abundant and ubiquitous chaperones function together as essential components of a multichaperone machinery, now called the Hsp90 chaperone machine (Pratt and Toft, 2003). Although Hsp90 has been shown *in vitro* to transiently associate with unfolded proteins to prevent their aggregation during refolding, it is apparently not required for *de novo* protein folding (Nathan et al., 1997), and it is now clear that unfolded proteins are eliminated *in vivo* via ubiquitination and proteasomal degradation rather than by undergoing Hsp90-dependent refolding. Indeed, there is no evidence that Hsp90 ever acts by itself *in vivo*, and it is likely that it acts only through its interaction with Hsp70 in the multichaperone machinery.

Hsp90 Acts at Protein Folding Clefts

The Hsp90 chaperone machine acts on proteins in a manner that is not dependent upon protein sequence, size or structure or any requirement that a protein be in an unfolded state. Indeed, in the best studied models, the Hsp90 machine interacts with major protein folding clefts in properly folded proteins (Pratt and Toft, 2003). Folding clefts determine regions where hydrophobic surfaces of the protein interior merge with the hydrophilic exterior, and they are a general topological feature of all proteins in native conformation. By focusing on the interaction of the Hsp90 machine with protein folding clefts we can understand how the machinery both regulates protein function and protein quality control.

Although there is clearly a gradient of behavior, protein folding clefts can be divided into two general categories: 1) clefts that more readily open, often to permit passage of ligands, such as steroids, ATP or heme, to the cleft interior (we will refer to these as *metastable* clefts); 2) clefts with stronger hydrophobic interaction

that maintain a more closed conformation (we will refer to these as *closed* clefts). The Hsp90 chaperone machine acts both to promote cleft opening and then to limit further cleft opening by forming stable Hsp90 heterocomplexes with the client protein. Access of ligands to their binding sites in the protein interior is essential to protein function and by promoting cleft opening the Hsp90 machinery can regulate protein function. In forming a stable heterocomplex with the open cleft state of the client protein, Hsp90 prevents further cleft opening that progresses to protein unfolding, leading to client protein ubiquitination and proteasomal degradation. Inhibition of Hsp90 by geldanamycin prevents this protein 'quality control' function of the chaperone machine, and proteins with metastable folding clefts that are not stabilized because of geldanamycin inhibition of Hsp90 proceed to more unfolded states that are readily ubiquitinated and degraded.

Hsp90 Stringently Regulates Proteins that are in a Metastable Folding State

The Hsp90 client proteins, such as RAF or ERBB2 (HER2), that are of particular interest in signaling pathways of oncogenesis have metastable clefts that, in the absence of heterocomplex assembly with Hsp90, have a high tendency to further unfold, leading to protein degradation. These metastable cleft proteins tend to form relatively stable heterocomplexes with Hsp90 that can be isolated and analyzed biochemically, and they are quite profoundly stabilized when they are in a heterocomplex with Hsp90. In contrast, proteins with more closed folding clefts have a much more dynamic interaction with the Hsp90 chaperone machine, and, when heterocomplexes with Hsp90 are formed in the cell, they rapidly disassemble. Thus, proteins with closed protein folding clefts have inherently longer half-lives, do not form stable heterocomplexes with Hsp90 that can be detected biochemically, and the effect of the Hsp90 machinery on their function and stability is less profound. As we will describe further, proteins can be converted by mutation from a closed cleft structure with this 'kiss-and-run' interaction with Hsp90 to a more metastable state with stable heterocomplex formation and stringently regulated Hsp90 client protein status.

In this chapter, we use two model Hsp90-regulated proteins to illustrate how the Hsp90 machinery interacts with protein folding clefts to regulate protein function and turnover. The glucocorticoid receptor (GR) is the most studied of the stringently-regulated Hsp90 client proteins, and because its high affinity steroid binding activity is strictly Hsp90-dependent, this receptor is an excellent model for showing how the Hsp90 machine regulates client protein function (Pratt and Toft, 2003). The dependence of steroid binding on Hsp90 has permitted detailed mechanistic study of the action of the Hsp90 machinery and its effects on different states of a protein folding cleft. Neuronal nitric oxide synthase (nNOS), an enzyme that is regulated through very dynamic interaction with Hsp90 (Bender et al., 1999), is a good model for studying how the Hsp90 machine regulates protein quality control. Catalytic activation of certain drugs and substrates to reactive metabolites by nNOS leads to covalent modification of the heme/substrate binding cleft with an opening of the cleft structure and ubiquitination of the enzyme. Thus, nNOS is a good example

of how cleft opening reflects an early stage of protein unfolding that triggers chaperone-dependent ubiquitination.

CLIENT PROTEIN•HSP90 HETEROCOMPLEX ASSEMBLY

When transcription factors, such as unliganded steroid receptors or some mutants of the p53 tumor suppressor, or oncogenic tyrosine kinases, such as SRC or RAF, are immunoadsorbed from cell lysates, they are recovered in biochemically stable heterocomplexes with a dimer of Hsp90 and variable but substoichiometric amounts of Hsp70 (reviewed in Pratt and Toft, 2003). The complexes also contain the Hsp90 cochaperone p23, which interacts dynamically with client protein-bound Hsp90 to stabilize the complex. As shown for the GR in (Figure 1), Hsp90 complexes with steroid receptors and p53 also contain one of several tetratricopeptide repeat (TPR) domain immunophilins that bind to a TPR acceptor site at the C-terminus of Hsp90. The immunophilins are characterized by their peptidylprolyl isomerase (PPIase) domains, which are binding sites for the immunosuppressant drugs FK506 (FKBPs) or cyclosporine A (CyPs). Steroid receptor and p53 (Galigniana et al., 2004b) heterocomplexes contain one of three TPR domain immunophilins (FKBP51, FKBP52, or CyP-40) or PP5, a protein phosphatase that contains both a TPR domain and a PPIase homology domain (Silverstein et al., 1997). In contrast to the transcription factors, protein kinase•Hsp90 heterocomplexes contain CDC37, a non-TPR domain protein that binds to a different site on Hsp90 from the immunophilins and binds directly to the catalytic domain of the kinase (Pratt and Toft, 2003).

The Hsp90 Chaperone Machine

The concept of an Hsp90/Hsp70-based chaperone machine evolved from studies of progesterone and glucocorticoid receptor•Hsp90 heterocomplex assembly by

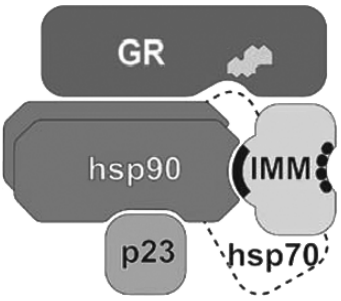

Figure 1. The core GR•hsp90•immunophilin complex as it is immunoadsorbed from cytosols prepared from hormone-free cells. One molecule of GR is shown bound to a dimer of hsp90 and one molecule of immunophilin. The immunophilin (IMM) binds via its TPR domain (black crescent) to a TPR acceptor site on hsp90. Immunophilin PPIase domain (dotted crescent). The steroid structure in the GR indicates that the ligand binding cleft is open and can be accessed by steroid

reticulocyte lysate (reviewed in detail in Pratt et al., 2006). In a simple assay, it was shown that immunoadsorbed receptors that were salt-stripped of Hsp90 became complexed with Hsp90 when they were incubated with rabbit reticulocyte lysate. Because the GR was converted to the high affinity steroid binding state, it was clear that the system in reticulocyte lysate was making the appropriate conformational change in the receptor when the complex with Hsp90 was formed. It rapidly became clear that all of the components required to make stable receptor•Hsp90 complexes, except p23, preexisted in a machinery in which the major chaperones, Hsp90 and Hsp70, were linked by a 60-kDa protein called Hop (Hsp organizing protein). Hop binds independently via an N-terminal TPR domain to Hsp70 and via central TPR domain to Hsp90 (Chen et al., 1996) to form an Hsp90•Hop•Hsp70 complex of stoichiometry 2:1:1 (Murphy et al., 2001) that also contains some of the Hsp70 cochaperone Hsp40 (Dittmar et al., 1998).

This multiprotein Hsp90 chaperone machine is ubiquitous in eukaryotes, and a variety of cochaperones of both Hsp90 and Hsp70 probably interact with it to modify assembly under various conditions in cells (Pratt and Toft, 2003). The same machinery in reticulocyte lysate appears to assemble Hsp90 heterocomplexes with both transcription factors and protein kinases (Nair et al., 1996), and the client proteins undergo a continuous cycle of assembly and disassembly of heterocomplexes (Smith, 1993).

The two key components of the machinery, Hsp90 and Hsp70, are both protein chaperones possessing nucleotide-binding sites that regulate their conformations. In each case, the ATP-bound conformation has a low affinity for binding hydrophobic peptide, and ATP hydrolysis resulting from intrinsic ATPase activity of the chaperone is accompanied by a conformational change to a state with high affinity for binding hydrophobic peptide (reviewed by Hartl and Hayer-Hartl, 2002 and by Picard, 2002). The ATPase activity of both proteins is essential for their biological function and is regulated by cochaperones. For example, DnaJ proteins (e.g. Hsp40) accelerate the ATPase activity of Hsp70 and Aha proteins are activators of Hsp90 ATPase activity. Hsp90 contains a strong dimerization domain at its C-terminus, and it functions biologically as a dimer.

Hsp90 in the chaperone machine has been reported to have a much higher affinity for geldanamycin than free Hsp90, and cancer cells were reported to have higher amounts of Hsp90 in the chaperone machine than normal cells (Kamal et al., 2003). However, it should be noted that Hsp90 binds Hop better when it is in the ADP (or unbound) conformation (Johnson et al., 1998), and these results could reflect metabolic differences (ATP levels) in the cells examined.

The Purified Five-Protein Assembly System

For mechanistic studies of client protein•Hsp90 heterocomplex assembly, reticulocyte lysate has been replaced by a minimal and efficient assembly system of five purified proteins – Hsp90, Hsp70, Hop, Hsp40, p23 (Dittmar et al., 1998, Kosano et al., 1998). As summarized in (Figure 2), only Hsp70 and Hsp90 are essential for

opening the ligand binding cleft to permit steroid access (Morishima et al., 2000a). The cochaperones Hop and Hsp40 are present at low stoichiometry with respect to Hsp90 and Hsp70 and are not essential for cleft opening, although they increase the efficiency of the process (Morishima et al., 2000a). p23 is not part of the Hsp90 chaperone machine and it is not required for cleft opening, but it acts both in the purified five-protein system (Dittmar et al., 1997) and *in vivo* (Morishima et al., 2003) to stabilize GR•Hsp90 heterocomplexes once they are formed by the machinery. p23 is present in cell lysates at very low stoichiometry with respect to Hsp90, but, as indicated in (Figure 2), we add it in very high concentration in the five-protein system. This is important, because the cell likes to maintain a more dynamic Hsp90 heterocomplex assembly/disassembly cycle, whereas we bias the purified system to more stable assembly. Also, as indicated in (Figure 2), p23 is

	Role in		Concentration (μM)	
Protein	Cleft opening	Stable complex	Retic lysate	5-protein system
Hsp90	Essential	Essential	~ 4	4.4
Hsp70	Essential	Essential	~ 6	4.2
Hop	Not essential	Not essential	0.6	0.2
Hsp40	Not essential	Not essential	0.7	0.2
p23	Not essential	Essential	0.6	**5.2**

p23 is limiting in reticulocyte lysate.

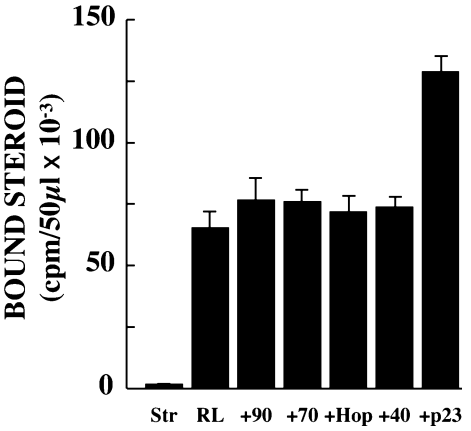

Figure 2. The five-protein heterocomplex assembly system. p23 is used in very high concentration in the 5-protein system, which biases this system towards stable client protein•Hsp90 heterocomplex assembly. The bar graph shows that p23 is limiting in reticulocyte lysate. Stripped GR immune pellets (Str) were incubated with reticulocyte lysate (RL) or RL plus purified Hsp90, Hsp70, Hop, Hsp40, or p23 in the amounts used in the 5-protein system. At the end of the incubation, pellets were washed and assayed for [^{3}H]dexamethasone binding activity. (Data from Morishima et al., 2003)

limiting in reticulocyte lysate (Morishima et al., 2003). Because it is also limiting *in vivo*, overexpression of p23 can have profound effects on client protein action (e.g. Freeman and Yamamoto, 2002). Although other mechanisms are possible, several studies suggest that overexpressed p23 is acting *in vivo* via its action as a cochaperone to increase Hsp90 effects, much as the high level of p23 in the five-protein system promotes stable heterocomplex assembly (Morishima et al., 2003; Oxelmark et al., 2003; Wochnik et al., 2004).

The Mechanism of Client Protein•Hsp90 Heterocomplex Assembly

Precise studies of the time course of progesterone receptor•Hsp90 heterocomplex assembly in reticulocyte lysate revealed an ordered series of events beginning with formation of a receptor complex with Hsp70 that was followed by assembly with Hsp90 (Smith, 1993). The study of stepwise assembly of receptor•Hsp90 complexes by purified proteins of the machinery has provided details supporting the mechanistic assembly scheme shown in (Figure 3). The first step in GR•Hsp90 heterocomplex assembly is the ATP-dependent priming of the GR to form a GR•Hsp70 complex that can be washed free of unbound Hsp70 and incubated with purified Hsp90 and Hop in a second ATP-dependent step (Morishima et al., 2000b). The very rapid priming step reflects a very focal interaction of 1 molecule of Hsp40 (Hernandez et al., 2002) and 1 molecule of Hsp70 (Murphy et al., 2003) with the ligand binding cleft of the receptor. This priming step is, thus, very different from the transient interaction of multiple chaperone molecules described for the refolding of unfolded proteins. The priming step requires a continuous high level of ATP and the ATPase activity of Hsp70, leading to the notion that client protein-bound Hsp70 may switch back and forth between ATP-bound and ADP-bound conformations during the priming steps (Morishima et al., 2001).

The GR•Hsp70 complex produced in this first step is primed to bind Hsp90, whereas the stripped receptor does not bind Hsp90 (Morishima et al., 2000b). After rapid Hsp90 binding, slow ATP-dependent opening of the steroid binding cleft is rate-limiting in the overall assembly process (Kanelakis et al., 2002). In order to have a cleft that is open to steroid access, the receptor-bound Hsp90 must assume its ATP-dependent conformation (Grenert et al., 1999), and it is only the ATP-dependent conformation of Hsp90 that binds p23 (Sullivan et al., 1997). It is clear that, during this second step, the receptor-bound Hsp90 must go through at least one complete ATPase cycle and continued ATPase activity of receptor-bound Hsp70 is required (Morishima et al., 2001). In considering how the cleft opening occurs, it seems reasonable that the client protein-bound Hsp90 and Hsp70 cooperate with each other in some sort of substrate handoff as they undergo ATP- and ADP-dependent conformational changes during the cleft opening process.

It should be emphasized that the five-protein system is a minimal system for efficient client protein•Hsp90 heterocomplex assembly, and there clearly are other

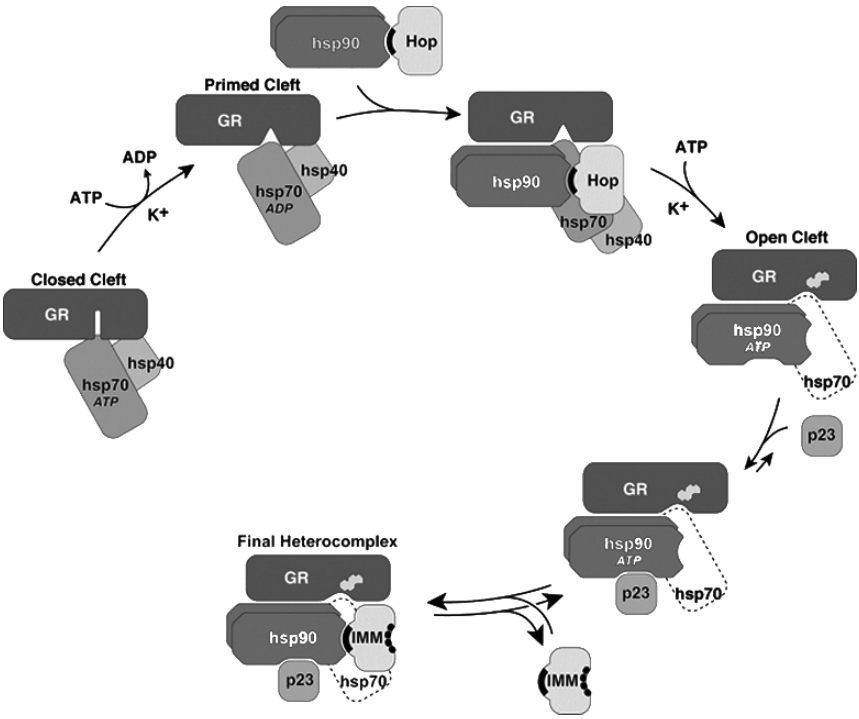

Figure 3. Mechanism of cleft opening and GR•hsp90•immunophilin heterocomplex assembly. The ATP-dependent conformation of hsp70 binds initially to the GR and in an ATP-, K^+-, and hsp40-dependent step(s) a GR•hsp70 complex is formed that is 'primed' to interact with hsp90. After hsp90 binding, there is a second ATP- and K^+-dependent step(s) that is rate-limiting and leads to opening of the steroid binding cleft, enabling access by steroid (indicated by the steroid structure). During GR•hsp90 heterocomplex assembly in cells and in reticulocyte lysate, Hop and some of the hsp70 dissociate during or at the end of the cleft opening step. The GR-bound hsp90 is now in its ATP-dependent conformation and can be bound by p23, which stabilizes the chaperone in that conformation, preventing disassembly of the GR•hsp90 heterocomplex. When Hop dissociates, the TPR domain immunophilins or PP5 can bind reversibly to the TPR acceptor site on GR-bound hsp90. The hsp40 and Hop components of the five-protein assembly system have been omitted from later steps for simplicity. TPR domains (black crescents)

factors in reticulocyte lysate that participate in the process. For example, in reticulocyte lysate and in cells, all of the Hop and some of the Hsp70 leave the client protein•Hsp90 heterocomplex at a late stage of assembly (Smith, 1993). However, most of the Hop is retained during assembly by the five-protein system, and we do not know what causes its release (Kanelakis et al., 1999). We also know nothing about the process of client protein•Hsp90 heterocomplex disassembly as it occurs during the normal assembly/disassembly cycle in the cell. Disassembly could be a purely stochastic process or it may be more complex and involve other cochaperones.

THE CONCEPT OF STABLE VERSUS DYNAMIC Hsp90 HETEROCOMPLEX CYCLING

There are two types of cycling of proteins into heterocomplexes with Hsp90. For want of a better term, we shall call the cycle of assembly/disassembly with the stringently regulated Hsp90 client proteins 'stable cycling'. The term stable cycling, thus, refers to the production of a p23-stabilized heterocomplex that will stay intact through normal biochemical separation procedures. In 'dynamic cycling', Hsp90 dissociates very rapidly after heterocomplex assembly, and there are no heterocomplexes to visualize. Dynamic cycling occurs when the Hsp90 machinery interacts with closed protein folding clefts, and it also occurs when Hsp90 complexes with metastable clefts are not stabilized by p23. Both stable and dynamic cycling may be seen under various conditions with the glucocorticoid receptor.

Under conditions where stable GR•Hsp90 heterocomplexes are not formed, one can demonstrate dynamic cycling by showing Hsp90-dependent generation of steroid binding activity. For example, dynamic cycling is seen when the stripped GR is incubated with the purified assembly system minus p23 (Dittmar and Pratt, 1997). By carrying out the assembly reaction in the presence of radiolabeled steroid, which enters the ligand binding cleft as soon as it is opened, we demonstrate the appropriate Hsp90-dependent conformational change despite the fact that GR•Hsp90 heterocomplexes cannot be visualized. With proteins that have closed clefts and undergo only dynamic cycling with Hsp90, Hsp90-dependent regulation of protein trafficking or turnover can be detected by geldanamycin inhibition of the process.

Stable versus Dynamic Cycling of Hsp90 with the GR

A variety of mutational studies on glucocorticoid receptors and protein kinases have demonstrated a segment that is required for stable client protein•Hsp90 heterocomplex cycling and stringent regulation by Hsp90. The discovery of a stable Hsp90 binding segment on the GR ensued from studies in the Simons laboratory that defined the N-terminus of the ligand binding domain (LBD). A variety of N-terminal truncations extending into the GR LBD were fused to another protein to maintain their stability in *vivo*, allowing definition of the N-terminus of a functional LBD with high affinity steroid binding activity (Xu et al., 1996). As indicated in (Figure 4), a seven amino acid segment at the N-terminus of the GR LBD was found to be required for stable Hsp90 heterocomplex assembly and for steroid binding activity (Xu et al., 1998). This region required for stable LBD•Hsp90 heterocomplex assembly lies near the opening of the steroid binding cleft (Giannoukos et al., 1999), and mutation of this seven-amino acid segment has revealed a lot about stable versus dynamic assembly and steroid access to the ligand binding cleft (Kaul et al., 2002; Dong et al., 2006).

Although deletion of amino acids 547-553 of the rat GR yields no stable Hsp90 binding (Xu et al., 1998), no specific amino acid sequence that is critical for binding seems to exist (Kaul et al., 2002). Because there is no specific sequence, we have

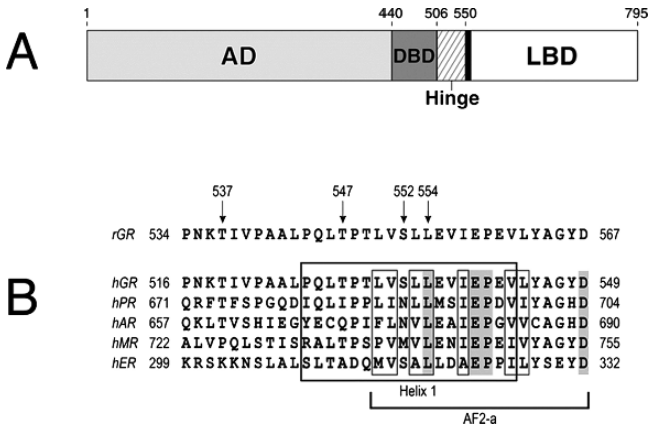

Figure 4. The seven-amino acid feature required for stable GR•Hsp90 heterocomplex assembly. A, Domains of the rat GR. AD, activation domain; DBD, DNA binding domain; LBD, ligand binding domain. The short segment in black under 550 at the N-terminus of the LBD is required for stable Hsp90 assembly with the GR. B, Sequence around the 7-amino acid segment 547-553 of the rat GR (rGR) aligned with comparable segments of the human GR (hGR), progesterone receptor (hPR), androgen receptor (hAR), mineralocorticoid receptor (hMR), and estrogen receptor (hER). The invariant residues are shaded and conserved hydrophobic residues are boxed. Helix 1 of the LBD is indicated by the large box and the autonomous transactivation domain, AF2-a, is indicated by the bold bracket

termed the seven-amino acid segment a structural 'feature' that is required for stable GR•Hsp90 heterocomplex assembly. Of particular interest with regard to dynamic cycling was the triple mutant, GR (P548A/T549A/V551A), which cycled dynamically with Hsp90 and had very low steroid binding in cells (Kaul et al., 2002). As shown in (Figure 5), this triple mutant exhibits a dose response for dexamethasone-dependent transcriptional activation that is shifted more than two orders of magnitude to the right.

In cell-free steroid binding assays it has been shown that the GR stripped of Hsp90 has a very low steroid binding activity (Nemoto et al., 1990) that is consistent with the rightward shift in dose response observed with the triple mutant. A similar rightward shift in the dose response curve was seen by Picard et al., (1990) when they constructed a strain of yeast in which Hsp90 expression could be downregulated. At a low level of Hsp90 expression, the steroid dose response curve for GR-dependent transcriptional activation from a reporter plasmid was shifted markedly to the right with respect to the response at a normal level of Hsp90 expression. Thus, stable assembly of GR•Hsp90 heterocomplexes does not switch the GR from a non-steroid-binding state to a steroid-binding state. Rather, stable heterocomplex assembly with Hsp90 prolongs the time that the ligand binding cleft is open to access by steroid, converting the receptor from a low affinity to a high affinity steroid binding state.

When there is dynamic cycling with Hsp90, the open state of the GR steroid binding cleft is not maintained and steroid binding affinity is low. This is clearly shown under conditions where Hsp90 is modified such that it engages only in

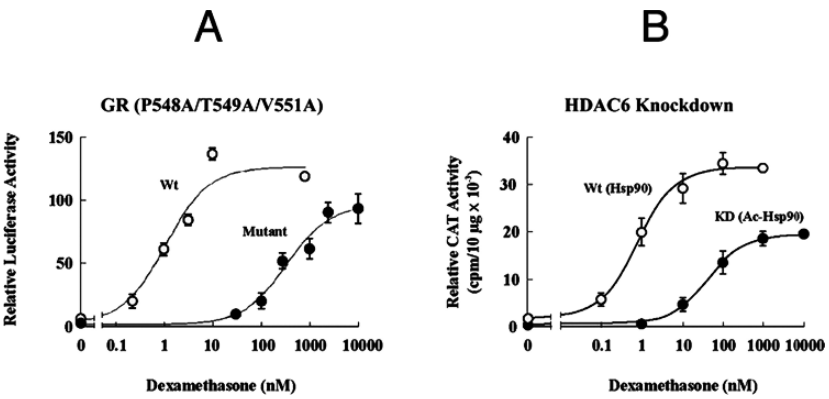

Figure 5. Conditions for dynamic GR•Hsp90 heterocomplex cycling shift the dose response curve for dexamethasone-dependent transactivation to the right. A, dexamethasone stimulation of transcription from a luciferase reporter in cells with wild type (Wt) and P548A/T549A/V551A (mutant) GR (from Kaul et al., 2002). B, Dexamethasone stimulation of transcription from a chloramphenicol acetyltransferase reporter in wild type (deacetylated Hsp90) and HDAC6 knockdown (KD) (acetylated Hsp90) cells (from Murphy et al., 2005)

dynamic cycling with metastable clefts. In wild type cells, Hsp90 is normally deacetylated because of the activity of histone deacetylase 6 (HDAC6), a cytoplasmic HDAC that is associated with microtubules (Hubbert et al., 2002). HDAC6 binds to Hsp90 and deacetylates it, and HDAC6 knockdown by specific small interfering RNA leads to hyperacetylation of Hsp90 (Kovacs et al., 2005). Acetylated Hsp90 engages in dynamic heterocomplex assembly with the GR but not in stable assembly, apparently because the acetylated Hsp90 does not interact properly with p23 (Murphy et al., 2005). Incubation of acetylated Hsp90 with purified HDAC6 converts the Hsp90 to wild type behavior. Thus, acetylation of Hsp90 results in dynamic GR•Hsp90 heterocomplex cycling, and as shown in (Figure 5), this is manifest in the cell as an approximately 100-fold shift to the right in the steroid dose response curve for gene activation (Murphy et al., 2005).

Ligand Binding Clefts and Hsp90 Heterocomplex Cycling

The effect of Hsp90 complex assembly on the steroid binding cleft in the GR ligand binding domain (LBD) is summarized by the cartoons of (Figure 6). Because the steroid binding cleft of the GR opens only very transiently during the course of normal molecular breathing in the absence of Hsp90 (Panel A), the steroid binding affinity is very low and high concentrations of steroid are required to initiate the hormone effect. This is the case in cells that have been engineered to express low levels of Hsp90 (Picard et al., 1990) or when GR•Hsp90 heterocomplex cycling is very dynamic because of GR mutation (Kaul et al., 2002) or Hsp90 acetylation (Murphy et al., 2005). When Hsp90 is not bound, the steroid binding cleft in the GR is predominantly in the closed, non-steroid-binding state. As illustrated in Panel B, the chaperone machinery facilitates cleft opening and p23-dependent stabilization

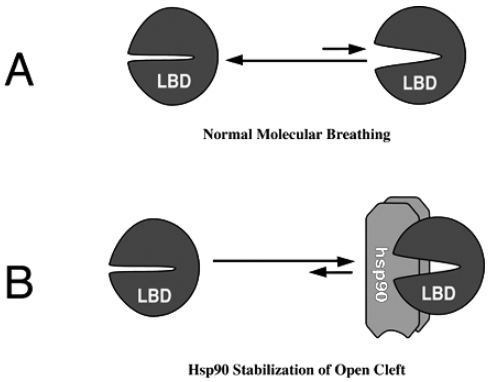

Figure 6. Behavior of the hydrophobic steroid binding cleft in the ligand binding domain (LBD) of the GR in the absence (A) or presence (B) of stable heterocomplex assembly with Hsp90

of the open state of the cleft. Low concentrations of steroid are now sufficient for binding and initiation of the hormone effect.

There are differences in the behavior of metastable ligand binding clefts with respect to their Hsp90 binding requirements. For example, the GR and the estrogen receptor (ER) behave quite differently. When Hsp90 is stripped from the GR, even at low temperature, it immediately reverts to the closed cleft form illustrated in Panel A of (Figure 6), and ATP-dependent cleft reopening by the chaperone machinery is required to regenerate high affinity steroid binding activity (Pratt and Toft, 1997). After its translation, the ER must assemble into a heterocomplex with Hsp90 to achieve its high affinity steroid binding conformation (Fliss et al., 2000). However, when the cleft in the ER LBD is opened, it is stabilized by water, and the receptor can be stripped free of Hsp90 and purified with maintenance of its high affinity steroid binding state. The cleft in the ER LBD opens much more extensively than that in the GR. The association rate constants for estrogen ligands are consistent with diffusion limited binding, whereas the association rate constants for glucocorticoid binding to the GR•Hsp90 heterocomplex are three orders of magnitude lower. Thus, even in the GR•Hsp90 heterocomplex when the steroid binding cleft is open and the receptor is in its high affinity binding state, the narrow cleft opening that is achieved still limits steroid access.

Ligand Binding Within the Cleft Alters Hsp90 Cycling

Steroids bind deep within the ligand binding clefts of the steroid receptors, facilitating a temperature-dependent collapse of the cleft around the ligand to the closed cleft state (Gee and Katzenellenbogen, 2001). Because this steroid bound, transformed form of the steroid receptors was not recovered from cells with Hsp90, it was originally thought that the liganded receptor no longer cycled into complexes with Hsp90 (Pratt and Toft, 1997). That model in which steroid binding triggers Hsp90 dissociation has been widely accepted and is presented in basic textbooks

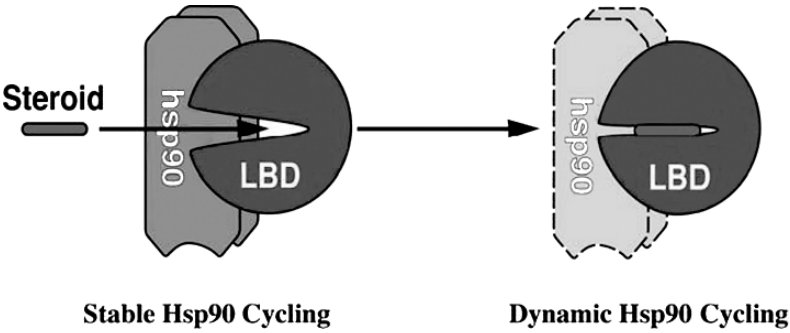

Figure 7. Steroid binding promotes a closed cleft state of the GR LBD, converting the receptor from stable to dynamic cycling into heterocomplexes with Hsp90

as the initial step in steroid hormone action. However, it is now clear that the model is wrong, and as diagramed in (Figure 7), binding of steroid to the GR favors the closed cleft conformation of the LBD, which engages in dynamic GR•Hsp90 heterocomplex cycling. This dynamic cycling of GR•Hsp90 heterocomplexes is important for the rapid, dynein-dependent movement of the receptor to the nucleus that is triggered by steroid binding (reviewed in Pratt et al., 2004). The dynein-dependent movement of steroid receptors (Pratt et al., 2004) and p53 (Galigniana et al., 2004) is inhibited by geldanamycin blockade of dynamic cycling with Hsp90.

Stable versus Dynamic Cycling of Hsp90 with p53

Probably the first example of conversion from more dynamic to stable cycling with Hsp90 was provided by natural mutations of the p53 tumor suppressor protein that were described in human tumors. p53 mutations occur in more than half of all human tumors (Hollstein et al., 1999), and inactivation of p53 is the most common alteration found in human cancer (Hollstein et al., 1991). Although most p53 mutations in human tumors are located in the DNA-binding domain and inactivate its transcriptional activity (Hollstein et al., 1999), p53 inactivation may occur in other ways. Like the GR, p53 shuttles between cytoplasm and nucleus, and one mechanism of p53 inactivation is retention in the cytoplasm. p53 mutants that are retained in the cytoplasm are in stable heterocomplex with Hsp90 (Blagosklonny et al., 1996). These mutant-p53•Hsp90 heterocomplexes are assembled by the Hsp90 chaperone machinery, and they have the same complement of associated proteins as GR•Hsp90 heterocomplexes (Galigniana et al., 2004b). Although native Hsp90 heterocomplexes with wild-type p53 have not been demonstrated, treatment of cells with geldanamycin does affect wild-type p53 function (Walerych et al., 2004). Thus, it seems that the wild-type p53 undergoes dynamic cycling with Hsp90 and that mutation of p53 to cytoplasmic localization reflects mutation to stable cycling with Hsp90.

A Common Feature Determines Stable Cycling of Hsp90 Client Kinases

A feature similar to the seven-amino acid segment required for stable cycling of GR•Hsp90 heterocomplexes has been identified within the αC-β4 loop of the catalytic domain of over 100 protein kinases (Citri et al., 2006). The first demonstrations of segments required for protein kinase•Hsp90 heterocomplex assembly were for LCK, a SRC family tyrosine kinase (Prince and Matts, 2004), and for the ERBB2 receptor tyrosine kinase (Tikhomirov and Carpenter, 2003; Citri et al., 2004). The story that has been developed for the ERBB kinases 1 and 2 is a magnificent demonstration of how dynamic and stable cycling with Hsp90 relate, respectively, to weak and stringent regulation of protein function.

The ERBB family of receptor tyrosine kinases (reviewed by Yarden and Sliwkowski, 2001) include the epidermal growth factor receptor ERBB1, a ligandless receptor ERBB2, and two heregulin binding receptors, ERBB3 and ERBB4, that have not been found to be regulated by Hsp90. The ERBB receptors are single transmembrane receptors, and binding of ligands causes hetero- and/or homodimerization with stimulation of their tyrosine kinase activity and phosphorylation of the C-terminal tail. Enhanced expression of ERBB family receptors is often observed in human cancers of epithelial and neuronal origin. Overexpression of ERBB2 occurs most frequently and is associated with poor prognosis of several types of carcinomas, in particular those of breast and ovary (Yarden and Sliwkowski, 2001). ERBB2 functions as a ligandless coreceptor that heterodimerizes with other members of the ERBB family to amplify ERBB signaling.

ERBB2 was shown to be stably associated through its kinase domain with Hsp90, but ERBB1 was not found in complex with Hsp90 (Xu et al., 2001). The turnover of ERBB2 is stringently regulated by Hsp90, whereas ERBB1 undergoes only modest ubiquitination upon treatment of cells with geldanamycin (Citri et al., 2002). Thus, ERBB1 seems to undergo dynamic cycling with Hsp90 whereas ERBB2 undergoes stable cycling. A comparison of the very homologous kinase domains of ERBB1 and ERBB2 resulted in identification of a segment within the kinase domain responsible for its geldanamycin-induced degradation (Tikhomirov and Carpenter, 2003). Figure 8 shows this motif, which lies in close association with the ATP binding cleft and the αC helix, a region regulating kinase activity (Huse and Kuriyan, 2002). The eight amino acid segments shown within the box in (Figure 8) were swapped between ERBB1 and ERBB2 to achieve the appropriate exchange of stable heterocomplex assembly with Hsp90 and geldanamycin sensitivity (Citri et al., 2004). Of particular importance within the swapped segment is the presence of the negatively charged aspartate in ERBB1, as shown by construction of an ERRB2 glycine to aspartate mutant that had decreased Hsp90 association and substantial resistance to geldanamycin-induced degradation (Xu et al., 2005). Citri et al. (2006) note that many non-Hsp90-dependent kinases appear to have a negative charge in the αC-β4 loop region, suggesting that this mitigates against Hsp90 regulation.

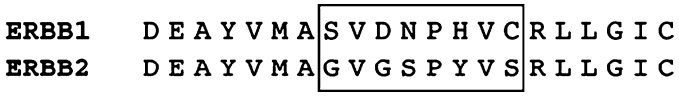

Figure 8. Sequence alignment of the region required for geldanamycin sensitivity and stable Hsp90 heterocomplex assembly of ERBB2 with the corresponding region of the ERBB1 kinase domain. The segments within the box were swapped with resulting change in heterocomplex cycling with Hsp90 (Citri et al., 2004)

How Does the Hsp90 Binding Feature Work?

The segments identified for stable Hsp90 heterocomplex assembly endow both steroid receptors and protein kinases with stringent regulation by the chaperone. There is no specific sequence for stable cycling, although it should be noted that a number of the stable Hsp90 cycling proteins preserve a proline and at least one leucine in a PXXXXLL portion of the stable assembly segment. The lack of a specific binding sequence complicates speculation as to how the stable assembly segments function. Investigators working with ERBB2 have suggested that the stable assembly segment is a region that interacts directly with Hsp90 (Citri et al., 2004; Xu et al., 2005; Citri et al., 2006). This is a reasonable proposal; however, there has, as yet, been no direct demonstration of stable Hsp90 binding to this segment.

Because we have approached the problem from the view point of explaining how the segment determines high affinity steroid binding activity, we have arrived at a different proposal. We have focused our attention on the notion that Hsp90 forms stable heterocomplexes with metastable clefts. Thus, the feature defined by the segments may allow the clefts to more readily open, endowing them with the metastability that is required for the Hsp90 chaperone machine to produce a stable client protein•Hsp90 heterocomplex. This notion that the feature defined by the segment allows a folding cleft to breathe more easily and assume a more open state could readily apply to the regulation of protein kinases. The fact that the segment maps to the αC-β4 loop is important because helix αC is an important mediator of conformational changes that take place within the ATP-binding cleft of many protein kinases (Huse and Kuriyan, 2002).

Stable Client Protein•Hsp90 Complex versus 'Kiss-and-Run'

To form a stable client protein•Hsp90 complex, Hsp90 must go through at least one complete ATPase cycle and then achieve its ATP-bound conformation in the final complex (Pratt and Toft, 2003). During this cycle, it passes through a state with preferred binding to hydrophobic peptide. It is thought that an exposed hydrophobic area in the central domain of Hsp90 interacts directly with the substrate protein (Meyer et al., 2003), and in our model of assembly with metastable clefts, Hsp90 would bind directly to hydrophobic amino acids in the cleft as they are exposed

during heterocomplex assembly rather than binding to the stable assembly segment itself. At the end of assembly when the cleft is open to steroid access, Hsp90 is in its p23-stabilized, ATP-dependent conformation. This final complex is salt-labile, and it is likely that, by this stage, the GR and Hsp90 have passed through their hydrophobic-hydrophobic interactions to a binding defined more by polar and charge interactions at the receptor surface. The client protein•Hsp90 heterocomplexes are further stabilized by Hsp90-binding proteins, such as the TPR domain immunophilins and CDC37, that interact directly with the steroid receptors and the protein kinases, respectively (Pratt and Toft, 2003).

We do not know much about the process of dynamic cycling of closed clefts with Hsp90. It is possible that in this 'kiss-and-run' interaction, Hsp90 does not pass through a complete ATPase cycle. Because the Hsp90 is not as exposed to hydrophobic amino acids of the cleft interior to the extent that it is when it interacts with metastable clefts, the Hsp90 may not be able to proceed completely through the third stage of assembly shown in (Figure 3). Although the duration of Hsp90 association with the protein during dynamic cycling is very short, protein functions are affected. We think it is possible that this 'kiss-and-run' interaction is the most common way in which the Hsp90 chaperone machinery interacts with proteins. It is likely that the Hsp90 chaperone machinery interacts stochastically with all proteins in the cytoplasm and nucleus, with three possible results being 1) no Hsp90 binding, 2) dynamic cycling, or 3) stable cycling. Thus, the proteins usually considered to be Hsp90 client proteins may be regarded as a select subclass of metastable cleft proteins that undergo stable cycling and stringent regulation.

THE Hsp90 CHAPERONE MACHINERY AND PROTEIN QUALITY CONTROL

Treatment of cells with an Hsp90 inhibitor, such as geldanamycin, increases the degradation rate of client proteins that undergo stable Hsp90 heterocomplex cycling (Neckers et al., 1999). In the absence of stabilization by Hsp90, these metastable cleft proteins are targeted for degradation via the ubiquitin/proteasome pathway (reviewed by Pickart, 2004). Not only does heterocomplex formation with Hsp90 protect client proteins against ubiquitin-dependent degradation, but two other components of the chaperone machinery, Hsp70 and Hsp40, are required for ubiquitin-dependent degradation of many proteins (Lee et al., 1996; Bercovich et al., 1997). In this section, we will develop the concept that the Hsp90 chaperone machinery is responsible for protein surveillance and quality control.

Chaperone-Dependent Ubiquitination

Although a number of substrate-specific E3 ligases have been identified that bring ubiquitin-charged E2 enzymes to proteins for ubiquitination, it has not been understood how proteins that are damaged and unfolding are targeted for ubiquitination. This problem is being resolved with the recognition that some E3 ligases utilize

chaperones as their specificity partners (Cyr et al., 2002). The chaperone-dependent E3 ligase CHIP (carboxy terminus of Hsc70-interacting protein) has been most studied with regard to the targeting of Hsp90 client proteins for ubiquitination. CHIP is a 35-kDa member of the RING-domain family of E3 ubiquitin ligases that binds via an amino-terminal TPR domain to both Hsc/Hsp70 and Hsp90 (Ballinger et al., 1999; Connell et al., 2001). CHIP possesses a carboxy-terminal U-box that interacts with the UBCH5 family of E2 ubiquitin-conjugating enzymes (Jiang et al., 2001).

It is thought that RING-type E3 ligases act as bridging proteins to bring the ubiquitin-charged E2 enzyme into the vicinity of the substrate (Pickart, 2004). However, it is not known if CHIP itself contacts the substrate, and it is thought that CHIP is targeted to the substrate by the chaperone (Hohfeld et al., 2001). Because the TPR domain of CHIP binds to Hsp90 as well as to Hsp70, both chaperones have been thought to target substrates for degradation (Connell et al., 2001; Cyr et al., 2002). However, it cannot be the case that CHIP binding to Hsp90 accounts for geldanamycin-induced degradation of client proteins that undergo stable Hsp90 heterocomplex cycling. Geldanamycin prevents Hsp90 binding to the client protein, and it is client protein-bound Hsp70 to which CHIP must bind to be effective. Both stable and dynamic cycling with Hsp90 mitigate against ubiquitination and degradation of the protein substrate, but the effect of geldanamycin in reducing protein half-life is much greater for stable cycling Hsp90 client proteins. This is because these proteins are intrinsically less stable in that their metastable folding clefts have a much higher propensity to unfold when further cleft opening is not limited by being in a stable heterocomplex with Hsp90. The way in which Hsp90 interacts with the substrate may affect its geldanamycin sensitivity, as higher drug concentrations appear to be required to promote degradation of dynamic cycling proteins than stable cycling proteins.

Overexpression of CHIP has been shown to increase ubiquitination and degradation of a variety of proteins, including those we discuss in this chapter: GR (Connell et al., 2001), nNOS (Peng et al., 2004), p53 (Esser et al., 2005), and ERBB2 (Zhou et al., 2003). Although CHIP has opened up a new way of understanding how the Hsp90 chaperone machinery functions in protein quality control, it is clearly not the only E3 ubiquitin ligase involved. We know, for example, that both the GR and nNOS undergo similar rates of proteasomal degradation in geldanamycin-treated $chip^{-/-}$ and $chip^{+/+}$ cells. There is undoubtedly some redundancy of action among the multiple E3 ligases, and it is likely that others will be found to be chaperone-dependent. For example, Hsp70 appears to target the parkin E3 ubiquitin ligase to misfolded proteins (Tsai et al., 2003), and the MDM2 E3 ligase directly binds Hsp90 (binding to Hsp70 was not examined) (Burch et al., 2004). Either of these E3 ligases could be involved in chaperone-dependent client protein degradation. In a curious intersection of two of the Hsp90 client proteins we have discussed here, dexamethasone induces ubiquitination and proteasomal degradation of the GR and p53 in hypoxic cells in a manner that is dependent upon the E3 ligase activity of MDM2 (Sengupta and Wasylyk, 2006).

Cleft Modification and Ubiquitination of nNOS

Neuronal nitric-oxide synthase (nNOS) has proven to be a useful model for exploring how specific conformational distortion of a hydrophobic cleft in a protein triggers ubiquitination. The nitric-oxide synthases are cytochrome P450-like hemoprotein enzymes that catalyze the conversion of L-arginine to citrulline and nitric oxide by a process that requires NADPH and molecular oxygen (reviewed by Marletta, 1993). As shown in Panel A of (Figure 9), NOS enzymes are bidomain in structure with an oxygenase domain, which contains the cysteine residue that is coordinated to the prosthetic heme as well as the tetrahydrobiopterin binding site, and a reductase domain, which contains the binding sites for FMN, FAD, and NADPH. nNOS is a highly regulated enzyme, requiring homodimerization and association with Ca^{2+}-calmodulin for activity. Another mechanism for regulation involves the selective ubiquitin-dependent degradation of dysfunctional nNOS (reviewed by Osawa et al., 2003).

nNOS undergoes quite dynamic cycling into heterocomplexes with Hsp90, with only trace amounts of the chaperone being coimmunoadsorbed with the enzyme (Bender et al., 1999). Nevertheless, Hsp90 inhibitors cause the loss of cellular enzyme activity and lead to nNOS degradation via the ubiquitin/proteasome pathway (Bender et al., 1999). Heme binding is required for dimerization of nNOS to the active enzyme, and Hsp90 inhibitors prevent heme binding and, thus, heme-mediated dimerization and activation of heme-deficient apo-nNOS in

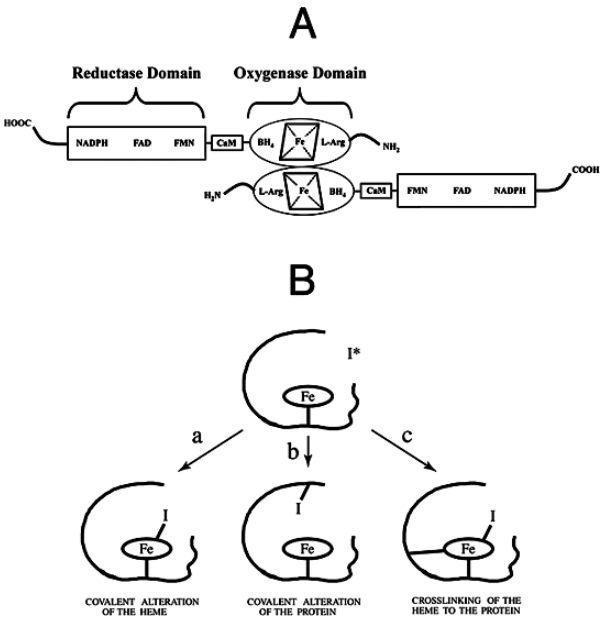

Figure 9. Domain structure of nNOS (A) and pathways for its metabolism-based inactivation (B). I*, reactive intermediate

Sf9 insect cells, which have a low level of endogenous heme (Billecke et al., 2002; 2004). A role for Hsp90 heterocomplex cycling in heme binding may apply to other heme proteins as well. For example, Hsp90 is required for the heme-mediated activation of the yeast transcriptional activator HAP1, which regulates genes involved in respiration and oxidant control (Lee et al., 2002). Geldanamycin has been found to inhibit heme-mediated reconstitution of microsomal CYP2B1, an action attributed to Grp94, a geldanamycin-sensitive member of the Hsp90 family localized to the endoplasmic reticulum (Zgoda et al., 2002). However, it should be noted that at least one of the rapid turnover cytochrome P450 enzymes, CYP2E1, is an Hsp90 client protein (Morishima et al., 2005), and it could be the cytoplasmic Hsp90 chaperone machinery that is facilitating opening of the hydrophobic cleft and heme entry.

For nNOS there is clear evidence that specific chemical attack within the hydrophobic heme/substrate binding cleft triggers its unfolding and ubiquitination (reviewed by Osawa et al., 2003). Certain mechanism-based or suicide inactivators, such as N^G-methyl-L-arginine, N^5-(1-iminoethyl)-L-ornithine, and the antihypertensive drug guanabenz, cause accelerated degradation of nNOS (Nakatsuka et al., 1998; Noguchi et al., 2000). Inasmuch as reversible inhibitors do not have this effect, it is clear that loss of enzyme activity alone is not sufficient to trigger degradation. As shown in Panel B of (Figure 9), mechanism-based inactivation can occur via alkylation of the heme moiety, covalent binding to amino acids in the enzyme active site, or by crosslinking of the heme to the apoprotein (Osawa and Pohl, 1989). Inactivation by the latter mechanism is especially important for triggering degradation (Osawa and Pohl, 1989), and it has been shown that the inactivators that cause accelerated degradation of nNOS crosslink the heme to the enzyme (Jianmongkol et al., 2000; Vuletich et al., 2002). Using myoglobin as a model, the heme-protein crosslink is known to lead to opening of the heme binding cleft (Osawa et al., 1991; 1993) to yield a more unfolded state of the protein (Osawa and Pohl, 1989). In the case of nNOS, the mechanism-based inactivation triggers ubiquitination and proteasomal degradation (Noguchi et al., 2000; Bender et al., 2000; Dunbar et al., 2004). CHIP appears to be an important E3 ligase for nNOS ubiquitination. Overexpression of CHIP causes proteasomal degradation of the enzyme and purified CHIP promotes nNOS ubiquitination by a purified E1, E2 ubiquitination system in a manner that is enhanced by Hsp70 (Peng et al., 2004). In the presence of lactacystin to inhibit proteasomal degradation, CHIP and Hsp70 are coimmunoadsorbed with nNOS, again indicating that CHIP is binding to the substrate via Hsp70 (Peng et al., 2004).

Observations made with CYP2E1 parallel observations made with nNOS. CCl_4 is a mechanism-based inactivator that crosslinks heme to CYP2E1 (Osawa and Pohl, 1989) to cause site-specific cleft damage followed by ubiquitination (Tierney et al., 1992) and proteasomal degradation (Yang and Cederbaum, 1997). As with nNOS, overexpression of CHIP causes CYP2E1 degradation, and CHIP functions as an E3 ligase for CYP2E1 ubiquitination in a purified ubiquitination system (Morishima et al., 2005). Proteasomal degradation of CYP2E1 is also caused by treatment with the Hsp90 inhibitor radicicol (Morishima et al., 2005).

Cleft Modification and Ubiquitination of ERBB2, Estrogen Receptor and p53

Several acrylamido-4-anilinoquinazoline compounds developed as specific inhibitors of ERBB2 have been shown to inactivate its kinase activity by alkylating a cysteine in the ATP binding cleft (Fry et al., 1998). These compounds are irreversible, site-specific inhibitors of ERBB1 and ERBB2 but are not active against a variety of other receptor tyrosine kinases or intracellular tyrosine kinases (reviewed by Fry, 2000). CI-1033 is an especially potent member of these irreversible ERBB1/ERBB2 kinase inhibitors. Treatment of ERBB2-expressing cells with CI-1033 induces ERBB2 ubiquitylation and degradation, whereas a reversible analog elicits lower ubiquitination and no evident degradation (Citri et al., 2002). Mutagenesis of the cysteine in the ATP binding site of ERBB2 to alanine abolishes sensitivity to CI-1033 but enhances sensitivity to geldanamycin (Citri et al., 2002). Thus, covalent interaction within the cleft triggers client protein ubiquitination, and the C to A mutation appears to render the cleft even more prone to opening (unfolding) in the absence of stable heterocomplex cycling with Hsp90. Treatment with geldanamycin causes a loss of Hsp90 and an increase in Hsp70 recovered with ERBB2 (Xu et al., 2001), and the effect of CI-1033 is the same but less pronounced (Citri et al., 2002). CHIP serves as an E3 ligase for ERBB2 ubiquitination (Zhou et al., 2003), and both CHIP and Hsp70 are coimmunoadsorbed with ERBB2 from geldanamycin-treated cells (Zhou et al., 2003). These observations are again consistent with the notion that CHIP binds to the substrate via Hsp70 to cause its ubiquitination.

The estrogen receptor (ER) provides another nice example where inhibitor binding within the hydrophobic ligand binding cleft distorts the cleft to trigger ubiquitination and proteasomal degradation. ICI 182780 (fulvestrant) is an antiestrogen that was developed for treatment of breast cancer and is devoid of any agonist activity (reviewed by Nicholson et al., 1995; Osborne et al., 2004). Pure antiestrogens of this class cause rapid degradation of the ER (Dauvois et al., 1992) by promoting ubiquitination and proteasomal degradation (Wijayaratne and McDonnell, 2001). Like other steroid receptors, the ER is an Hsp90 client protein, and the CHIP E3 ligase plays an important role in geldanamycin-induced degradation (Fan et al., 2005). Complexes containing ER, Hsp70 and CHIP have been isolated from cell lysates, but curiously, treatment of cells with either hydroxytamoxifen or ICI 182780 was found to abrogate the binding of CHIP to the ER, leading the authors to suggest that ligand binding switches the ER from CHIP-dependent degradation to an alternative pathway (Tateishi et al., 2004). Estrogen treatment induced degradation of the ER in $chip^{-/-}$ cells to the same extent as in $chip^{+/+}$ cells (Tateishi et al., 2004), suggesting again that other chaperone-dependent E3 ligases may function in redundant manner to ubiquitinate the Hsp90 client.

The example of p53 underlines the notion that Hsp90 clients may utilize multiple E3 ligases for degradation. Although interest has focused on the controlled regulation of p53 through p53-dependent induction of the RING-domain E3 ligase MDM2 and subsequent MDM2-dependent p53 ubiquitination, p53 may be targeted

by other E3 ligases (Michael and Oren, 2003). CHIP can induce the ubiquitination and proteasomal degradation of both wild-type p53 and mutant p53 that undergoes stable cycling with Hsp90 (Esser et al., 2005). Importantly, in U2OS cells depleted of CHIP, the amount of p53 increases significantly, suggesting that chaperone-mediated, CHIP-dependent degradation plays a role in maintaining low concentrations of p53 under physiological conditions (Esser et al., 2005).

The Hsp90 Chaperone Machinery: A Machinery for Protein Surveillance

The studies with geldanamycin and site-specific cleft modifiers have yielded considerable insight as to how the Hsp90 chaperone machinery functions as a machinery for protein surveillance. Hsp70 and Hsp90 are extraordinarily abundant soluble proteins, and they likely contact most, or all, proteins in the cytoplasm and nucleus in a stochastic manner. As illustrated in (Figure 10), when the machinery interacts with a folding cleft in a protein, it can either proceed towards Hsp90 heterocomplex assembly and client protein preservation or towards chaperone-dependent degradation. The GR is used to illustrate the quality control decision in (Figure 10) because of its well described cleft status and because it was one of the first proteins shown to undergo geldanamycin-induced proteasomal degradation (Whitesell and Cook, 1996; Segnitz and Gehring, 1997) and CHIP-mediated ubiquitination (Connell et al., 2001). If the protein contacted by the chaperone machinery is undamaged and in native conformation, then it can proceed to either dynamic or

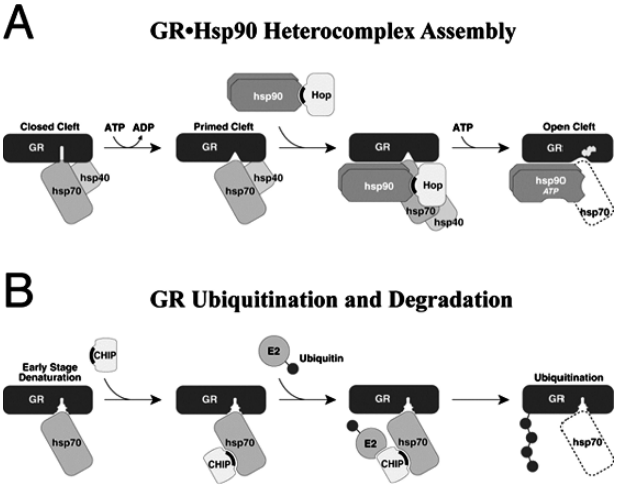

Figure 10. The triage decision between protein rescue and protein degradation. A, Interaction of the machinery with the normally folded client protein (indicated by the GR) leads to either dynamic or stable heterocomplex cycling with Hsp90. B, When protein damage leads to opening of the cleft in a degree of unfolding that can no longer be recognized by Hsp90, a chaperone-dependent E3 ligase, such as CHIP, directs ubiquitin-charged E2 enzyme to the damaged substrate. Solid crescents represent TPR domains

stable cycling into heterocomplexes with Hsp90 (Panel A). However, as we have illustrated with the examples of site-specific cleft damage above, if the damage to a protein is sufficient, cleft opening will occur as hydrophobic amino acids are exposed to the solvent during early stages of unfolding. In this case, the protein proceeds towards chaperone-dependent ubiquitination and degradation (Panel B).

A major question is what essential part of the machinery, Hsp70 or Hsp90, makes the triage decision. We have previously speculated that Hsp70 may bind in different conformations (i.e., ATP-dependent or ADP-dependent conformation) to proteins that are in native or nascent unfolding states (Pratt et al., 2006). However, experiments we have subsequently performed on Hsp70/Hsp40 priming of native nNOS and nNOS that has undergone mechanism-based inactivation suggest that this speculation is wrong. Thus, although Hsp70 binds before Hsp90, it does not direct the decision to proceed to Hsp90 heterocomplex assembly and client protein preservation or to proceed to ubiquitination and proteasomal degradation. The fact that geldanamycin inhibition of Hsp90 heterocomplex assembly results in substrate ubiquitination and degradation suggests that the assembly of heterocomplexes with Hsp90 mitigates against Hsp70-dependent ubiquitination by chaperone-dependent E3 ligases. On the basis of studies with denatured luciferase, it has been suggested that binding of geldanamycin shifts Hsp90 from refolding to degradation mode by preventing release of Hsp90 from the polypeptide substrate (Schneider et al., 1996). This is entirely the opposite of what we propose. Rather, inhibition of Hsp90 heterocomplex assembly permits unrestrained activity of chaperone-dependent E3 ligases binding to substrate-bound Hsp70.

Thus, it is Hsp90 stabilization of the folding cleft that limits further unfolding and determines the stability of the substrate. Although we have presented Hsp90 binding and CHIP binding as separate pathways in (Figure 10), it is clear that the whole process of protein quality control proceeds in an integrated manner. As Hop leaves complexes during assembly, the TPR binding site on substrate-bound Hsp70 is available to bind CHIP. Thus, CHIP has been recovered in client protein•Hsp90 heterocomplexes, and CHIP has even been shown to undergo ligand-activated retrograde movement with steroid receptors that is dependent upon dynamic cycling with Hsp90 (Galigniana et al., 2004a; Thomas et al., 2004). It is best to think of the Hsp90 chaperone machine as being very dynamic and plastic in the sense that it can be used for different purposes, such as protein quality control and protein trafficking, depending upon the accessory proteins (cochaperones) that are binding to substrate-bound Hsp90 and Hsp70.

OVERVIEW

Interaction of the Hsp90 Chaperone Machinery with Protein Folding Clefts

The Hsp90 chaperone machinery interacts with and regulates a wide variety of proteins, independent of their size or sequence, by interacting with protein folding clefts, a topological feature of all natively folded proteins. In many proteins, these

clefts provide access of ligands, such as steroids, heme or ATP, to hydrophobic binding sites in the protein interior. These clefts are metastable in the sense that they must readily open to permit ligand access, but this propensity for cleft opening imposes an instability on the protein, in that cleft opening can easily proceed to protein unfolding. It is these metastable cleft proteins that form stable heterocomplexes with Hsp90 and are the most stabilized to degradation via the ubiquitin/proteasome pathway. Other proteins have folding clefts that tend to remain closed because they are stabilized by strong hydrophobic interactions in the cleft interior. These proteins are inherently more stable, they have longer half lives, and they cycle dynamically with Hsp90. The function and stability of these closed cleft proteins is much less affected by their dynamic cycling into heterocomplexes with Hsp90 than client proteins with metastable clefts, which are stringently regulated by Hsp90. It is the metastable cleft signaling proteins that have attracted the keen interest of cancer researchers because they are profoundly affected by Hsp90 inhibitors, such as geldanamycin.

The client protein•Hsp90 heterocomplexes are assembled by a multichaperone machinery in which Hsp70 and Hsp90 are the essential components. This machinery facilitates cleft opening and then, by forming stable heterocomplexes with Hsp90, it limits further cleft unfolding. Although chaperones are usually regarded as interacting with unfolded states of proteins to facilitate refolding, the Hsp90 chaperone machinery does quite the opposite in that it interacts with natively folded proteins to effect a partial unfolding (cleft opening). The ability of the Hsp90 chaperone machinery to recognize and bind to protein folding clefts has a number of consequences, such that it can be regarded as a general machinery for protein management in the cell. The different effects that ensue from the assembly of Hsp90 heterocomplexes involve the interaction of substrate-bound Hsp70 and Hsp90 with a variety of cochaperones, like TPR domain immunophilins, PP5, CDC37, and CHIP.

How Interaction of the Chaperone Machinery with Protein Folding Clefts Accounts for Different Hsp90 Actions

- *Protein Function:* Opening of protein folding clefts by the Hsp90 chaperone machinery facilitates ligand entry to hydrophobic binding sites in the protein interior. In the case of steroid and dioxin receptors, this permits them to respond at low ligand concentrations. In the case of enzymes, such as NOS and the Hsp90-regulated protein kinases, the site affected is the site of catalysis and enzyme activity is affected. It is also possible that client protein function is affected by TPR proteins that bind to Hsp90, such as the immunophilins with their peptidylprolyl isomerase activity and PP5 with its serine/threonine phosphatase activity.
- *Protein Quality Control:* Treatment of cells with an Hsp90 inhibitor, like geldanamycin, results in rapid degradation of Hsp90 client proteins, like RAF,

ERBB2 and AKT, by the ubiquitin/proteasome pathway. These are all proteins with metastable clefts that are profoundly stabilized when they are in heterocomplex with Hsp90. How their association with Hsp90 inhibits their ubiquitination is not known. But, when Hsp90 association is inhibited by geldanamycin, chaperone-dependent E3 ubiquitin ligases that associate with client protein-bound Hsp70 target ubiquitin-charged E2 enzymes to the substrate for ubiquitination. Thus, in the case of metastable cleft proteins that are stringently regulated by Hsp90, inhibition of Hsp90 heterocomplex assembly permits chaperone-dependent E3 enzymes to function unabated through their interaction with client protein-bound Hsp70, and they are rapidly degraded by the 26S proteasome.

The Hsp90 chaperone machine may be the major mechanism in the cell for recognizing and processing damaged proteins. It has not been known how proteins that have undergone oxidative or toxic damage are shunted to the ubiquitin/proteasome pathway of degradation. It is reasonable to propose that, as proteins undergo such damage, their folding clefts begin to open as the unfolding process proceeds. As long as Hsp90 can form even transient heterocomplexes with the opening cleft, ubiquitination directed by chaperone-dependent ubiquitin E3 ligases, like CHIP, is inhibited. When unfolding of the cleft has progressed to the point where Hsp90 heterocomplexes cannot form, the E3 ligases bound to client protein-bound Hsp70 target ubiquitin-charged E2 enzyme to the substrate.

- *Protein Trafficking:* The Hsp90 chaperone machine may be the major mechanism by which protein cargo is linked to molecular motors for protein trafficking along cytoskeletal tracks. Because the Hsp90 chaperone machinery interacts with protein folding clefts, it has the potential for capturing proteins, however transiently, into Hsp90 heterocomplexes. The Hsp90-binding TPR domain immunophilins link the client protein•Hsp90 heterocomplex to the dynein/dynactin motor protein system for retrograde movement (reviewed by Pratt et al., 2004). Myosin-binding UNC proteins that bind to the TPR acceptor site on Hsp90 may perform a similar linking function for anterograde movement. It is also thought that Hsp90 serves to 'hand off' proteins from these trafficking systems to Hsp90-binding TPR domain proteins that function as components of protein import machineries for organelles, such as mitochondria and peroxisomes (Owens-Grillo et al., 1996; Pratt et al., 2001; Young et al., 2003).
- *Assembly of Protein Complexes:* It is becoming clear that the Hsp90 chaperone machinery plays an important role in the assembly of some stable multisubunit protein complexes. For example, the Hsp90 chaperone machinery is required for the assembly and maintenance of the 26S proteasome (Imai et al., 2003). We speculate that by facilitating cleft opening, hydrophobic amino acids that are otherwise buried are exposed to solvent, facilitating the formation of hydrophobic interactions between protein subunits. Thus, protein-protein interactions are strengthened by the addition of van der Waals bonds that would not normally be formed.

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CHAPTER 2

IMPLICATIONS OF HEAT SHOCK PROTEINS IN CARCINOGENESIS AND CANCER PROGRESSION

DANIEL R. CIOCCA[1,*], MARIEL A. FANELLI[1],
F. DARIO CUELLO-CARRIÓN[1] AND STUART K. CALDERWOOD[2,3]

[1]*Oncology Laboratory, Institute of Experimental Medicine and Biology of Cuyo (CRICYT-CONICET), and Argentine Foundation for Cancer Research (FAIC), Mendoza, C. C. 855, 5500 Mendoza, Argentina, e-mail: dciocca@lab.cricyt.edu.ar*

[2]*Division of Molecular and Cellular Radiation Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215*

[3]*Department of Medicine, Boston University School of Medicine, MA 02118, USA, e-mail: scalderw@bidmc.harvard.edu*

Abstract: Heat shock proteins (Hsp) participate in many events related to cancer as molecular chaperones, starting from the very beginning of carcinogenesis. Several etiological factors involve the Hsp family in their mechanisms of action, including oncogenic viruses, hereditary and non hereditary alterations in tumor suppressors or oncoproteins, hypermethylation, radiation and carcinogenic agents. All of them produce changes in the Hsp response with consequences in cell proliferation, differentiation, inflammation, apoptosis, DNA repair, angiogenesis, metastasis, and drug resistance and in the immunological response mounted by the host. In this chapter we will examine the participation of the Hsp response in tumor cell transformation, either by up-regulation or down-regulation of specific Hsp. This can explain the variations in Hsp expression found in pre-neoplastic and neoplastic human tumors in different tissues and organs. These variations have important clinical consequences in cancer progression, and the exploitation of such knowledge may improve anticancer treatment strategies

Keywords: Heat shock proteins, cancer, etiology, carcinogenesis, metastasis, drug resistance, immunity, cancer progression, DNA repair, prognosis

INTRODUCTION

Many of the functions attributed to the heat shock proteins (Hsp) have been obtained through the study of the role of these proteins in tumor cell biology. For example, the chaperone activity of Hsp90 is critical to maintain many client proteins that

*Corresponding author: Oncology Laboratory, IMBECU, CRICYT, Casilla de Correo 855, Mendoza 5500, Argentina, Tel: +54-261-5244045, Fax: +54-261-5244001, e-mail: dciocca@lab.cricyt.edu.ar

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are essential for cancer cell growth and survival (See: *Pratt et al*, Chapter 1, this volume). This has led to the study of a large array of Hsp90 inhibitors that interfere with the chaperone function of this protein causing destruction of mutated proteins, oncoproteins, and also altering the stabilizations of "normal" proteins important for tumor progression/vascularization like hypoxia-inducible factor 1, via ubiquitination and proteasomal degradation (See: *Neckers*, Chapter 12, *Whitesell*, Chapter13, *Kamal et al*, Chapter 14, *Workman*, Chapter 15). This form of cancer therapy is under active investigation (Chiosis et al 2006). On the other hand, some Hsp have been unexpectedly linked to cancer, for instance during the search for estrogen-induced proteins in MCF-7 human breast cancer cells, a new 24 kDa protein was identified which later proved to be a member of the Hsp family, Hsp27 (reviewed by Ciocca et al 1993). This protein has been related to anticancer drug resistance, to the prognosis of certain cancer types, and to the inhibition of specific apoptotic pathways (reviewed by Ciocca and Vargas-Roig, 1997, Ciocca and Calderwood 2005, Calderwood et al 2006). In other instance researchers were identifying genes regulated by an antitumor (apoptotic) and anti-angiogenic agent in myeloma cells using oligonucleotide arrays, and among the modulated genes were included several members of the Hsp family (Chauhan et al 2003). Thus, through employing different approaches and techniques, the area of Hsp involvement in tumor biology has expanded to many crucial aspects of tumor development, embodying cell growth and differentiation, apoptosis, metastasis, drug resistance, and anticancer therapies including immunotherapy. These aspects will be covered in the different chapters of this book.

HEAT SHOCK PROTEINS AND CARCINOGENESIS

There is a cascade of molecular events that mediate the transformation of a normal cell into a cancer cell. Several etiological agents and factors have been identified as responsible for initiating the carcinogenic process, including are viruses, radiation, carcinogenic compounds, and hereditary and non-hereditary genetic alterations. Thus we aim to know how these cancer etiological agents can modify the molecular milieu of the transformed cells and, in our case, to the role of the Hsp.

Understanding the consequences of such changes are paving the way to cancer prevention, diagnosis and treatment strategies. Among the identified oncogenic viruses are adenovirus, HPV, HBV and HCV; all of them have been related with changes in the expression of certain Hsp (Burdon 1986, Ciocca et al 1991, Ciocca et al 1992, Lim et al 2005). Consequences of such alterations are cytoskeleton modifications characteristic such as seen in HPV infection (collapse of the chaperone-cytoskeleton complex to a perinuclear location seen in koilocytes). The cytoskeleton is very sensitive to different stressors, and the Hsp contribute to cytoskeleton organization (Quinlan 2002). Figure 1 shows the relationship between the oncogenic agents and the Hsp. Another microorganism linked with carcinogenesis is Helicobacter pylori that affects expression of PCNA, p53, c-erbB-2 and Bcl-2 in the human gastric mucosa (Jorge et al 2003). The infection of MKN7

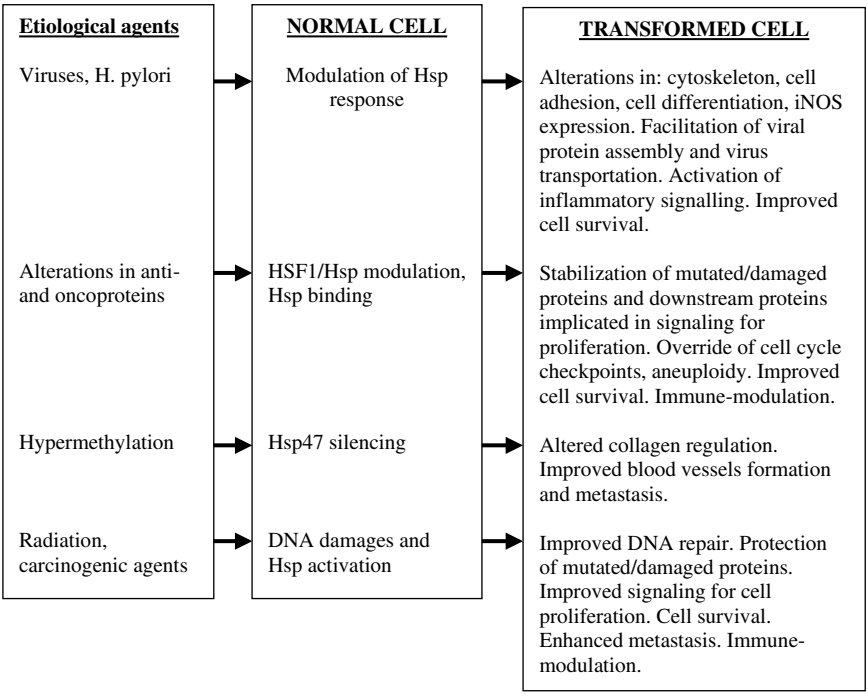

Figure 1. Schematic representation of the relationships between the oncogenic agents/events and the heat shock response. The etiological factors of cell transformation act on a normal cell, modulating on the Hsp response. These changes in Hsp expression can then produce a number of alterations in the transformed cell contributing to the carcinogenesis

human gastric epithelial cells by this bacterium inhibited or attenuated the expression of Hsp70, and this response was dependent on the bacterial strain used (Tragosz et al 2006). This inhibitory effect on Hsp70 has been linked to a weakening of the gastric mucosa, facilitating the pathogen infiltration through the epithelial tight junctions and posterior activation of inflammatory signalling by leukocytes. The restoration of Hsp70 levels in cultured cells suppressed the expression of iNOS (a major factor of gastric cell damage induced by Helicobacter pylori) (Yeo et al 2004).

Anti-oncogenic proteins can also interact with Hsp, for example the mutant form of p53 binds Hsp72/Hsc73 more firmly than the non-mutant p53 (Finlay et al 1988, Lehman et al 1991); the retinoblastoma gene product Rb110 can selectively bind Hsc73 (Nihei et al 1993), and the breast cancer susceptibility gene-1 (BRCA1) protein can increase the expression of Hsp27 while cancer cell lines exposed to heat shock showed a rapid disappearance of BRCA1 (Xian Ma et al 1993). Thus, the heat shock response and the Hsp participate in the pathways of certain anti-oncogenic proteins.

On the other hand, we might ask: Are there links between oncogenic proteins and Hsp? Early during carcinogenesis we observe activation of key molecules linked to cell growth and survival, including heregulin (HRGβ1) that binds to c-erbB receptors on the cell surface of transformed cells. This causes the induction of Hsp with the consequences of cell protection against apoptosis and anchorage-independent growth (Khaleque et al 2005). The role of this pathway in HSF1 activation as well as the consequences of this activation are presented in chapter 3 of this book by *Calderwood et al*. In a recent review we have analysed the interactions of estrogen receptors and Her-2/neu in breast cancer cells (Ciocca et al 2006). Briefly, the Her-2/neu and membrane estrogen receptor mediated PI3K pathway activation via HSF1 can stimulate the synthesis of Hsp with important clinical consequences on cell growth and survival, bringing resistance of tumor cells to anticancer therapies. In fact, the breast cancer patients that expressed both estrogen receptors and Her-2/neu positive phenotype have shown a shorter disease-free survival and overall survival, even when treated with the anti-estrogen tamoxifen alone or in combination with chemotherapies, and the Hsp seem to be at least in part responsible of this resistance to the anticancer drugs (Ciocca et al 2006). Zhang et al (2005) performed a proteomic of overexpressed proteins in Her-2/neu-positive breast cancer; they found that several proteins involved in metabolic and detoxification pathways are up regulated, including Hsp27. The proto-oncogene c-Myc (which may be activated by heregulin and Her-2/neu) is another example of an oncogene implicated in Hsp regulation, specifically with Hsp70, by interaction of c-myc and cbf/nf-y, causing alterations in the cell cycle (Taira et al 1999).

Hypermethylation can cause gene inactivation and has been linked to an altered Hsp response, due to silencing of Hsp47, a collagen specific molecular chaperone that regulates collagen type I and IV production in human neuroblastoma cells (Yang et al 2004). These authors reported that treatment with demethylating agent 5-Aza-dC caused Hsp47 up-regulation (>80-fold), inhibition of neuroblastoma growth, and that the tumorigenicity of this cell line was inversely correlated with the level of collagen. In line with this, there is evidence that Hsp47 plays a role in tumor progression mediating the endogenous processing of collagen XVIII, a precursor of endostatin, in head and neck tumor cells (Ollins et al 2002).

DNA is exposed to endogenous (e.g., oxidative stress) and exogenous damaging agents. Examples of the latter include mutations, viruses, hypermethylations, and radiation and exposure to carcinogenic compounds (including anti-neoplastic drugs) may also produce DNA alterations. DNA damage is detected by sensor proteins that recognize the specific damage or the alterations at the level of the chromatin, with subsequent activation of several specific signalling pathways that form part of the DNA damage responses (Barzilai and Yamamoto 2004). The ability to repair DNA lesions is critical for cell survival and cells with such damage can arrest the cell cycle and repair the DNA or they can activate the apoptotic pathways. If some of the DNA damage response pathways malfunction, the transformed cell will enter into a cascade of DNA and chromatin changes. Therefore, the ability

to repair the DNA lesions is strongly associated with individual susceptibility to cancer. There is growing evidence that the Hsp participate in some of these DNA damage responses (See: *O'Callaghan and Gabai*, Chapter 9). We have reported that the stress response participates in DNA repair when human peripheral blood mononuclear cells where exposed to DNA damage induced by doxorubicin (Nadin et al 2003). We found high levels of Hsp27 and Hsp70 with nuclear translocation of these proteins when the cells were exposed to a heat shock (with a resting period of 24 hours) before drug exposure. Under these circumstances cells showed high expression of the mismatch repair proteins hMLH1 and hMSH2 and increased numbers of undamaged cells. In addition, immunofluorescence studies and confocal microscopy revealed that hMLH1 and hMSH2 co-localize with Hsp27 and Hsp70 suggesting that these proteins may contribute with the DNA mismatch repair function (Nadin et al 2006). Xiao et al (2002) report that in lymphocytes of workers exposed to chemical carcinogens, high levels of Hsp72 are associated with lower genotoxic damage. In line with the implications of Hsp in DNA repair, others report the association of Hsp70 with a key base excision repair enzyme stimulating endonuclease activity at abasic sites (Kenny et al 2001, Bases 2005). Hsp70 is also induced by DNA damage induced by ultraviolet C in a human lung adenocarcinoma cell line (Niu et al 2006). Therefore, there is growing evidence for the involvement of Hsp in the molecular mechanisms of DNA damage and repair.

HEAT SHOCK PROTEINS IN MALIGNANT AND NON-MALIGNANT HUMAN TISSUES

We have seen that several cancer etiological agents alter/modulate many molecular pathways during cell transformation, and that the Hsp response participates in tumor cell transformation usually by up-regulation but also by down-regulation of specific Hsp. This can explain the variations in Hsp expression found in preneoplastic and neoplastic human tumors in different tissues and organs. At the histopathological level, in most cases the transition from a normal tissue to a cancer tissue is accompanied by an increase in Hsp expression. During the carcinogenesis of the uterine cervix, the subcolumnar cells of the endocervix showed strong expression of Hsp27 during the process of transformation into *foci* of metaplasia (Ciocca et al 1986). It is known that in the majority of cases cervical intraepithelial neoplasia arises from a metaplastic epithelium, and in human cervical samples with intraepithelial neoplasia as well as in invasive carcinomas Hsp27 is persistently expressed at high levels in an elevated number of cases (Puy et al 1989). A similar situation has been reported for Hsp70 and this protein was differentially overexpressed in human squamous cell carcinomas of the uterine cervix compared to normal cervix or pre-malignant lesions (Ralhan and Kaur 1995). Hsp27 showed higher expression levels in endometrial carcinomas than in the normal and non-malignant counterparts (Ciocca et al 1989). Lim et al (2005) reported that expression of Hsp27, Hsp60, Hsp70, Hsp90, GRP78, GRP94 increased along with the stepwise progression of

HBV-related hepatocarcinogenesis. Another example occurs in the mammary gland where GRP78 mRNA and protein are detected in normal and benign human lesions but are overexpressed in malignant lesions (Fernandez et al 2000). In addition, expression of Hsp90 was significantly higher in human breast cancer biopsy samples than in non-cancer tissues (Yano et al 1999). In renal cell carcinoma Hsp27, Hsp70 and Hsp72 expression was higher than in adjacent non-cancerous human renal tissue (Erkizan et al 2004, Atkins et al 2005), while in melanomas Hsc73 expression was higher than in melanocytic nevi (Deichmann et al 2004). These authors found that in addition to melanoma, gastric and uterus cancer tissues showed higher Hsc73 mRNA expression on a matched tumor/normal cDNA array than their normal counterparts. Hsp90 has also been found at higher levels in melanomas and melanoma metastases than in melanocytic nevi (Becker et al 2004).

In lymphomas, Hsp90 and Hsp60 showed higher expression in Reed-Sternberg cells of Hodgkin's disease than in the background reactive lymphoid cells, while Hsp27 was positive in malignant cells and absent in all lymphoid cells and histiocytes (Hsu and Hsu 1998). In contrast, these authors found that Hsp27, Hsp60 and Hsp90 were absent or expressed at low levels in malignant cells in anaplastic large cell lymphoma and in most high-grade lymphomas. Thus the Hsp response is differentially regulated during the carcinogenesis; in other words, these proteins may participate in the pathogenesis of certain cancers but not in others. Of interest are the studies of Czarnecka et al (review, 2006) and of Cappello et al. (2006) regarding mitochondrial chaperones Hsp60 and Hsp10 in bronchial, colorectal, prostatic and exocervical carcinogenesis. They observe differential expression of these two Hsp according to the organ studied. For example Hsp60 and Hsp10 were overexpressed in colorectal, prostatic and exocervical carcinogenesis, while both proteins were down-regulated during bronchial epithelial carcinogenesis in smokers with chronic obstructive pulmonary disease. This is a clear example that not all of the molecular chaperones are overexpressed during tumor development/formation, suggesting that there are specific molecular changes that produce the up- or down-regulation of specific Hsp. On the other hand, certain Hsp, like Hsp105 do not appear in the early stages of malignant transformation but in more advanced stages of tumor development (Kai et al 2003), implicating a role in the late molecular stages of tumor pathogenesis such as metastasis. Indeed, Hsp105 expression correlates with survival in patients with lung adenocarcinomas (Ullmann et al 2004). In addition, during tumor formation, cells may suffer a number of different stresses such as low oxygenation, glucose depletion and acidosis, awakening specific responses: hypoxia inducible factor-1 (stabilized by Hsp90) and glucose-regulated protein GRP78, which are implicated in angiogenesis and drug resistance (Osada et al 2004, Dong et al 2005). The new microenvironment of the cancer cells is highly heterogeneous, not only from tumor to tumor but also within the different areas of a given solid tumor and the metabolism of tumor cells is not the same in the periphery were they grow and invade the normal tissue, and in the central core of the tumor. These contrasting factors may explain the heterogeneity of Hsp expression found in the cancer tissues.

DRUG RESISTANCE

Drug resistance is a serious problem for the treatment of cancer patients, and there are many molecular hypotheses to explain the innate or *de novo* resistance to anticancer treatments (Bernal 1997, Ciocca et al 2005). This is an important research field which aims to: a) block the molecular pathways that interfere with cell death, and b) identify useful markers that can predict the response to the treatment(s). The differential expression of Hsp in cancer tissues has impact in the response of the tumor cells to anticancer agents. Both basic and clinical data support the role of Hsp in drug resistance and we have already mentioned Hsp90, and the relationships between estrogen receptors, heregulin and the Hsp; Hsp27 and Hsp70 have been implicated in counteracting death signals and we have previously reviewed this topic (Ciocca and Vargas Roig 1997, Ciocca and Calderwood 2005, Calderwood et al 2006). In a recent study Lee et al (2006) established Hsp27 overexpression in a cancer cell line and showed cellular resistance to heat shock, *cis*-platin, staurosporin and H_2O_2. However, similar resistance was not found for irradiation or serum starvation. In addition, Hsp27 overexpressing cells showed a delay in cell growth suggesting that this may contribute to the chemoresistance. This is consistent with a previous study in human breast cancer biopsy samples showing that Hsp27 overexpressing cells have a lower proliferation rate (Vargas Roig et al 1997). A decreased proliferation of trastuzumab (a therapeutic monoclonal antibody against Her-2/neu) resistant breast cancer cell line was obtained when these cells were treated with an Hsp90 inhibitor (17-AAG, a derivative of geldanamycin) (Zsebik et al 2006). In addition, the authors found decreased Her-2/neu levels, suggesting that 17-AAG might be useful in trastuzumab resistant Her-2/neu overexpressing tumors.

Another stress-induced protein is GRP78/BiP, a major endoplasmic reticulum chaperone and Hsp70 family member, implicated in drug resistance (anti-apoptotic). Many anticancer drugs can induce apoptosis. Interference with the GRP78 expression levels sensitizes human breast cancer cells to etoposide-mediated cell death (Dong et al 2006). In addition, using GRP78 overexpression and siRNA techniques, it has been found that this protein facilitates tumor growth and confers drug resistance to cancer cells (reviewed by Li and Lee 2006).

One strategy to evade the Hsp response in cancer cells is through administration of small interfering RNA (siRNA) to silence HSF1. This has been explored in HeLa carcinoma cells (Rossi et al 2006) which show a dramatic increase in sensitivity to concomitant hyperthermo-chemotherapy (but not when the treatments were administered separately). Thus, silencing of HSF1 might be useful to block the Hsp response increasing the sensitivity of cancer cells to this combination of anticancer treatments.

One recent example of the possible value of Hsp detection to predict the response to chemotherapy has been reported in non-small cell lung cancer patients. Berrieman et al (2006) studied several proteins correlating their expression levels with the response to chemotherapy with the single agent vinorelbine, they found that Hsp27-positive tumors showed more cancer progression. The predictive value of Hsp to anticancer treatments has been reviewed (Ciocca and Calderwood 2005).

METASTASIS

The final step of tumor progression involves the invasion of tumor cells into surrounding tissue and their dissemination to form colonies (metastases) in distant organs. Mechanisms involved in this complex, multistage process include: disruption of cell-cell junctions, digestion of the extracellular matrix, migration to and to crossing of the walls of blood / of the lymphatic vessels, penetration of the circulation, survival of a fraction of such cells in the blood the circulation and finally colonization of distant organs re-using many of the processes that catalyzed invasion. Approximately >90% of cancer related deaths are due to metastasis (Germanov et al 2006). The detailed molecular regulation of the metastatic behavior is not well understood, however, there is a consensus that cell-to cell and cell-to- substrate interactions are profoundly altered in cancer cells reaching these final stages of cancer progression (Cavallaro et al 2004). Hsp have the capacity to interact with proteins involved in the regulation of cell adhesion, invasion and migration. Several studies point to the ability of Hsp27 to increase metastatic potential of tumor cells in nude mice and enhance their resistance to therapy (Blackburn et al 1997, Katoh et al 2000). Much evidence indicates an essential role for the MAPKAP-kinase/Hsp27 pathway during cancer progression and cell migration. Phosphorylation of Hsp27 can be catalyzed by MAPKAPK-2 and MAPKAPk-3 (Landry et al 1992, Ludwig et al 1996), protein kinase C (PKC) (Maizels et al 1998), cGMP-dependent protein kinase (Butt et al 2001), and protein kinase D (Doppler et al 2005). Recently, in human breast cancer cells Hp27 was shown to promote migration, and such effects are inhibited by blocking PKC-dependent phosphorylation of Hsp27 (Ki Deok et al 2005). Hsp27 is highly up-regulated in prostate cancer cells and cell invasion is mediated through a pathway in which p38/MAP kinase activates MAPKAPK2, which leads to phosphorylation of Hsp27 which mediates matrix metalloproteinase type 2 (MMP-2) activation and cell invasion (Xu et al 2006). The MMP-2 protease is important for digestion of many of the major components of the extracellular matrix surrounding tumor masses and for subsequent invasion of primary tumor cells (Irah et al 2001).

Naturally occurring compounds that inhibit Hsp90 action, such as geldanamycin and radicicol, have provided evidence of the importance of Hsp90 in cancer cell growth and progression (Maloney et al 2002, Soga et al 2003) (Chapters 12, 13, 14, 15, this volume). Moreover, a number of Hsp90 clients, such as c-Src, epidermal growth factor receptor, and MMP-2 (Neckers et al 2003, Workman et al 2004) play a role in bone metastasis. Indeed, the inhibition of Hsp90 increases osteoclast formation and potentiates bone metastasis (Price et al 2005). Sanderson et al (2006) identify Hsp90 inhibitors as important regulators of many aspects of tumor angiogenesis (and potentially lymphangiogenesis). Recently, an *extracellular role* for Hsp90 was demonstrated in the pathological process of tumor cell invasion and metastases (Eustace et al 2004). The Hsp90α isoform, but not Hsp90β, is expressed extracellularly where it interact with MMP-2, assists its activation and leads to increased tumor invasiveness. Thus Hsp90 drugs decrease invasiveness without inhibiting intracellular Hsp90 (Eustace et al 2004).

The cadherin-catenin system is an important molecular pathway that undergoes crosstalk with Hsp expression; both processes are regulated by phosphatidylinositol 3 kinase (PI-3K). In breast cancer cells, this pathway is activated by the membrane estrogen receptors and/or by heterodimerization of the Her-2/neu oncoprotein, PI-3K can then positively regulate Hsp transcription through activation of HSF-1 (Ciocca et al 2006). On the other hand, the PI-3K/Akt signal transduction cascade also regulates intracellular β catenin levels. Akt is a downstream target of PI-3K, phosphorylating and inactivating glycogen synthase kinase 3-β (GSK3β), thereby, accumulating unphosphorylated β-catenin (Monick et al 2001). β catenin is a truly dual-functioning protein involved in cell adhesion and cell signaling. Initially, it was discovered for its role in cell adhesion by binding to the intracellular domain of the cadherin and linking cadherin to the actin cytoskeleton through the adaptor protein α catenin. The adhesion function is based on a subcellular pool of β catenin that is membrane-associated and stable. In contrast, the signaling function of β catenin is conferred by a soluble cytoplasmic pool that is highly unstable in the absence of a *Wnt* signal, undergoing phosphorylation by casein kinase 1 and glycogen kinase 3 β that mark it for proteasome-mediated degradation (Bienz 2004). Upon *Wnt* signal, mutations of β catenin, or deletion of the APC gene, β catenin accumulates in the cytoplasm and translocates to the nucleus promoting the transcription of *Wnt* target genes by binding to TCF transcription factors in the nucleus. The cell signaling function of β catenin is coupling to a loosening of adhesion between epithelial cells during developmental processes and also during cancer progression. Several β-catenin associated proteins such as androgen receptor, estrogen receptor α and galectin have been described and related to the β catenin signaling pathways in cancer. Recently we observed a novel interaction of β catenin with Hsp27 and HSF1 by immunoprecipitation and co-localization studies. Moreover β catenin was observed in the same tumor area and in the same tumor cells that expressed Hsp27 (paper submitted). The association was strong when β catenin was expressed in the cytoplasm of tumor cells. In addition, β catenin co-localized with HSF1 (D. R. Ciocca, in preparation). Finally, the prognostic significance of cadherin-catenin proteins was examined by immunohistochemistry in breast cancer patients and we found that the disease free survival and the overall survival were significantly shorter for patients with cytoplasmic β catenin positive tumors (Fig. 2). The interaction of β catenin with Hsp27 and HSF-1 may explain some of the molecular pathways that influence tumor cell survival and the clinical significance in the prognosis of the breast cancer patients.

IMMUNOLOGICAL RESPONSES

The general "protective" functions of Hsp occur at single cell level, but these proteins are also involved in protective functions at the systemic level (e. g. during the development of an immune response against foreign agents such as bacteria, viruses, parasites, fungus or toxins) (Mizzen 1998). In addition, cancer cells may also elicit an immune response in the host due to the presence of tumor associated

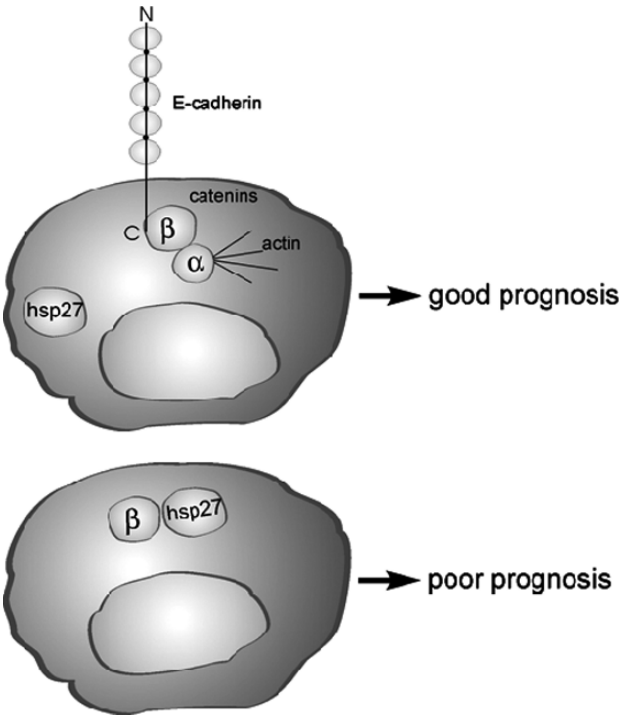

Figure 2. Schematic representation showing possible situations present in tumor cells when the Hsp27 interacts with β-catenin

antigens (TAAs) which are sensed as non-self. The mammalian immune response has two broad components: (i) a first line of protection against common molecular components of disease ("danger signals"). This is *innate immunity* and does not depend on previous contact with the antigen. At this stage, phagocytic processes play a crucial role; macrophages apprehend and degrade stranger molecules, infected cells or tumoral target cells. In addition, another cell type that actively takes part in innate immunity, is the natural killer (NK) cell. (ii) The second defensive line that vertebrates elicit against a persistent (sustained) attack is to turn on a specific system of recognition and of aggressor agents called specific, *adaptive* or *acquire immunity*. This type of immunity differs from the innate response by the ability of specific identification of the alien agent by receptors on the surface of T and B cells. Basically, this response is mediated by two kinds of cells: a) antigen presenting cells (APCs) such as macrophages, monocytes and dendritic cells (DCs) which participates in uptake, processing and presentation of antigens, and b) lymphocytes which are enabled to recognize specifically a naive antigen processed by the APCs. Several members Hsp play active and crucial roles in the development of both innate and adaptive immune responses (See: *Bonorino & Souza*, Chapter 10, *Gong et al*, Chapter 18, *Tamura et al*, Chapter 19).

The first evidence of the immunogenic capacity of Hsp came with the observation that certain factors purified from tumors can elicit specific anti-tumor immune responses against a second challenge with a same tumor (Srivastava et al 1986). The group of P. Srivastava, pioneered in this field, identified this factor as glycoprotein 96 kDa (gp96), a member of Hsp90 subfamily. Subsequently, other experiments have shown that the immunogenicity observed is due to tumor antigens chaperoned by the Hsp. Studies *in vitro* and in animal models have supported this affirmation (Udono et al 1993, Ciupitu et al 2002). The immunogenicity of the Hsp-peptide complex results from at least two major characteristics: (i) peptide-independent triggering of *innate immunity*, and (ii) a peptide-dependent capacity to elicit an adaptive response including cytotoxic T lymphocytes (CTLs) response. Gp96, Hsp70 and Hsp90, purified from solid tumors have immunogenic capacity and are strong inductors of an immune response elicited by $CD8^+$ T cells (Doody et al 2004, Janetzki et al 2000, Schild et al 1999, Udono et al 1993, Udono et al 1994). At present, it is well known that CTLs plays an important role like cellular effectors in the immune response against several pathogens including immunogenic tumors. During the cell-mediated immune response, APCs process proteins and present antigenic peptides fragments through the major histocompatibility complex (MHC) class II molecules to $CD4^+$ T helper (Th) lymphocytes. The activated $CD4^+$ T cells assist the effector cells through the generation of cytokines. Although tumor cells can directly present endogenously processed antigenic peptides in the surface of MHC class I molecules to $CD8^+$ T cell precursors, initiation of tumor-specific CTL response involves indirect presentation of tumor antigens by specialized APCs. Immature DCs residing in the periphery have a high capacity for endocytosis, facilitating antigen uptake. Hsp induce DC maturation as determined by their up-regulation of MHC class II and the co-stimulatory molecules CD83, CD86 (B7.2) and CD40. Mature DC lose their capacity for endocytosis and migrate into the draining lymph node, a process that can be mediated by Gp96, Hsp70, Hsp90 and Hsp110 (Singh-Jasuja et al 2000, Kruppner et al 2001). The APC then present endogenous or exogenous peptide antigens expressed on the cell surface with MHC class I or II antigen, respectively, to naive T cells, thereby initiating an antigen-specific immune response. On account of its capacity for acting as carriers of antigen peptides, Hsp are considered as biological and potent adyuvants in "cross-presentation" of antigenic peptides. This process consists in the transfer of exogenous peptides to MHC class I molecules via endosomal pathway in APCs and exposition to naive $CD8^+$ T cells. In order to enter in this molecular pathway, the antigenic HSP-chaperoned peptides must be recognised and internalised by adaptor molecules present in the surface of APCs (Singh-Jasuja et al 2000, Castellino et al 2000, Doody et al 2004). Recently, the mechanism of uptake has been elucidated and some receptors implicated in this process identified (Binder et al 2004). Moreover, it is well known that DCs internalise Hsp by receptor-mediated endocytosis that activates DC and enhances antigen presentation. Hsp also interact with some different classes of receptors including members of the Toll-like receptor (TLR) family (Sonderman et al 2000, Vabulas et al 2002). The TLR are

mammalian homologues of *Drosophila* Toll protein responsible for both embryonic development and the immune response of the fly against fungal infections (reviewed by Akira and Takeda 2004). TLRs are type I transmembrane proteins concerns to interleukin-1 (IL-1) superfamily receptor, with an ectodomain consisting of leucine rich repeats (LRRs), and one or two cysteine-rich regions. The intracellular domain contains a Toll/IL-1 receptor (TIR) domain, based on homology of the region with a similar intracellular domain of IL-1R. The TIR domain governs the hetero- and homo-dimerization between TLRs and the association between TLRs and TIR domain-containing adaptor molecules such as MyD88, IRAK, TRAF6. The intracellular interactions between these molecules activate the nuclear factor-κB (NF-κB) transcription factors, as well as the mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase. The final effect is the expression and release of pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1 beta (IL-1β). Innate immune recognition also induces secretion of effector cytokines, such as IL-12, which control $CD4^+$ T-cell differentiation, induction of co-stimulatory molecules on APCs that are necessary for T-cell activation, and signals that induce the activation of B-cell. In this manner, the innate immune response to pathogens assists the immune system to produce an adaptive response.

In higher vertebrates, TLRs play a crucial role in the innate immune response against microorganisms by recognition of specific motifs in determined microbial components called pathogen-associated molecular patterns (PAMPs). Hsp such as Hsp60, Hsp70 and gp96 from different sources behave in a similar way to PAMPS and induce pro-inflammatory cytokines TNF-α, IL-1, IL-6, and IL-12, release of nitric oxide (NO) and CC chemokines such as MCP-1, MIP-1 and RANTES by APCs (Asea et al 2000, Asea et al 2002, Wang et al 2002). They also induce maturation of DC by up-regulation of surface molecules as MHC class I and II, and co-stimulatory molecules such as CD40, CD80, CD83 and CD86 (Singh-Jasuja et al 2000, Kruppner et al 2001). Studies have shown that uptake and processing of Hsp60 and Hsp70 activates surface receptors such as LPS receptor CD14, and signalling receptors TLR-2 and TLR-4 leading to maturation of APC (Ohashi et al 2000, Asea et al 2000). In addition, Hsp70 from *Mycobacterium tuberculosis* activates a calcium-dependent pathway cellular signalling and the production of pro-inflammatory cytokines, CC chemokynes and NO (Panjwani et al 2002, Wang et al 2002). Hsp70 were also shown to interact with the key receptor CD40 (Becker et al 2002). Another family of receptors involved in recognition and processing of Hsp-chaperoned peptides are scavenger receptors (reviewed by Facciponte et al 2005): CD91 (Basu et al 2001), LOX-1 (Delneste et al 2002), SR-A (Berwin et al 2003), SREC-1 (Berwin et al 2004) and CD36. Scavenger receptors are known to bind to modified lipoproteins and bacterial products. It is possible that TLR and SR are co-receptors and participate in intracellular crosstalk.

In addition to acting as ligands to receptors on APC, Hsp also are ligands for NK cells (reviewed by Multhoff 2006). NK cells are important components of

the innate immune system and with macrophages constitute the front line against bacteria, viruses, parasites and also cancer cells (Kelly et al 2002). The NK cells do not require primary stimulation, but their cytolytic activity can be increased by cytokines including IL-2, IL-15, or alarm signals including LPS and Hsp. Although tumor cells with altered or missing MHC expression pattern are ideal targets for NK cells, activatory ligands are also indispensable in their activation. Distinct MHC class I allele groups, including HLA C, were identified as negative regulatory ligands for NK cells. Until recently CD16 and CD56 have been recognized as unique surface markers specific for NK cells and stimulatory/inhibitory receptors were identified. These receptors belongs to different groups: killer cell immunoglobulin-like receptor (KIR), the immunoglobulin-like transcript (ILT), C-type lectin receptor, and natural cytotoxic receptor (NCR) and interact with MHC class I molecules on target cells. HLA-E molecules are a non-classical HLA class I molecule that interacts with inhibitory receptor complex CD94/NKG2A. Immediately after a stress, an up-regulated surface expression of HLA E-Hsp60 complexes was not recognized by the inhibitory CD94/NKG2A receptor leading to a diminished capacity to inhibit a principal NK cell population. These data indicate that external stress can modulate the immune response by NK cells (Michaelsson et al 2002). Complexes gp96-peptides induce the production of perforin, a protein responsible of apoptotic membrane breaking by NK cells in innate and adaptive immune response. (Strbo et al 2003).

Surface expression of Hsp70 on membranes of tumor cells stimulates the cytolytic and migratory ability of NK cells and induces death (Chen et al 2002, Gastpar et al 2004). NK cells are necessary for Hsp70-mediated induction of CTL immune response against lung metastases in animals (Massa et al 2005). Gross et al. (2003) have identified Hsp70 as an activatory ligand for CD94-expressing NK cells. Multhoff et al. (2000) have showed that the injection of Hsp70-secreting tumors genetically engineered to mice elicited an increasing number of DCs mediating a potent CD8+ T cell response and elevated tumor recognition by NK cells. Gross et al. also have demonstrated that the interaction between Hsp70 and NK cells is saturable and dose-dependent such as APCs because it is possible to speculate the existence of specific receptor for Hsp70.

Hsp appear to be immunogenic *per se* independently if they are bearing peptides. Hsp70 and Hsp90 interact with receptors on APC, including TLR2, TLR4 and CD14. Thus Hsp at the systemic level is acting as "signals of alarm" in the presence of dangerous agents (pathogens and tumors) (Todryk et al 2000). In the last case it is known that during transformation from normal to malignant cells, Hsp expression progressively increases. Several studies report the over-expression of Hsp in a wide variety of human solid tumors. They may thus become targets for the immune system (Bonorino & Souza, Chapter 10). Some tumors can inhibit the immune response through: (i) Tumor-derived suppression through release of tumor-derived soluble factors (TDSF) including IL-10, transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF). TDSFs induce immature myeloid cells and regulatory T cells (Treg) which would favor tumor progression through

inhibition of DC maturation and T cell activation (Zou et al 2005, Yang et al 2004). (ii) Absence of danger signals. If the tumor does not emit alarm signals, APCs may not express sufficient co-stimulatory molecules to activate T cells. These T cells will be tolerant and fail to kill the tumor cells. (iii) Inhibition by NKT cells. If NKT cells are activated through their TCR with CD1d, they produce several cytokines. Many of these are anti-inflammatory and can inhibit the action of $CD4^+$ and $CD8^+$ T cells and may impair the capacity of APCs to address the adaptive immune responses. (iv) Inhibition by $CD25^+CD4^+$ T cells (Treg). Antigen-specific $CD25^+CD4^+$ T cells can blockade tumor-specific immune responses by interfering with the ability of APCs to activate $CD4^+$ and $CD8^+$ T cells. In a recent work, researchers have shown that Hsp60 can down-regulate an adaptive immune response by up-regulating Tregs through TLR2 pathway. Interestingly, no APCs were necessary to this hsp60 coactivation (Zanin-Zhorov et al 2006).

On the other hand, Hsp could be not detected by the immune system. Classically, Hsp are cytosolic proteins carrying out tasks in the inner of the cell, but there is abundant evidence of the presence of soluble forms of Hsp in the extracellular milieu. Cytosolic Hsp do not posses leader peptides to obtain membrane localization, however these Hsp participate in the transport across membranes of another proteins. Also has been reported Hsp in the serum of healthy individuals after physical exercise (Walsh et al 2001), in cancer patients, during infections or autoimmune diseases, and in the medium of tissue cultures. However, as yet unclear the mechanism by which the cytosolic Hsp reach the intercellular space. Is known that Hsp are released by necrotic but not by apoptotic cells (Basu et al 2000). Necrosis is a type of cell death very frequent in solid tumors. However, Barreto et al. (2003) have demonstrated that IFN-γ induces an active release of Hsc70 in tumor cells without suffering apoptotic or necrotic cell death. Neither is clear if Hsp are released as free soluble form or exported in detergent-soluble membrane vesicles named exosomes.

Many human solid tumors and bone marrow samples from chronic myeloid leukemia patients have shown elevated Hsp expression on the plasma membrane of the tumor cells. In general, normal tissues are Hsp70 membrane-negative. This over-expression of Hsp70-membrane bounded correlates positively with an increase sensitivity of these tumors to cytolytic NK-activity. This observation also has been looked on the plasma membrane of tumor cell lines (Shin et al 2003). Arispe et al (2004) have demonstrated a direct interaction of Hsp70 with phosphatidylserine, a lipid of plasmatic membrane, in PC12 tumor cells. Triantofilou & Triantofilou (2004) have demonstrated that Hsp70 associated with TLR-4 clusters in lipid rafts after addition of bacterial LPS. The presence of Hsp70 bounded to plasma membrane was found to be dependent of lipids rafts. Other researchers found the presence of Hsp70 in detergent-soluble microdomains, which are enriched with sphingolipids. (Broquet et al 2003).

All of these findings over the interplay between Hsps and the components of the immune system are opening the doors for pre-clinical and clinical use of Hsp in cancer immunity.

CONCLUSIONS

The Hsp response participates in tumorigenesis through specific changes in Hsp expression levels, involving in most instances up-regulation which makes the cancer cells more resistant to adverse growing conditions, including to anticancer therapies. There is also evidence that certain Hsps participate in DNA repair. Moreover, the molecular interactions of the Hsp with oncogenic and anti-oncogenic proteins as well as with proteins involved in cell signalling, in cell adhesion and cytoskeleton help to understand the participation of the Hsp in the metastatic process and in the immunological response. These interactions are providing clues to attack the cancer cells by disruption of the Hsp-peptide associations (e.g., with 17-AAG), or by favouring or manipulating the Hsp-tumor peptide associations to mount an immunological response. Therefore, the Hsp are in an active stage of translational research to improve the diagnosis and treatment of cancer patients.

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CHAPTER 3

HSF1: AN EMERGING FACTOR IN CANCER

STUART K. CALDERWOOD[1,2,*], Md ABDUL KHALEQUE[1], AJIT BHARTI[2] AND DANIEL R. CIOCCA[3]

[1]*Division of Molecular and Cellular Radiation Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215*

[2]*Department of Medicine, Boston University School of Medicine, MA 02118, USA, e-mail: scalderw@bidmc.harvard.edu*

[3]*Oncology Laboratory, Institute of Experimental Medicine and Biology of Cuyo (CRICYT-CONICET), and Argentina Foundation for Cancer Research (FAIC), Mendoza, C. C. 855, 5500 Mendoza, Argentina, e-mail: dciocca@lab.cricyt.edu.ar*

Abstract: Heat shock factor1, the transcriptional activator of the heat shock protein (*hsp*) genes is expressed to high level in a number of types of malignancy. HSF1 elevation is coupled to the activation of the c-*erb-B2* pathway, a common change associated with breast cancer. HSF1 may exert pro-malignant effects through the activation of *hsp* gene transcription. The resulting elevated levels of Hsp promote cancer growth through inhibition of apoptosis and promotion of autonomous growth. In addition, HSF1 possesses properties other than those of a transcriptional activator. HSF1 is a gene repressor and binds to the gene co-repressor MTA1 (metastasis associated protein 1). HSF1 may thus function in cancer due to repression of genes that deter malignancy. HSF1 activation may also occur secondarily to treatment with current agents used in chemotherapy such as Hsp90 inhibitors and proteasome inhibitors. Such HSF1 activation may be a confounding effect in chemotherapy. HSF1 thus plays a significant role in tumor growth and response to therapy

Keywords: Heat shock transcription factor, protein, apoptosis, autonomous growth, glycogen synthase kinase 3, metastasis associated protein one, gene co-repressor, estrogen, receptor, chromatin, heregulin, c-*erb-B2*, hsp 90 inhibitor, chemotherapy, proteasome, mammary carcinoma

*Corresponding author: Beth Israel Deaconess Medical Center, Avenue, 21–27 Burlington Room 553B, MA 02215, Tel: 617-632-0628, Fax: 617-632-063, e-mail: scalderw@bidmc.harvard.edu

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INTRODUCTION

Heat shock transcription factor 1 (HSF1) and the heat shock protein (*hsp*) genes which it regulates play pivotal roles in the stress response in ensuring the quality of intracellular proteins in eukaryotic organisms and protecting cells from stress-induced death (Bukau et al., 2006; Lindquist and Craig, 1988) (*Brunet et al*, Chapter 11). HSF1 and the Hsp are essential for cell survival through the dual mechanisms of (1) repair of protein damage and (2) inhibition of the pathways leading to programmed cell death (PCD). HSF1 was recently shown to play a significant role in malignant transformation and the factor is activated and overexpressed in a number of types of cancer. Elevated Hsp levels are essential for the growth of many tumor types and inhibition of hsp90 or hsp70 function leads to the induction of the death program and tumor regression (Calderwood et al., 2006; Whitesell and Lindquist, 2005) (*Neckers*, chapter 12, *Whitesell*, Chapter 13, *Kamal* et al, Chapter 14, *Workman*, Chapter15, *Calderwood* et al, Chapter17). Elevated Hsp levels signal a poor prognosis in many cancers and are associated with resistance to surgery and chemotherapy (Ciocca and Calderwood, 2005) (*Ciocca* et al, Chapter 2). Current investigations suggest a role for HSF1 in malignant transformation involving its ability to mediate Hsp expression as well as HSF1 properties in cell cycle regulation and metastasis that are independent of Hsp transcription. In this chapter, we will examine the regulation and properties of HSF1 and explore mechanisms that explain how these properties are subverted in cancer.

PROPERTIES AND REGULATION OF HSF1 AS A TRANS ACTIVATOR

HSF1 is the transcriptional activator of the *Hsp27*, *49*, *60*, *70*, *90*, *and 110* genes in response to protein stresses (including heat, oxidative, ischemic stress) and its full activation leads to the elevation of Hsp to levels that dominate cellular gene expression (Voellmy, 1994; Wu, 1995). Aggregated, denatured and damaged proteins are the common proximal inducers of HSF1 activity during stress (Ananthan et al., 1986; Kampinga, 1995; Kampinga et al., 1995). Disruption of the *hsf1* gene leads to a profound loss of thermotolerance and markedly increased susceptibility to heat-induced apoptosis (McMillan et al., 1998). However, *hsf1* also mediates additional functions in addition to cell survival, and its inactivation leads to lethality at the embryonic stage and growth retardation associated with disruption of developmental programs. Two additional members of the mammalian *hsf* family *hsf2* and *hsf4* have also been isolated, although their role in cancer is not entirely clear. One isoform of HSF2 (HSF2A) cooperates with HSF1 in *HSP* gene transcription (He et al., 2003) while HSF4 functions as an HSF1 repressor (Nakai et al., 1997).

HSF1 Regulation

In normal cells under non-stress conditions, HSF1 is inactive and does not participate in basal *hsp* transcription to any appreciable degree (Hensold et al., 1990; Price and

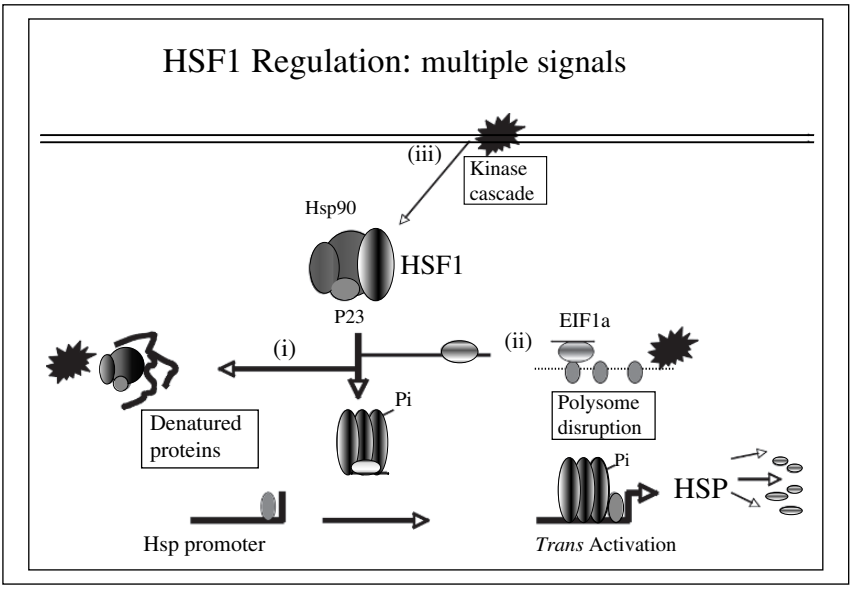

Figure 1. Regulation of HSF1 in the stress response

Calderwood, 1991). Such inactive HSF1 is found in a large complex containing Hsp90 and a group of accessory co-chaperones (Figure 1). Within this complex, HSF1 is in a highly coiled monomeric state and lacks the ability to bind the *cis*-acting heat shock elements (HSE) in the proximal promoters of *hsp* genes (Figure 1) (Baler et al., 1993; Rabindran et al., 1993; Wu, 1995). Cell stress results in breakdown of the inhibitory complex containing the inactive monomer and formation of a DNA-binding trimeric form of HSF1 that can bind HSE in the promoters of *hsp* genes (Baler et al., 1993; Rabindran et al., 1993; Sarge et al., 1993; Westwood and Wu, 1993) (Figure 1).

In non-stress conditions, HSF1 is maintained in an inactive complex containing Hsp90, immunophilins such as Cyp40, FKBP1, FKBP2 and P23. Heat shock (i) leads to activation, which involves the escape from the Hsp90 complex, sequestration Hsp90 by denatured proteins, and HSF1 trimerization. HSF1 trimers then bind HSE in Hsp genes. Additional signals also lead to activation. These include: (ii) the binding To HSF1 trimers of the EIF1a/HR1 complex released from heat damaged polysomes and (iii) the induction of a kinase cascade that involves upstream tyrosine kinases and downstream HSF1 phosphorylation on serine residues. Active trimers bind to *hsp loci* that are already in an open conformation with bound RNA polymerase II. Additional on chromatin events involving ATP dependent remodeling proteins and histone acetylation are also involved.

HSF1 activation of transcription is a multi-step process, involving trimerization, acquisition of HSE-binding activity and inducible phosphorylation (Hensold et al., 1990; Price and Calderwood, 1991; Voellmy, 1994; Wu, 1995). Hsp90 is a major regulator of HSF1 in unstressed cells and mediates constitutive repression. Indeed

the majority of such HSF1 is bound in a complex with hsp90 and the co-chaperones FKBP52 and p23, a complex is reminiscent of those in which steroid hormones are found (Figure 1). HSF1 trimerization during stress is accompanied by the remodeling of HSF1-Hsp90 complexes an due to sequestration of HSP90 within protein aggregates (Figure 1) (Zou et al., 1998) (Guo et al., 2001). In addition, the regulatory protein 14-3-3e binds to HSF1 and mediates its nuclear exclusion and repression when growth stimuli induce HSF1 phosphorylation by the protein kinases ERK1 and GSK3 (He et al., 1998; Wang et al., 2003; Wang et al., 2004b). HSF1 thus resembles a number of factors in being subject to regulation by phosphorylation and 14-3-3 dependent alterations in nucleocytoplasmic shuttling (Brunet et al., 2002). A further mechanism in HSF1 regulation involves the translational elongation factor eIF1a and a non-coding RNA sequence known as HR1, although the exact details of this type of regulation are unknown as yet. It is proposed that eIF1a is release from polysomes disrupted by heat shock and that its binding in association with HR1 positively activates HSF1 activation (Figure 1).

Role of Phosphorylation in HSF1 Regulation

Early studies suggested that HSF1 activation involves a profound retardation in electrophoretic mobility caused by phosphorylation (Hensold et al., 1990; Sorger and Nelson, 1989). HSF1 hyperphosphorylation in mammalian cells is correlated with a second step in HSF1 activation in addition to the DNA binding step and may couple promoter occupation to *trans* activation (Price and Calderwood, 1991; Voellmy, 1994). Evidence for this hypothesis is that exposure of cells to compounds that cause HSF1 to bind DNA without inducing transactivation (sodium salicylate and menadione) along with agents that induce hyperphosphorylation without inducing DNA binding (phorbol esters and phosphatase inhibitor calcyulin), fully active, hyperphosphorylated HSF1 is generated (Xia and Voellmy, 1997). However, HSF1 activation and Hsp synthesis can occur in the absence of hyper-phosphorylation and the exact mechanisms that couple stress signaling signaling and phosphorylation to *trans* activation are not clear. In addition, the nature of the protein kinases involved in HSF1 activation and the sites on HSF1 impacted by such kinases are still not fully characterized.

Phosphorylation Sites within HSF1

Analysis of HSF1 by phosphoaminoacid analysis and 2-dimensional phosphopeptide mapping indicated multiple sites of phosphorylation largely on serine, perhaps to be expected in a protein containing 20–25% serine and threonine residues (Chu et al., 1996). Characterization of these sites by analytical chemistry approaches and their role in HSF1 regulation has begun (Guettouche et al., 2005). The first such sites to be characterized were in proline-rich regions of the protein; it was shown that HSF1 contains inhibitory phosphorylation sites at serines 303, 307 and 363

that are targeted by, respectively, the protein kinases GSK3, ERK1 and PKC and these studies have been confirmed in part by others (Chu et al., 1996; Chu et al., 1998; Kline and Morimoto, 1997; Knauf et al., 1996) (Figure 2). In the case of phosphoserines 303 and 307, at least a portion of these inhibitory effect is due to recruitment of the phosphoserine binding protein 14-3-3ε and nuclear export of HSF1 (Wang et al., 2003; Wang et al., 2004b) (Figure 2). Interestingly, heat shock causes ERK1 to bind stably to HSF1 and phosphorylate serine 307, and thus mediates 14-3-3ε association (Wang et al., 2003; Wang et al., 2004b).

HSF1 contains multiple phosphorylation sites that exert both positive (T142, S195, S230, S326) and negative (Ser121, Ser303, Ser307, Ser363) effects on transcription. This permits HSF1 to respond both to stress and to more physiological inputs in order to adjust to the metabolic state of the cell.

We have recently discovered a novel inhibitory phosphorylation site, serine 121; Ser 121 phosphorylation promotes the binding of Hsp90 and leads to inhibition of HSE binding by HSF1 (Wang et al., 2006). Positively acting phosphorylation sites in HSF1 that stimulate *HSP* gene transcription, are now beginning to be analyzed and threonine 146 in the leucine zipper domain and serine 230 in the regulatory domain are required for *trans* activation of HSP genes (Soncin et al., 2003) (Holmberg et al., 2001). Threonine 146 is a target for casein kinase 2 and though essential for full HSF1 activity its exact role in unclear (Soncin et al., 2003). HSF1 is also phosphorylated by Polo-like kinase 1 a modification that enhances its nuclear localization (Kim et al., 2005). The understanding of how HSF1 phosphorylation is coupled to *trans* activation of HSP promoters is currently incomplete and much still remains to be learned regarding this. However, a recent

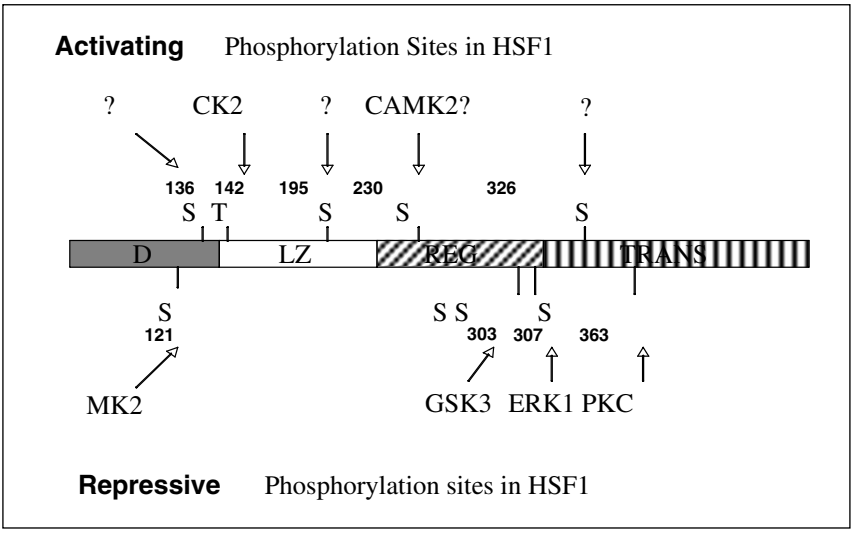

Figure 2. Regulatory phosphorylation sites in HSF1

study points to another phosphorylation site at Ser 326 as important in stress-induced transactivation (Guettouche et al., 2005). In terms of upstream signaling, one thing that is clear is that HSF1 mediated Hsp gene transcription mediated by stress requires the activation of a tyrosine kinases that act upstream of HSF1 (Price and Calderwood, 1991) (Figure 1). As HSF1 is not phosphorylated on tyrosine before or after heat shock, direct effects of the tyrosine kinase on HSF1 are unlikely (Chu et al., 1996). Emerging studies now indicate that changes in malignant cells can subvert some of these signaling pathways and lead to HSF1 activation and elevated Hsp expression under non-stress conditions (Ciocca et al., 2006; Khaleque et al., 2005).

Hsp Gene Promoters- Events in the Nucleus and the Influence of Chromatin

After HSF1 has been activated, it migrates to the nucleus and encounters the promoters of heat shock gene. *Hsp* promoters all possess one or more arrays of HSE, with each array containing three repeats of the sequence nGAAn – reviewed by (Wu, 1995). Arrays of HSE are the cognate binding sites for HSF trimers and confer stress/HSF inducibility on the promoter (Wu, 1995). In uninduced cells, *hsp* promoters are poised for a rapid activation of transcription by assembly in an accessible chromatin structure bound to RNA polymerase II which initiates a short transcript from the *hsp70* gene (Lis and Wu, 1993) prior to pausing. Following stress, HSF trimers are rapidly targeted to the the promoter, relieve the pause in transcription polymerase II and initiate multiple rounds of HSP gene transcription. Promoter proximal pausing also occurs in the human *Hsp70* gene as well as other rapidly induced genes (c-*fos*, c-*myc* and igk) (Brown and Kingston, 1997; Fivaz et al., 2000; Schneider et al., 1999). Transcription from *hsp* promoters also require the resolution of chromatin structure in the promoter regions and in the structural gene. Covalent modification of histones is involved in *Hsp70* activation and histone phosphorylation and acetylation increases globally in heat shock genes on activation by heat (Nowak and Corces, 2000; Smith et al., 2004). The activation domains of human HSF1 also recruit human BRG1, the ATPase domain of SWI/SNF complexes that mediate ATP dependent remodeling of chromatin (Corey et al., 2003; Sullivan et al., 2001). The SWI/SNF complex resolves the nucleosome structures in front of the elongating Pol II on the *HSP70* promoter, and may aid in overcoming the transcriptional pause and stimulation of *hsp*70 RNA synthesis (Corey et al., 2003). Recent studies have shown HSF1-dependent alterations in histone acetylation in the mouse *hsp70* gene (Thomson et al., 2004). As we discuss later, interaction of HSF1 with chromatin remodeling proteins and modulators of histone acetylation may play a key role in cancer. Thus, both ATP-dependent remodeling and histone modification are required for efficient Hsp transcription. The hair-trigger nature of HSF1 mediated transcription might make it particularly susceptible to the loss of regulation that occurs in cancer and may mediate some of the changes seen in *hsp* gene transcription.

HSF1 IS A *TRANS* REPRESSOR OF NON-HSP GENES

In addition to its role as a powerful transcriptional activator, HSF1 acts as an inhibitor of transcription of non-*hsp* genes. We have shown that HSF1 inhibits transcription of many inducible genes, including interleukin 1 beta (*IL-1β*), tumor necrosis factor alpha (*TNFα*), c-*fms* through the mediation of HSF1 (Cahill et al., 1997; Cahill et al., 1996; Singh et al., 2000; Singh et al., 2002; Xie et al., 2002a; Xie et al., 2002b) c-*fos* and urokinase plasminogen activator (*uPA*) (Chen et al., 1997). Indeed repression by HSF1 is a conserved property in eukaryotes as evidenced by *Drosophila* studies(Westwood et al., 1991). This capacity for gene repression is specific for HSF1 within the *HSF* family in mammalian cells and is not a property of HSF2 (Chen et al., 1997; Xie et al., 2002a).

Some clues as to the factors involved in repression are afforded by a study showing that HSF1 repression of *IL1B* is inhibited by the histone deacetylase inhibitor trichostatin A, suggesting a role for histone deacetylases (HDACs) (Xie et al., 2003). Indeed, most current hypotheses for short-term gene repression stress the role of modification of histones and nucleosome structure on target promoters (Davis and Brackmann, 2003). The modification of residues in the tails of histones H3 and H4 by acetylation, methylation and phosphorylation influences transcription by two main mechanisms: (1) altering histone association with DNA (acetylation being associated with a relaxation in DNA binding, open chromatin conformation and active transcription) and (2) the pattern of histone modification gives rise to protein binding sites and is known as the histone code (Davis and Brackmann, 2003; Marmorstein, 2001). In addition, short range changes in nucleosome structure that affect transcription are carried out by ATP-dependent chromatin remodeling proteins (often associated with transcription factors) that permit RNA polymerase complexes to move processively along nucleosome containing DNA (Vignali et al., 2000). We searched for potential co-repressor molecules involved in HSF1 dependent repression and in a proteomic screen were able to find tight association with the gene co-repressor MTA1 (Khaleque et al, in press). MTA1 belongs to an unique co-repressor complex that contains both the ATP-dependent chromatin remodeling protein Mi2 and histone deacetylases 1 and 2 (Bowen et al., 2004; Mishra et al., 2003; Xue et al., 1998). We have shown that HSF1-MTA1 complexes bind to the promoters of target genes and repress them in a manner that depends on the HDAC activity of the co-repressor complex (Khaleque et al, in press; Figure 3).

In the ACTIVE state, DNA is in an open conformation due to recruitment of histone acetylases and ATP dependent remodeling complexes (SWI/SNF), and RNA polymerase II is able to transcribe the sequence of the structural gene. HSF1 recruitment by, for instance exposure to heregulin brings associated MTA1 and histone deacetylases to the promoter, leading to histone deacetylation, nucleosome recruitment and the formation of a compacted chromatin conformation that contributes to gene repression. We show HSF1/NuRD complexes binding the activating protein still associated with DNA This is in line with our experiments that indicate that repression involves HSF1 association with other transcriptional

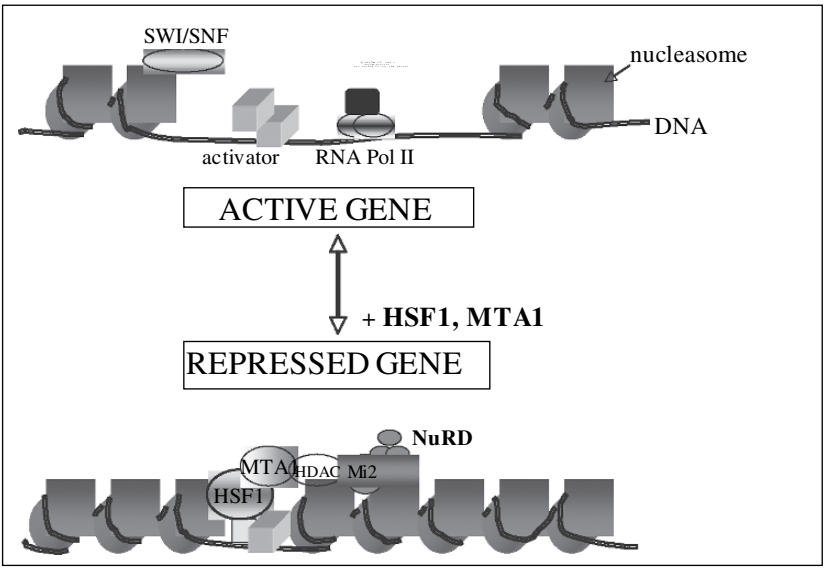

Figure 3. Model for gene repression by the HSF1/MTA1/NuRD complex

activators such as NF-IL6 on target promoter. In addition however, MTA1 can itself bind directly to ER and recruitment to promoters may involve this interaction.

NON-TRANSCRIPTIONAL ROLES FOR HSF1 IN THE CELL CYCLE AND CANCER

HSF1 also plays some cellular roles unrelated to transcription that may also be involved in its pro-malignant influence. HSF1 is activated to a trimeric form during mitosis (Bruce et al., 1999). Interestingly although trimer formation is at least as great in magnitude as during heat shock, does not lead to *hsp* transcription or the increased expression of Hsp (Bruce et al., 1999). Some clue to the meaning of these findings may be gleaned from recent studies showing that in PC-3 prostate carcinoma cells in which HSF1 is overexpressed, polyploidy is observed (Wang et al., 2004d). In addition, overexpression of HSF1 increases polyploidy, while an HSF1 dominant negative protein reverts the cells to normal diploidy (Wang et al., 2004d). In this context, HSF1 appears to play a role at the mitotic spindle checkpoint. This checkpoint couples DNA replication to the successful completion of anaphase through regulation of the destruction of cell cycle genes such as securin and Cyclin B1. Cyclin B1 destruction is essential for exit from mitosis and is inhibited in cells containing high levels of HSF1 (Wang et al., 2004d). Dominant negative HSF1 increases cyclin B1 destruction suggesting a key role for HSF1 at the mitotic spindle checkpoint (Wang et al., 2004d). Cyclin B1 levels are regulated

by the ubiquitin E3 ligase APC/C which directly ubiquitinates Cyclin B1 and other substrates and fosters their destruction (Peters, 2006). Polyploidy is known to be related to genomic instability and thus elevated HSF1 concentrations may lead to polyploidy and genomic instability through their effects on the mitotic spindle checkpoint and may play a permissive role in malignant progression (Agarraberes et al., 1997; Carroll et al., 1999; Matzke et al., 1999; Rasnick and Duesberg, 1999). HSF-1 may thus participate in the malignant phenotype through its permissive role in the dysregulation of ploidy in p53 null cells.

Non-transcriptional roles for HSF1 may also mediate resistance to apoptosis. HSF1 has been found to bind tightly to the DNA damage response kinase DNA dependent protein kinase (DNA-PK) and stimulate its kinase activity (Peterson et al., 1995). This scaffold function of HSF1 which results in binding and stimulating DNA-PK activity leads to apoptosis resistance (Nueda et al., 1999). The role of these interactions in cancer has not yet been explored.

In addition, HSF1 is implicated in the *multiple drug resistance* phenotype and resistance to doxorubicin though its role in activating the multidrug resistance gene 1 (MDR-1). This also appears to be an effect independent of its normal transcriptional functions and occurs in the absence of Hsp expression (Tchenio et al., 2006). Targeting HSF1 may therefore take on greater urgency.

ACTIVATION OF HSF1 DURING MALIGNANT TRANSFORMATION

HSF1 levels become elevated in a number of cancers, and activation in this way may influence malignant behavior through Hsp transcription, gene repression or some of the non-transcriptional functions listed above(Hoang et al., 2000) (Ciocca, D.R. & Calderwood, S.K., in preparation). One mechanism for HSF1 activation could involve the deterioration of the milieu of the tumor leading to stresses such as hypoxia that activate the heat shock response as in Figure 1. However, little evidence exists to support such a notion and many tumor cell lines grown in the idealized conditions of tissue culture continue to express high levels of HSF1 (Calderwood et al., 2006). Indeed, recent studies indicate that HSF1 is activated independently of stress by pro-malignant stimuli. We have shown that HSF1 is induced by heregulin β1 (HRGβ1), a protein released into the tumor milieu that, on binding to receptors on adjacent cells, induces transmembrane signals that mediate the complete malignant program in mammary tissues (Khaleque et al., 2005). Our experiments show a novel *hsf1* dependent pathway for HRGβ1 induction of anchorage independent growth and resistance to apoptosis as well as a potential mechanism for the elevation of Hsp in cancer (Figure 4). HRGβ1 increases Hsp 60, 70 and 90 expression in cancer cells through the activation of HSF1.HSF1 activation by HRGβ1 involves some of the phosphorylation sites shown in Figure 2: inhibition of the constitutive kinase GSK3 through a pathway that includes the sequential activation of the protein kinases c-*erb*B-2, PI-3-K and Akt by heregulin binding leads to HSF1 activation (Figure 4). Previous studies showed that GSK3 is a potent repressor of HSF1 through phosphorylation within a regulatory domain that constitutively represses the

trans-activation domains while strongly activating the same domains during stress Figure 2; (Chu *et al*., 1996; Green *et al*., 1995; Newton *et al*., 1996). HRGβ1 also activates the ERK cascade shown previously to phosphorylate HSF1 on Ser307 and lead to its repression (Chu *et al*., 1996; Wang *et al*., 2003). Recent studies indicate a mechanism through which HRGβ1 bypasses the inhibitory effects of ERK. ERK1 has been shown to bind HSF1 and repress through phosphorylation on serine 307, secondary phosphorylation on serine 303 by GSK3, recruitment of 14-3-3ε and cytoplasmic sequestration (Chu *et al*., 1996; Chu *et al*., 1998; Wang *et al*., 2004b). High affinity binding to 14-3-3ε requires the dual phosphorylation of HSF1 at serines 303 and 307 (Wang *et al*., 2003). Activation of the PI-3K/Akt pathway by HRGb1 appears to override the repressive effects of the ERK cascade through its ability to inhibit GSK3 and presumably to cause HSF1 dephosphorylation at serine 303 (Figure 4). Similar events result from heat shock in which simultaneous activation of ERK and PI-3K may permit transcription of HSP genes through GSK3 inhibition (Wang *et al*., 2004b). Despite these similarities, however, the mechanisms involved in pro-malignant signaling through HRGβ1 and stress-induced HSP expression diverge, as HRGβ1 leads to increased intracellular HSF1 levels while stress activation occurs by a strictly posttranslational pathway (Sarge *et al*., 1993; Wu, 1995). In addition, activation by HRGβ1 does not lead to a major shift in the electrophoretic mobility of HSF1, contrasting with heat shock which causes a quantitative shift in HSF1 mobility associated with hyperphosphorylation (Chu *et al*., 1996; Sarge *et al*., 1993). The functional role of hyperphosphorylation in HSF1 activation is however not clear (Sarge *et al*., 1993). GSK3 plays the role of constitutive repressor of many pro-malignant pathways including β-catenin, BCl-3 and androgen receptor signaling and targets its substrates towards proteosomal degradation via the binding of E3 ubiquitin ligases (Cardozo and Pagano, 2004; Ha et al., 2004; Karim et al., 2004; Viatour et al., 2004; Wang et al., 2004a). Activation of pro-malignant signaling through heregulin or Wnt relieves such repression through Akt phosphorylation of GSK3 on inhibitory domains, thus permitting target proteins to escape degradation and to accumulate (Karim *et al*., 2004; Viatour *et al*., 2004; Wang *et al*., 2004a). HSF1 may be regulated in a similar way, as both HRGβ1 and LiCl inhibit GSK3 and lead to enhanced HSF1 accumulation However, HSF1 turns over slowly with or without heregulin and major involvement of a pathway involving stabilization is not clearly predicted (M.A. Khalique & S. K. Calderwood, in preparation). This was the first demonstration of a direct pathway leading to HSF1 activation in malignant cells and clearly much is yet to be learned regarding this mechanism (Figure 4). Other factors that lead to GSK3 activation including the RET receptor also cause Hsp accumulation in cells (Myers and Mulligan, 2004). Tyrosine receptor kinases such as *c-erb-B2* or *c-Erb-b3* are thus good candidates for mediating the tyrosine kinase step long thought to be at the head of the cascade that leads to stress-induced HSF1 activation (Figure 1). In addition alternative pathways for Hsp induction in cancer also exist. Hsp genes are subject to repression by members of the p53 pathway, and loss of p53 or p63 function in cancer also leads to Hsp accumulation (Agoff et al., 1993; Jung et al., 2001; Madden et al., 1997).

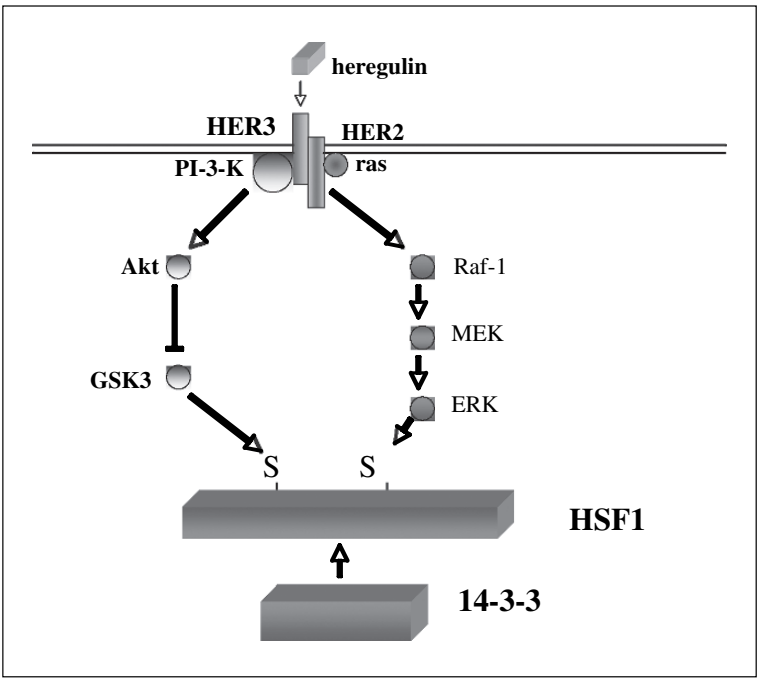

Figure 4. Activation of HSF1 by heregulin

Heregulin binds to HER3/HER2 dimers on the cell surface and activates transcription. Receptor activation leads to bivalent signaling. HSF1 is repressed by dual phosphorylation on Ser307 (by ERK1) and Ser303 (by GSK3). Heregulin leads to activation of Akt, an inhibitor of GSK3 and thus to dephosphorylation and de-repression of HSF1.

HSF1 AND ELEVATED HSP CONCENTRATIONS OPPOSE PROGRAMMED CELL DEATH PATHWAYS

HSF1 is an essential inhibitor of cell death after stress (McMillan et al., 1998). It has been further shown that HSF1 mediates cytoprotection downstream of HRGβ1 (Khaleque et al., 2005). HRGβ1 is a potent inducer of tumorigenesis and metastasis and this effect is correlated with ability to support anchorage independent growth *in vitro* (Aguilar *et al*., 1999; Tsai *et al*., 2003). *hsf1* plays a significant role in HRGβ1-induction of anchorage independent growth (Khaleque et al., 2005). Other studies show that Hsp70 overexpression in fibroblasts leads to anchorage independent growth, suggesting a role for Hsp70 in these effects of HSF1 (Volloch and Sherman, 1999). Indeed, *hsf1*-/- cells show no evidence of anchorage independent growth without or with HRGβ1 (Khaleque et al., 2005). *hsf1* could therefore permit anchorage independent growth in HRGβ1treated cells through increased cell

survival. The triggering of anti-apoptotic pathways is a common by-product of tumor progression pathways (Hanahan and Weinberg, 2000; Schulze-Bergkamen and Krammer, 2004) and HRGβ1 treatment protects cells from pro-apoptotic signals and requires *hsf1* for full protection (Khaleque et al., 2005). Previous studies have outlined a network of additional pro-apoptotic pathways downstream of heregulin, commonly involving the activation of PI-3 kinase and Akt (Bhat-Nakshatri et al., 2002; Downward, 2004; Gottlieb et al., 2002; Venkateswarlu et al., 2002; Yarden and Sliwkowski, 2001). These include the induction of the NFkB and MDM2 pathways, and repression of FOXO3a and the pro-apoptotic BCl2 homologues BAX and BAD (Bhat-Nakshatri et al., 2002; Downward, 2004; Gottlieb et al., 2002; Li et al., 2001; Venkateswarlu et al., 2002). The HSF1 pathway constitutes another anti-apoptotic mechanism emanating from HRGβ1 – receptor binding (Khaleque et al., 2005). Hsp 27, Hsp70 and Hsp90 which suppress both intrinsic and extrinsic pathways of apoptosis at a number of steps may therefore number among the downstream effectors of HRGβ1 cytoprotection (Beere, 2004). However, HSF1 possesses properties over and above those of an activator of transcription and these two may also be important in its pro-malignant influences.

HSF1, GENE REPRESSION AND TUMOR DEVELOPMENT

The HSF1 binding protein and gene co-repressor MTA1 has the added significance of being closely associated with breast cancer progression and metastasis (Kumar et al., 2003; Mazumdar et al., 2001; Nicolson and Moustafa, 1998; Toh et al., 1994). Gene repression by histone deacetylation plays an important role in the development of many types of cancer and histone acetylases function as tumor suppressors (Davis and Brackmann, 2003). Indeed Kumar and co-workers have shown that MTA1 is induced by heregulin and that MTA1 induced in this manner can repress estrogen receptor (ER) regulated genes and thus increase metastasis (Kumar et al., 2003; Mazumdar et al., 2001; Tsai et al., 2003). We have found that elevated levels of HSF1 repress estrogen receptor (ER) dependent transcription and become associated with MTA1 at ER binding *loci* on the chromatin of estrogen dependent breast carcinoma cells (Khaleque et al, in preparation) (Figure 3). HSF1 appears therefore to play an integral role in at least a subset of breast cancers and its ability to recruit the Mi2 /NuRD co-repressor complex and override estrogen induced transcription may be significant in cancer development. HSF1 binds avidly to MTA1 in a range of breast cell lines and the proteins are co-associated in human breast tissue (Ciocca, D.R. and Calderwood, S.K., in preparation). HSF1 appears to participate in breast cancer at the intersection of two essential but competing signal transduction pathways, the estrogen-ER pathway and the c-*erb*B2 pathway (Ciocca et al., 2006). Although expression of ER promotes breast carcinoma growth, ER expression is antagonistic to invasion and metastasis (Bewick et al., 1999; Kumar et al., 2003; Neubauer et al., 2003). The influence of ER expression can however be overridden by c-*erb*B2 overexpression. Our studies show that stimulation of the c-*erb*B2 pathway by heregulin promotes HSF1 association with MTA1 and repression

of ER dependent promoters. Interaction of HSF1 with MTA1 additionally leads to increased *HSP70* transcription and Hsp expression in response to heregulin (Khaleque et al., 2005). As mentioned above, Hssp70 is a potent PCD inhibitor and thus antagonizes the effects of cytotoxic therapy (Beere, 2004; Khaleque et al., 2005). HSF1 may also participate in progression of gastrointestinal cancers by repressing the promoter of the XAF1 gene. XAF1 is a suppressor of XIAP, a caspase 3 inhibitory protein and loss of XAF1 thus leads to protection from caspase dependent apoptosis (Wang et al., 2006). Elevation of HSF1 during malignant transformation may thus be implicated in tumorigenesis through multiple mechanisms, including expression of anti-apoptotic proteins Hsp70 and XIAP and repression of ERE regulated genes including pro-apoptotic c-*Myc* (Khaleque et al., 2005; Wang et al., 2004c).

HSF1 AND THERAPY

The elevated HSF1 expression in cancer and its rather pleiotropic effects in the malignant phenotype suggests the possibility of directly targeting the factor in cancer therapy. In addition HSF1 activation is proving to be a complication of a number of novel therapies, including proteasomal inhibitors and hsp90 inhibitors (Zaarur et al., 2006). Both of these inhibitors induce HSF1 and lead to elevated Hsp expression through mechanisms indicated in Figure 5 (Zaarur et al., 2006). In fact at least two other drug families which might find use in cancer therapy, the non-steroidal anti-inflammatory drugs and cyclopentanone prostaglandins also

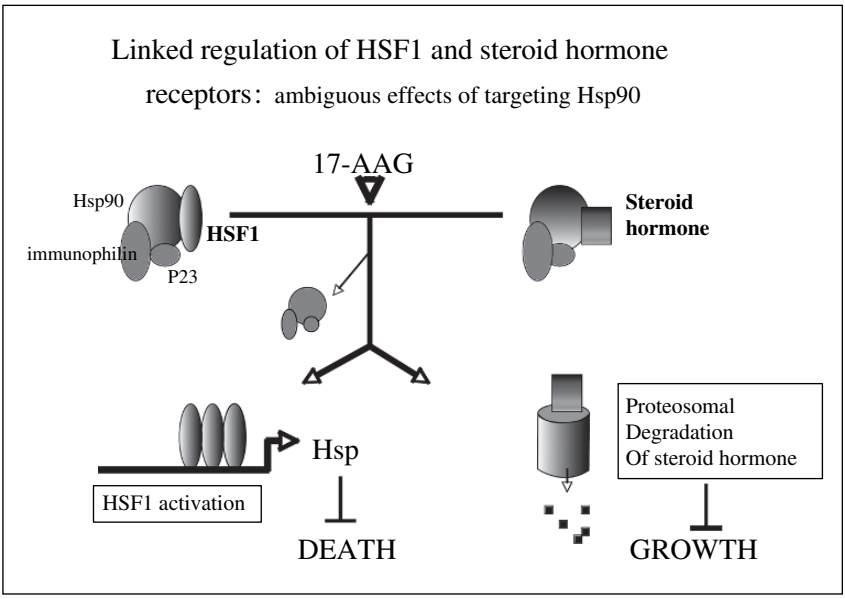

Figure 5. Hsp90 inhibitors produce competing effects on cancer cells

induce HSF1 activation and Hsp expression (Ciucci et al., 2006; Stevenson et al., 1999). Our recent studies indicate that inhibiting the heat shock response in cancer cells treated with proteasome inhibitors and hsp90 inhibitors leads to a markedly enhanced response to treatment (Zaarur et al., 2006).

Inhibitors such as 17-AAG prevent the stabilizing effects of Hsp90 on clients such as steroid hormones, oncogenic kinases and transcription factors and lead to destruction of these proteins by the proteasome. However, the drugs also lead to the release of HSF1 from the Hsp90 complex. In contrast to other Hsp90 clients, HSF1 is stable in free form and is free to activate transcription of Hsp genes leading to a profoundly resistant state.

CONCLUSIONS

HSF1 is normally under tight regulation and is not active in growing, unstressed cells from normal tissues. However, in cancer HSF1 control is subverted, resulting in its elevated concentration and constitutive transcriptional activation. Elevated HSF1 levels play a role in cancer through protection from programmed cell death, switching of gene expression towards a more metastatic phenotype and resistance to therapy.

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CHAPTER 4

ANTI-APOPTOTIC, TUMORIGENIC AND METASTATIC POTENTIAL OF HSP27 (HSPB1) AND αB-CRYSTALLIN (HSPB5): EMERGING TARGETS FOR THE DEVELOPMENT OF NEW ANTI-CANCER THERAPEUTIC STRATEGIES

ANDRE-PATRICK ARRIGO

Stress, Chaperons and Cell Death Laboratory, CNRS UMR 5534, Centre de Génétique Moléculaire et Cellulaire, Université Claude Bernard, LYON 1, 16 rue Dubois, 69622 Villeurbanne Cedex, France, e-mail: arrigo@univ-lyon1.fr

Abstract: Human Hsp27 (also denoted HspB1) and αB-crystallin (also denoted HspB5) are major heat-inducible small heat shock proteins (sHsps). These polypeptides share dynamic phosphorylation and oligomeric properties suggesting that different functional forms of these proteins exist. Elevated levels of expression of these sHsps counteract both necrotic and apoptotic cell deaths induced by various stimuli including heat shock, oxidative stress, inflammatory cytokines, death-receptor agonists and apoptotic inducers. In cells exposed to agents or conditions that unfold proteins, such as heat shock, these proteins act as ATP-independent molecular chaperones that can, in concert with other members of the heat shock protein family (i.e. Hsp70, Hsp40 and Hsp90), facilitate the repair or promote the degradation of damaged proteins which are potentially produced in stressed cells. In addition, Hsp27 and αB-crystallin limit the formation of oxidized macromolecules by stimulating the anti-oxidant defences of cell. Hsp27 and αB-crystallin also negatively regulate apoptosis through their ability to interact with several key components of the apoptotic signalling pathway. Moreover, particular oligomeric forms of these polypeptides stabilize the cytoskeleton. The high level of constitutive expression of Hsp27 and αB-crystallin in a wide range of tumors resistant to cancer therapeutics as well as their tumorigenic and metastatic potential designate these proteins as potential targets for future anti-cancer therapeutic strategies

Keywords: Hsp27 (HspB1), αB-crystallin (HspB5), apoptosis, anti-cancer agents, tumorigenic potential, aptamers

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HSP27 AND αB-CRYSTALLIN: INVOLVEMENT IN THE PROTEIN REFOLDING MACHINERY

Heat Shock Proteins (Hsps, denoted also stress proteins) are characterized by their stimulated synthesis in response to heat shock. On a more general point of view, when the environment of a cell becomes deleterious Hsps are expressed and act as molecular chaperones that interfere with the formation of mal-folded polypeptides and promote the refolding of proteins whose structure has been altered (particularly newly made polypeptides). Hsps also act at the level of intracellular transport of proteins, cytoskeleton architecture, mutations masking, intracellular redox and protection against spontaneous or stimulated cell death. Small stress proteins (or sHsps) are low molecular weights (20–30 kDa) Hsps characterized by a common C-terminal domain (about 40% of the protein: the alpha-crystallin domain) (Ingolia and Craig, 1982; de Jong et al., 1993; Arrigo and Landry, 1994). sHsps are in the form of oligomers of heterogenous native sizes (between 50 to 800 kDa) (Arrigo and Landry, 1994). Some sHsps, such as human Hsp27 and αB-crystallin are phosphorylated on serine residues by either MAPKAPK2 kinase (Hsp27, αB-crystallin) (Rouse et al., 1994) and p42/p44 MAPK (αB-crystallin) (Kato et al., 1998). Today, the molecular function of most high molecular weight Hsps (Hsp70, Hsp90, Hsp60) is relatively well known (i.e. ATP-dependent chaperones), while that of the sHsps was, until recently, rather obscure. Recent studies, including ours, have revealed that the high molecular weight oligomeric structures formed by sHsps have an ATP-independent chaperone activity (Jakob et al, 1993; Rogalla et al, 1999). These structures have short half-life and are in dynamic equilibrium with oligomers of smaller sizes (Lelj-Garolla and Mauk, 2005). The phosphorylation of Hsp27 and αB-crystallin leads to the formation of oligomers of small sizes that negatively regulate the chaperone activity of these proteins (Kato et al., 1994; Mehlen et al., 1997a; Kato et al., 1998; Préville et al., 1998b; Rogalla et al., 1999). Large oligomers are supposed to act as reservoirs that store mal-folded or oxidized polypeptides (also denoted molten globules) until they are either processed for refolding by ATP-dependent chaperones (type Hsp70 and co-chaperones) or degraded by the proteasome (Ehrnsperger et al., 1997; Lee et al., 1997; Arrigo, 2005a; Parcellier et al., 2003b). αB-crystallin also modulates the ubiquitin/proteasome pathway in a phosphorylation- and cell cycle-dependent manner (den Engelsman et al., 2003). Hence, both polypeptides participate in the so-called "protein triage" that occurs in cells recovering from stress.

Contrasting with the chaperone activity associated with the large oligomers of Hsp27, the small oligomers formed by this protein act towards cytoskeleton integrity (Mounier and Arrigo, 2002). Indeed, the small oligomers of Hsp27 (such as those observed in heat shock treated cells) help the cell to maintain cytoskeleton architecture (Lavoie et al., 1995; Huot et al., 1996). αB-crystallin is also deeply involved in the maintenance of cytoskeleton integrity, particulary the desmin cytoskeleton of muscle cells (Vicart et al., 1998; Bova et al., 1999).

In heat shock treated cells, Hsp27 also favors translation inhibition of non heat shock induced mRNAs through its ability to dissociate cap-initiation complexes and bind eIF4G initiation factor (Cuesta et al., 2000). During the recovery period

subsequent to heat shock, Hsp27 was also reported to stimulate the recovery of RNA and protein synthesis as well as RNA splicing (Carper et al., 1997; Marin-Vinader et al., 2005). Hence, cells expressing Hsp27 and αB-crystallin are characterized by a strong survival advantage.

HSP27 AND αB-CRYSTALLIN AS REDOX MODULATORS

In eukaryots, respiratory energy production usually generates side products such as reactive oxygen species (ROS) that can induce an oxidative stress if their level is too high. Mild oxidative stress are usually buffered by the cell through sophisticated defence mechanisms (i.e. detoxifiant enzymes, vitamines C and E and thiol-containing molecules, such as glutathione). If the intensity of the stress exceeds the buffering capacity of the cells, apoptosis or even necrosis occurs. Apoptosis is usually induced by a moderate oxidative stress probably through glutathione depletion that triggers the mitochondrial apoptotic pathway (Zucker et al., 1997). In contrast, drastic oxidative stress induces necrosis as a consequence of irreversible oxidations that inhibit caspases and decrease ATP intracellular levels (Jacobson, 1996; Samali et al., 1999).

A decrease in the deleterious effects induced by oxidants, such as hydrogen peroxide, menadione, doxorubicin and tumor necrosis factor (TNFα), is induced by the expression of mammalian Hsp27 and αB-crystallin (Huot et al., 1991; Mehlen et al., 1993; Mehlen et al., 1995b; Mehlen et al., 1996a; Wang et al., 1996; Park et al., 1998; Arrigo et al., 2005a) or *Drosophila* sHsps (Mehlen et al., 1995b; Mehlen et al., 1996a; Morrow et al., 2004). The expression of these sHsps usually correlates with decreased ROS and nitric oxide ($NO^{\bullet}$) levels (Mehlen et al., 1995b; Preville et al., 1999; Rogalla et al., 1999; Firdaus et al., 2006). Consequently, cells expressing these sHsps are characterized by reduced lipid peroxidation, protein oxidation (Mehlen et al., 1996a), DNA damages, and F-actin architecture disruption (Huot et al., 1996; Huot et al., 1997; Préville et al., 1998a). sHsps expression also upholds mitochondrial membrane potential ($\Delta\Psi m$) in cells exposed to oxidative challenges (Preville et al., 1999; Paul and Arrigo, 2000). ROS level decrease is particularly intense when sHsps are overexpressed in cells normally devoid of constitutive sHsps expression (such as murine fibroblasts). Of interest, the antioxidant activity of Hsp27, αB-crystallin and *Drosophila* Hsp27 is reduced glutathione (GSH) dependent (Mehlen et al., 1996a). This implies that sHsps do not provide a protection against the oxidative stress induced by agents that alter GSH reducing activity such as buthionine sulfoximine (BSO) (a specific and essentially irreversible inhibitor of γ-glutamyl-cysteine synthetase) or diethyl maleate (DEM) which chelates the free sulfhydryl group of GSH. We have also observed that an expression vector-mediated increase in sHsps levels upregulates GSH level and/or upholds this redox modulator in its reduced form (Mehlen et al., 1996a; Preville et al., 1999; Baek et al., 2000; Paul and Arrigo, 2000; Arrigo et al., 2005b). However, the phenomenon can be difficult to detect in cells that already express a high level of sHsps (i.e HeLa cells). The glutathione antioxidant power mediated by Hsp27 is a consequence of the up-regulation (expression

and/or activity) of several enzymes involved in the ROS-glutathione pathway, such as glucose-6-phosphate dehydrogenase (G6PDH), glutathione reductase and glutathione transferase (Preville et al., 1999). Indeed, G6PDH, the first enzyme of the pentose phosphate cycle, reduces NADP+ in NaDPH(H)+ and is therefore the key enzyme that provides the reducing power of the cell. It is also important to note that the modulation of the intracellular redox state by sHsp is not restricted to cell cultures but also occurs in whole animal. Indeed, the decrease in the constitutive expression of Hsp27 and αB-crystallin observed in cardiac cells of *hsf 1 (-/-)* mice (72% in the case of Hsp27 and 34% in the case of αB-crystallin) was found to correlate with 34% decreased G6PDH activity, 40% decrease in the GSH to GSSG ratio and in a 43% increase in the level of ROS (Yan et al., 2002). In addition, the expression of either Hsp27 (Arrigo et al., 2005b; Chen et al., 2006) or αB-crystallin (our unpublished observation) decreases iron intracellular levels. This suggests that sHsps can down-regulate the formation, through iron-dependent Fenton reaction, of hydroxyl radicals (OH^O). This assumption was confirmed by oxyblot analysis of proteins present in cells expressing Hsp27 (Preville et al., 1999; Firdaus et al., 2006) or αB-crystallin (our unpublished results). The Oxyblot technique detects carbonyl residues in oxidatively modified proteins and is used to estimate the damages caused by the very potent oxidant OH^O (Levine et al., 1990).

In cells undergoing oxidative stress, the cellular locale, oligomerization and phosphorylation of Hsp27 are modified in a dynamic way. The active form of Hsp27 is represented by its large unphosphorylated oligomers (Mehlen et al., 1997a; Rogalla et al., 1999) which hold *in vitro* chaperone activity (Ehrnsperger et al., 1997; Lee et al., 1997; Rogalla et al., 1999; Ehrnsperger et al., 2000). Concerning irreversibly oxidized polypeptides, Hsp27 large oligomers may participate in their recognition and degradation by the ubiquitin-independent 20S proteasome; a proteolytic machine that has high affinity for oxidized proteins (Sitte et al., 1998). No informations are yet available concerning the oligomeric structure of αB-crystallin responsible of its anti-oxidative activity.

Hence, Hsp27 and αB-crystallin appear functionally linked to the maintenance of redox state homeostasis (Arrigo and Landry, 1994; Arrigo, 1998; 2001; Arrigo et al., 2002b; Arrigo et al., 2005b: Arrigo, 2005 #2002). Consequently, these sHsps can easily interfere with the killing efficiency of redox state dependent anti-cancer drugs, such as 17AAG, or physical challenges, such as X-rays irradiation (see below).

ANTI-APOPTOTIC POTENTIAL OF HSP27

Hsp27 expression increases the cellular resistance to several pro-apoptotic agents or conditions (Mehlen et al., 1996b; Samali and Cotter, 1996; Arrigo, 2000; Garrido et al., 2001; Arrigo et al., 2002a; Concannon et al., 2003; Parcellier et al., 2003a; Arrigo, 2005b; a; Parcellier et al., 2005; Garrido et al., 2006; Schmitt et al., 2006). On the opposite, inhibition of Hsp27 expression by anti-sense or RNAi technology sensitizes cells that constitutively express this protein to apoptosis (Paul et al., 2002; Bausero et al., 2006; Rocchi et al., 2006).

In the case of an apoptotic process which depends on the mitochondrial pathway, three sites of action of Hsp27 have already been discovered: the first one is upstream of the mitochondria and modulates the signals that trigger the release of cytochrome c (Paul et al., 2002) or Smac/DIABLO (Chauhan et al., 2003a) from mitochondria. In this respect, we have described that, in etoposide and cytochalasin treated cells, Hsp27 interfers with Bid translocation to mitochondria (Paul et al., 2002). These studies also led to the discovery of a signaling pathway linking F-actin disruption to mitochondria. This pathway which induces Bid intracellular redistribution is negatively regulated by Hsp27 ability to protect F-actin network integrity (Paul et al., 2002). The second site of action of Hsp27 is located down-stream of the mitochondria at the level of cytochrome c and apoptosome (Garrido et al., 1999; Bruey et al., 2000a). The third site of action of Hsp27 is at the level of pro-caspase 3 activation (Pandey et al., 2000). Hsp27 also negatively interferes with Fas transduction pathway through an interaction with DAXX (Charette et al., 2000). This protein also interacts with cellular factors involved in the oncogenic signaling pathway. One such factor, member of the family « Signal Transducer and Activator of Transcription »: STAT3, is constitutively active in numerous tumors and controls the expression of numerous key genes that modulate the transformed phenotype or prevent apoptosis. For example, STAT3 is a transcriptional activator of genes encoding Bcl-xL or survivin (Gritsko et al., 2006). Hsp27-STAT3 complexes have been identified in breast cancer cells (Song et al., 2004) and members of the STAT family are now considered as putative target in cancer studies (Diaz et al., 2006; Libermann and Zerbini, 2006). Another factor modulated by Hsp27 is Akt (Rane et al., 2003). Of interest, Hsp27 is a target of the tumor-suppressor p53 polypeptide, however, the nature of Hsp27-p53 interactions in the regulation of apoptosis are not yet fully defined (Gao et al., 2000). Moreover, several of the genes that are upregulated by c-Myc in cells expressing wild-type p53 encode chaperones related to cell death protection as Hsp27 (Ceballos et al., 2005).

In vivo, the anti-apoptotic property of Hsp27 has been demonstrated using virus bearing Hsp27 coding sequence (Brar et al., 1999; Latchman, 2005). Moreover, Hsp27 and αB-crystallin are expressed during development and cell differentiation processes (Arrigo, 1995; Mehlen et al., 1997b; Arrigo, 2000). In this respect, the lethality of *hsp27* gene knocked-out mice is probably a consequence of the fact that embryonic stem cells unable to express Hsp27 during their early differentiation undergo apoptosis (Mehlen et al., 1997b). Apoptosis during early differentiation probably occurs as consequence of impaired protein triage (Mehlen et al., 1999; Arrigo, 2005b).

Changes in the oligomeric structure as well as in the phosphorylation of Hsp27 are observed in HeLa cells exposed to apoptotic inducers (Paul C, Arrigo A.-P. in preparation). These dynamic changes are inducer-specific and their kinetics do not resemble those occuring in cells exposed to either heat shock (Arrigo et al., 1988) or oxidative stress (Mehlen et al., 1995a; Mehlen et al., 1997a). For example, etoposide induces the transient formation of large Hsp27 oligomers. In contrast, staurosporine and cytochalasin D induce the transient formation of two populations of oligomeric Hsp27 structures characterized by large and small molecular masses. An other example is the inhibition of DAXX-mediated cell death which depends of the unphosphorylated dimers of Hsp27 (Charette et al., 2000). Moreover, the

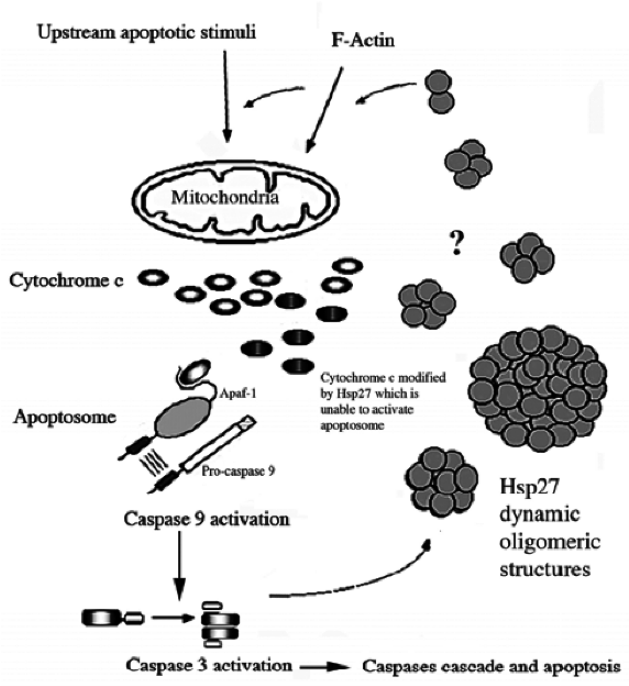

Figure 1. Hsp27 structural organization in the mitochondrial apoptotic pathway. Hsp27 in the form of large unphosphorylated oligomers interferes with the in vitro activation of pro-caspase-9 and pro-caspase-3. Medium sized or small oligomers of Hsp27 may bind cytochrome c and only small oligomers appear to act at the level of F-actin.The structure of Hsp27 at the level of the other (upstream or downstream) events is unknown

F-actin to mitochondria pathway which induces Bid intracellular redistribution is negatively regulated by the ability of Hsp27 small oligomers to protect F-actin network integrity (Paul et al., 2002). In contrast, the mechanism that negatively modulates apoptosis during early differentiation requires the rapid formation of large Hsp27 oligomers. *In vitro* caspase activation in cell free extracts stimulated by cytochrome c and dATP revealed a predominant role for the large oligomers of Hsp27 suggesting a link with the chaperone activity of the protein. Hence, different oligomeric forms of Hsp27 are active in the multiple steps of the apoptotic pathway (see Figure 1). This suggests that activities other than the chaperone function of Hsp27 are involved.

Hsp27 EXPRESSION IN CANCER CELLS: TUMORIGENIC AND METASTATIC POTENTIAL

Hsps have been studied for many years by researchers in the cancer field because of the well known hypersensitivity of cancer cells to heat shock. Some studies have proposed hyperthermia as a putative treatment of some human tumors (Hahn and Li,

1982a). However, in spite of some success, particularly in combined treatments with X-rays or chemotherapeutic agents, hyperthermia is nowdays less used because of thermotolerance-related problems and difficulty to heat shock treat the tumors (Hahn and Li, 1982b). However, recent observations suggest that mild heat shock treatments can still be useful to treat some types leukemia. One example is the heat shock mediated sensitization of leukemia T cells (Moulin and Arrigo, 2006) and chronic lymphocytic leukemia cells (Moulin et al., 2006) to TRAIL apoptosis. The molecular mechanism of cancer cell thermosensitivity is still unclear but could be related to the delayed expression of some Hsps, such as Hsp27, observed in oncogene-transformed cells exposed to heat shock (Fabre-Jonca et al., 1995; Gonin et al., 1997).

A fascinating property of Hsp27 is related to its high level of constitutive expression in several cancer cells, particularly those of carcinoma origin (breast, prostate, ovary and colon) (See: *Ciocca* et al, Chapter 2; *Budram-Mahadeo*, Chapter 5, *Bausero*, Chapter 6, *Brinet* et al, Chapter 12). Recently, publications reporting the implication of Hsp27 in cancer pathologies have grown exponentialy (Ciocca and Calderwood, 2005; Calderwood et al., 2006). For example, in breast cancer tumors, interesting correlations have been made suggesting links between Hsp27 expression and the level of estrogen receptors as well those of nodal metastasis, T avanced status, lymphatic/vascular invasion and patient survival (Ciocca et al., 1996). Hsp27 is also detected in endometrial cancer, oral cancer as well as in cancers from ovary, uterine cervix, prostate, salivary glands, œsophage, urinary system, skin, bone and nervous sytem (Ciocca and Calderwood, 2005). A high level of Hsp27 expression also occurs in hepatocarcinoma (Feng et al., 2005; Luk et al., 2006) as well as in kidney cancers (Atkins et al., 2005). Hsp27 expression is also considered as a prognosis marker for predicting the outcome succes or failure of chemotherapies based on different drugs such as vinorelbine in case of the treatment of non-small cells lung cancer (Berrieman et al., 2006). Hsp27 is also a marker of hormono-refractatory prostate cancer cells (Cornford et al., 2000; Rocchi et al., 2005) and urological tumor tissues (Takashi et al., 1997; Takashi et al., 1998). The presence of cell-free Hsp27 as well as Hsp27-cytochrome c complex in the lower genital track of women with ovarian and endometrial cancers has been proposed to be used in the early diagnosis of these malignancies (Korneeva et al., 2002). Finally, Hsp27 as well as its phosphorylated isoforms are up-regulated in acute leukemia (Xiao et al., 1996) and pre-B lymphoblastic leukemia (Strahler et al., 1991).

Hsp27 level can be used as prognostic factor in some types of cancer but not all. For example, Hsp27 is not a very useful marker in breast cancer (Oesterreich et al., 1996) while its expression clearly correlates with poor prognosis in ovarian, gastric, liver and prostate cancers as well as osteosarcomas. It is even associated with good prognosis in the case of oesophageal cancer, endometrial adenocarcinomas and malignant fibrous histiocytomas (Ciocca and Calderwood, 2005). Hence, the cellular context and more importantly the structural organization of Hsp27 are key parameters to take into account to determine the consequences of the expression of this protein in a cancer cell.

The molecular mechanism that triggers the expression of Hsp27 inside solid tumor is not yet solved. However, several lines of evidence suggest that the phenomenon could be hypoxia-related. Indeed, it is well known that the inside of solid tumors is characterized by hypoxic events (Kimbro and Simons, 2006). As a consequence, cancer cells react to hypoxia by stimulating HIF-1 (hypoxia induced factor-1) activation and subsequently Hsp27 accumulation since this stress protein has recently been described to be up-regulated by hypoxic signaling through HIF-1 activation (Whitlock et al., 2005). Moreover, it is well known that transgenic mice overexpressing Hsp27 are protected against ischemia/reperfusion induced injury (Hollander et al., 2004). Hence, It is possible that some cancer cells accumulate high loads of Hsp27 to protect themselves against hypoxia-induced cell death.

Several studies, including ours, have shown that the expression of Hsp27 induces a cellular resistance towards anticancerous drugs used in the clinic such as, cisplatin, the anthracycline derivative doxorubicin (Huot et al., 1991; Ciocca et al., 1992; Oesterreich et al., 1993; Garrido et al., 1996), vincristine (Verrills et al., 2006) and paclitaxel (Rocchi et al., 2004). In addition, some of these drugs, particularly cisplatin, vincristine and colchicine enhance Hsp27 expression (Oesterreich et al., 1991; Oesterreich et al., 1993; Bielka et al., 1994). Hsp27 expression is therefore correlated with cellular resistance to cytostatic drugs, a phenomenon which impairs the efficiency of the clinical treatments using these chemotherapeutic agents.

An important property of Hsps, such as Hsp70 (Jäättelä, 1993) and Hsp27 (Mehlen et al., 1995b), concerns their ability to modulate cell sensitivity to inflammatory cytokines. Moreover, our studies (Garrido et al., 1998; Arrigo, 2000) as well as those of Jäättellä (1999) suggest that Hsps play a major role in the immunological survey and tumorigenic potential of cancer cells. We have analyzed the role of Hsp27 in colorectal tumorigenesis (Figure 2). Two cell lines, named PROb and REGb, derived from a rat colorectal tumor, were used. PROb cells, injected in syngenic rat, formed large tumors that killed the animal. In contrast, REGb cells induced only small size tumors that regress. In contrast to REGb cells, PROb cells express a high level of Hsp27. Genetically manipulated REGb cells that overexpress Hsp27 were tested. It was observed that these cells behaved as PROb cells and formed large tumors as a consequence of apoptosis inhibition, hence demonstrating the tumorigenic potential associated to Hsp27 (Garrido et al., 1998). Analysis of Hsp27 structural organization in tumors has revealed a high tendancy to form chaperone-like large oligomers (Bruey et al., 2000b). The phenomenon is probably related to the fact that cell confluency (Garrido et al., 1997) and inflammation (i.e TNFα) (Mehlen et al., 1995b), two phenomena that occur inside tumors, favor the formation of large oligomers of Hsp27. It is also intriguing to note that several cancer cells express a high level of argpyrimidine modified Hsp27, a protein modification which stabilizes Hsp27 large oligomers (van Heijst et al., 2006). Hypoxia inside tumor (Kimbro and Simons, 2006), which promotes the accumulation of Hsp27 through HIF-1 activation (Whitlock et al., 2005), may also favor the formation of large oligomers. Hence, the tumorigenic potential of Hsp27 depends on specific changes in its oligomeric structure, and particularly the

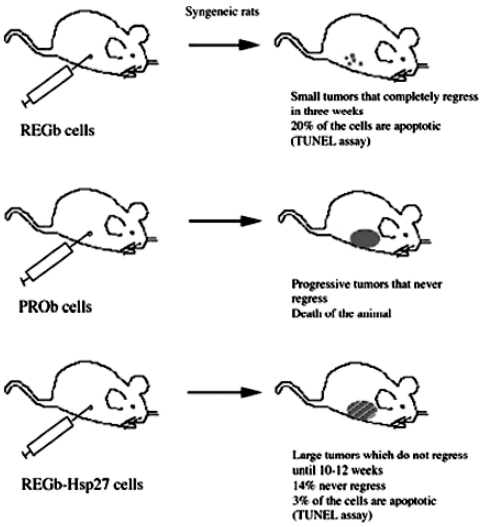

Figure 2. Cartoon describing the tumorigenic potential of Hsp27. Rat colon carcinoma REGb, PROb and Hsp27-expressing REGb cells were injected in syngenic rats. REGb cells are devoid of Hsp27 expression while PROb and Hsp27-expressing REGb cells contain similar levels of Hsp27. It was concluded that the presence of Hsp27 stimulates the formation of tumors. For further details see (Garrido et al., 1998)

formation of large unphosphorylated oligomers (Arrigo, 2000; Bruey et al., 2000b; Garrido, 2002).

It can therefore be concluded that Hsp27 plays a fondamental role in the ability of some cancer cells to evade from the apoptotic processes mediated by the immune system.

Recent proteomic studies have revealed that the expression of Hsp27 occurs during metastasis formation, hence suggesting that this protein plays a key role in this phenomenon (Ciocca and Calderwood, 2005). For example, hepatocarcinoma are characterized by an overexpression of Hsp27 in all metastatic tissues compared to non metastatic tissues (Song et al., 2006). Hsp27 is also detected in the case of gastric cancers producing metastasis (Chen et al., 2004). In prostate cancer, Hsp27 and the kinase (MAPKAPK2) responsible of its phosphorylation are necessary for TGFβ stimulation of cell invasion (Xu et al., 2006). Moreover, KRIBB3, a biphenyl isoxazole derivative, which inhibits Hsp27 phosphorylation, blocks the cellular migration that precedes the formation of metastasis (Shin et al., 2005).

HSP27 AS A POTENTIAL TARGET FOR FUTURE ANTI-CANCER THERAPIES

Experiments have been performed to inhibit Hsp27 overexpression in cancer cells using anti-sense and RNAi technologies. In this respect, we observed that a 40% decrease in Hsp27 level sensitized HeLa cells to apoptotic inducers (Paul et al.,

2002). Moreover, in the case of prostate cancer, recent studies have revealed that androgen depletion induces cytoprotection and increases Hsp27 level, phenomena dependent on Hsp27 interaction with STAT3 transcription factor (Rocchi et al., 2005). Of interest, small interference RNA (siRNA) targeting Hsp27 inhibits the growth of prostatic cell lines and induces apoptosis via caspase-3 activation (Rocchi et al., 2006). Hsp27 decreased level also reduced the tumorigenic potential of prostate cancer cells and enhanced their chemosensitivity to anticancer drugs, such as paclitaxel (Rocchi et al., 2004). An other example is related to murine 4T1 cells where silencing the *hsp27* gene eliminates the migration capability of these highly metastatic breast adenocarcinoma cells (Bausero et al., 2006). It has also recently been shown that overexpression of Hsp27 increases cell resistance to the inhibitor of Hsp90 chaperone activity and anti-cancer drug 17AAG whereas down-regulation of Hsp27 by siRNA increased sensitivity (McCollum et al., 2006). Moreover, cell line selected for stable resistance to 17AAG relative to parent cells showed increased Hsp27 expression. 17AAG resistance was dramatically diminished when 17AAG-resistant cells were transfected with Hsp27 siRNA. It was then observed that 17AAG resistance was dependent on the ability of Hsp27 to up-regulate GSH (McCollum et al., 2006). In our hand, we observed that RNAi directed decrease in Hsp27 levels senzitized radio-resistant human SQ20B head-neck cancer cells to gamma irradiations (Arrigo-Rodriguez-Lafrasse, manuscript in preparation) suggesting that anti-Hsp27 therapy is a potential addition to irradiation-based therapies. Since ROS are produced in X-rays treated cells (Rugo et al., 2002), a protective mechanism of Hsp27 against X-rays induced cell death may also be related to the ability of this protein to up-regulate GSH and decrease ROS level. Hence, our previous observations that Hsp27 regulates the intracellular redox state through modulation of GSH, ROS, $NO^{\cdot}$ and G6DPH (see paragraph b) (Mehlen et al., 1996a; Preville et al., 1999; Firdaus et al., 2006) explain why Hsp27 can inhibit the cytotoxicity induced by redox sensitive anti-cancer drugs or physical challenges. Since αB-crystallin shares Hsp27 anti-oxidant properties, it is probable that it also interfers with 17AAG or X-rays induced cell death.

ALPHA-BCRYSTALLIN AS POTENTIAL TARGET FOR ANTI-CANCER DRUGS DEVELOPMENT

Recent observations suggest that the property of inducing apoptosis resistance in tumors is not restricted to Hsp27. Indeed, it has been reported that αB-crystallin is a novel oncoprotein (Gruvberger-Saal and Parsons, 2006) that predicts poor clinical outcome in breast cancer patients and that apoptosis resistance conferred by αB-crystallin may contribute to the agressive behavior of basal-like carcinomas (Moyano et al., 2006). It was first discovered, through the analysis of breast cancer cDNA microarray and proteomic data (Perou et al., 2000; Sorlie et al., 2001) that αB-crystallin is constitutively expressed in basal-like breast carcinomas. It was also found that both αB-crystallin and Hsp27 are highly overexpressed in preinvasive ductal carcinoma compared with matched normal breast tissue

(Wulfkuhle et al., 2002). Constitutive expression of αB-crystallin is also detected in other human cancers, such as gliomas and prostate and renal cell carcinomas (Aoyama et al., 1993; Takashi et al., 1998; Chelouche-Lev et al., 2004). As for Hsp27, the presence of αB-crystallin in some tumors may be related to hypoxia-dependent events (Nefti et al., 2005). Several facts support the hypothesis that the presence of αB-crystallin contributes to the aggressive behavior of cancer cells: first αB-crystallin is a small heat shock protein and as so it can act as a stress-induced molecular chaperones that counteracts the aggregation of denatured proteins, thereby promoting cell survival. Indeed, overexpression of exogenous αB-crystallin confers protection against apoptotic stimuli such as those induced by heat shock, UV irradiation, staurosporine, TNF-α, TNF-related apoptosis-inducing ligand (TRAIL), etoposide, growth factor deprivation and oxidative stress while silencing of αB-crystallin expression sensitizes cells to apoptosis (Mehlen et al., 1996b; Kamradt et al., 2001; Kamradt et al., 2002; Kamradt et al., 2005). Second, neoplastic changes and invasive properties of breast cells appear related to the phosphorylation state of αB-crystallin (Chelouche-Lev et al., 2004). Indeed, phosphorylation at serine 59, the major phosphorylation site of αB-crystallin, attenuates the anti-apopototic activity of the protein, a phenomenon associated with a reduced oligomerization (and probably chaperone activity) of the protein (Webster, 2003).

Regarding to its mechanism of action, αB-crystallin has been reported to act at several different sites of the apoptotic pathway. For example, αB-crystallin interferes with procaspase-3 activation, whereas a pseudophosphorylation mutant of αB-crystallin that mimics stress-induced phosphorylation fails to suppress caspase-3 activation and apoptosis (Kamradt et al., 2001; Kamradt et al., 2002; Kamradt et al., 2005). It has also been reported that αB-crystallin binds the proapoptotic polypeptides Bax and Bcl-x_S and prevents their translocation to the mitochondria (Mao et al., 2004). However, no informations are yet available concerning the oligomeric structures of αB-crystallin that are active in the apoptotic pathway. Hence, apoptosis resistance conferred by αB-crystallin may contribute to the aggressive behavior of cancer cells such as the basal-like breast carcinoma cells. Moreover, because of its anti-oxidant properties, αB-crystallin will probably share Hsp27 ability to interfere with 17AAG or X-rays induced cell death.

HOW TO KNOCK-DOWN HSP27 AND ALPHAB-CRYSTALLIN ANTI-APOPTOTIC, TUMORIGENIC AND METASTATIC PROPERTIES?

As described above a high level of expression of Hsp27 or αB-crystallin enhances the resistance of some cancer cells to death while a reduced level of these proteins sensitizes them. Hence, one way to sensitize cancer cells that express high loads of Hsp27 or αB-crystallin is to reduce the level of these proteins. However, since anti-sense and RNAi technologies are not easy to handle *in vivo*, new approaches have to be discovered, such as the use of specific inhibitory drugs that inhibit the activity of Hsp27 or αB-crystallin without targeting their levels of

expression. Moreover, it will be of prime importance that these drugs be inocuitous towards normal cells expressing the targetted protein. Indeed, new canti-cancer drugs such as bortezomib/Velcade™ (a proteasome inhibitor used in the clinic) (Ludwig et al., 2005) or 17AAG (a geldanamycyne derivative inhibitor of Hsp90 chaperone activity, actually in clinical trial phases) (Georgakis and Younes, 2005) share the ability to sensitize cancer cells without inducing deleterious effects in normal cells. One approach to inhibit the activity of a specific protein is based on the action of RNA aptamers that specifically recognize, bind and inhibit the activity of the targetted protein. This approach is effective to target and inhibit the activity of the prostate-specific membrane antigen (PMSA) (Farokhzad et al., 2006a; Farokhzad et al., 2006b). In this respect, drug-encapsulated polymeric nanoparticle-aptamer bioconjugates is an emerging technology that can facilitate the delivery of chemotherapeutics to primary and metastatic tumors. Hydroxyapatite ceramic particles are also promising tools to deliver therapeutic drugs inside tumors (Ciocca et al., 2007) Peptide aptamers is an other approach (Colas et al., 1996). One advantage of small inhibitory peptides (8–10 amino acids) to RNA aptamers is the easier possibility to elucidate their 3-dimentional structure. This may lead, using peptido-mimetic drug design approaches, to the discovery of chemical drugs that mimic specific peptide structures and act as synthetic inhibitors of the targetted protein.

PERSPECTIVES

The approach of my laboratory toward inhibitors of Hsp27 and αB-crystallin is based on the search of aptamer peptides that specifically recognize, bind and inhibit the activity of Hsp27 or αB-crystallin by interfering with their oligomerization properties. Indeed, it is well known (less informations are available concerning αB-crystallin) that the protective mechanism of Hsp27 inside tumors necessitates complex changes in its oligomerization profile (particularly its presence in the form of large oligomeric structures). Consequently, small peptides or peptido-mimetic drugs that specifically recognize key domains in Hsp27 or αB-crystallin polypeptides may probably interfere with the formation of oligomeric structures that bear the chaperone, anti-oxidant, anti-apoptotic and tumorigenic properties of these proteins. Such peptides or peptido mimetic drugs will probably sensitize Hsp27-expressing cancer cells to apoptosis and/or overcome the activity of anti-cancer drugs that are inhibited by Hsp27, such as bortezomib/ Velcade™ (Chauhan et al., 2003b), 17AAG (McCollum et al., 2006) or paclitaxel (Rocchi el al., 2004).

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CHAPTER 5

HEAT SHOCK PROTEIN-27 (HSP-27) IN BREAST CANCERS: REGULATION OF EXPRESSION AND FUNCTION

VISHWANIE S. BUDHRAM-MAHADEO[1,*] AND RICHARD J. HEADS[2]

[1]*Medical Molecular Biology Unit, Institute of Child Health, University College London, 30 Guilford Street, London, WC1N 1EH*

[2]*Cardiovascular Division, King's College London School of Medicine, Department of Cardiology, The Rayne Institute, St Thomas's Hospital, Lambeth Palace Road, London SE1 7EH, UK*

Abstract: The small heat shock protein, Hsp27, is elevated in a significant proportion of breast cancers. Over-expression of Hsp27 in breast cancer cells increases anchorage-independent growth, invasiveness and resistance to chemotherapeutic drugs and is associated with poor prognosis and shorter disease free survival in a proportion of cancer patients. Hsp27 acts in a complex manner to elicit diverse effects e.g. increasing survival in response to many stresses by acting either as a molecular chaperone, by association with components of the apoptotic machinery and/or by increasing cellular glutathione to regulate the redox state of the cells. Hsp27 also regulates cytoskeleton organization and stability. Therefore, factors that increase the expression and/or alter the functions of Hsp27 in breast cancer cells can affect disease progression and outcome following treatment. Although Hsp27 expression can be mediated via the classical Heat Shock response following stress, its elevation in breast cancer cells is associated with independent positive regulators which include the estrogen receptor (ER) and transcription factors such as Brn-3b and Sp1. HET/SAF-B negatively regulates Hsp27 in these cells. The effects of Hsp27 are also regulated post-translationally by phosphorylation at specific residues, which alter the oligomerization state of the protein and thus its effects in the cells. The expression, effects and mechanisms by which Hsp27 acts in breast cancer cells are described in this chapter

Keywords: Hsp27, breast cancer, transcription regulation, promoter, estrogen receptor, Brn-3b transcription factor

Abbreviations: HSP, heat shock protein; HSE, heat responsive element; HSF, heat shock factor; ROS, reactive oxygen species; MAPK, mitogen activated kinases; ER, estrogen receptor; ERE,

*Corresponding author: Medical Molecular Biology Unit, Institute of Child Health, University College London, 30 Guilford Street, London, WC1N 1EH, Tel: 44-(0)20 7242 9789 ext 0761/0701, Fax: 44-(0)20 7905 2301, e-mail: v.mahadeo@ich.ucl.ac.uk

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estrogen responsive element; E-2, 17β-estradiol; TIC, transcriptional initiation complex; HET/SAF-B- Hsp27ERETATA binding/nuclear matrix protein/scaffold attachment factor

INTRODUCTION

Breast cancer is one of the most common female cancers in the western world affecting one in nine women at some stage in their life. It is a disease with complex etiology, that is influenced by many factors which include genetic, biologic, lifestyle and environmental (Cleator and Ashworth, 2004; Veronesi *et al*., 2005). However, the precise mechanisms which precipitate cellular transformation during tumourigenesis, maintain the malignant phenotypes, and allow cells to metastasize to distant sites, away from the tumour, are poorly defined and still being intensively studied. However, it is apparent that complex changes in the expression and/or function of specific cellular proteins contribute to the development and progression of cancers by conferring growth advantages and survival properties to tumour cells. Several heat shock proteins (Hsps) are elevated in a number of different cancers and, by virtue of their inherent functions in cells, these proteins often contribute to changes in the properties of tumour cells either during transformation, progression or in response to treatment.

HEAT SHOCK PROTEINS IN BREAST CANCERS

Hsps were first identified as proteins that were induced in cells subjected to heat stress but have since been shown to increase in response to many other cellular stresses and are now often referred to as Stress Response Proteins (Srp) (Lindquist and Craig, 1988; Welch, 1993). Hsps exist as several multigene families that have been classified according to their molecular size and large heat shock proteins include Hsp100, Hsp90, Hsp70, Hsp60 and Hsp56. The small heat shock protein family include Hsp27, α-A crystallin, α-B crystallin, p20, HspB2/HspB3 and HspB8, HspB9 and cvHsp and are typically between 15–30 kDa [reviewed in (Hightower, 1991; Ciocca *et al*., 1993)].

Hsps are ubiquitously expressed proteins that are highly conserved through evolution, suggesting that these proteins perform important functions, within diverse cell types in different organisms (Welch, 1992; Morimoto *et al*., 1997; Christians *et al*., 2003). In general, many Hsps share common properties, in that they can be induced in response to a number of different signals, e.g. environmental stresses such as heat stress, heavy metals and pro-oxidants, as well as pathophysiological stresses such as viral/microbial infection, inflammation, ischemia and drugs (Morimoto, 1993). Some Hsps also respond to growth signals and hormones and thus may have a physiological role in the normal development and/or in the differentiation of specialized cells (Morimoto, 2002). The effects of Hsps are diverse and largely depend on which of these proteins are induced in specific cell types and the growth conditions or stress signals that are present. Interestingly, many of the Hsp families

consist of multiple isoforms that can differ in their localization within different compartments of cells or can be expressed in distinct cell types or they may be induced under different cellular conditions (Arrigo, 2001; Yenari, 2002; Christians *et al*., 2003; Calderwood *et al*., 2006).

Elevated levels of different families of Hsps have been found in many types of cancers with distinct etiology and diverse cellular and molecular signatures [reviewed in (Ciocca and Calderwood, 2005; Calderwood *et al*., 2006)] (See: *Ciocca et al*, Chapter 2, this volume). In breast cancer, three main groups of Hsps, are elevated in a significant proportion of tumours namely, Hsp 27, 70 and 90 (Ciocca and Calderwood, 2005; Calderwood *et al*., 2006). Table 1 summarizes the common properties and putative functions of each of these Hsps and some implications of their over-expression in breast cancer cells.

The complexity of these proteins is increased by the findings that specific forms can give rise to distinct effects. Thus, two isoforms of Hsp90 have been identified, with Hsp90α being the inducible form that is implicated in breast cancer whereas Hsp90β is regarded as the constitutively expressed but relatively minor form of this family (Csermely *et al*., 1998) (See: *Pratt et al*, Chapter1). In the case of Hsp70, at least 8 distinct isoforms have so far been identified and these differ in terms of their expression patterns in different cell types as well as their localization within specific cellular compartments (Tavaria *et al*., 1996; Son *et al*., 1999; Rohde *et al*., 2005). Closer inspection revealed that specific Hsp70 isoforms, Hsp70-2 and Grp78 (Hsp70-5 or Bip), are over-expressed in many breast cancers. In this family, HSP70-2, in particular, seems to be critical for conferring growth changes in breast cancer cells since loss of this protein using short interfering RNA to silence gene expression, resulted in growth arrest and apoptosis (Rohde *et al*., 2005). In the case of Hsp27, post-translational modification gives rise to distinct phosphorylated forms of this protein that can be distinguished on the basis of their isoelectric point on two-dimensional gel electrophoresis (Landry *et al*., 1992). These modifications change the organization of the Hsp27 complexes and also alters the function of this protein and this will be discussed in more detail, later.

Elevated expression of Hsps in cancers can confer protection against many stresses that these cells encounter during the development or progression of tumourigenesis. The protective effects arise from the ability of Hsps to act in different capacities in various cells. For instance, many Hsps act as chaperones that participate in protein synthesis, folding and translocation and they also help to maintain the integrity of cellular proteins under stressful conditions (Tavaria *et al*., 1996; Hartl and Hayer-Hartl, 2002; Nollen and Morimoto, 2002). Some Hsps also enhance cell survival more directly by modulating the apoptotic machinery (Latchman, 2002b; Concannon *et al*., 2003; Farooqui-Kabir *et al*., 2004). Small Hsps, such as Hsp27, also associate with structural elements of the cytoskeleton and so confer protection against stress-induced changes, thus helping to maintain cellular integrity (Landry and Huot, 1999). Because Hsps have such diverse mechanisms for conferring protection on cells, it is not surprising that elevated expression of Hsps in tumour cells can enhance survival, growth and/or alter the behaviour of these cells.

Table 1. Summary of the properties, functions and effects of Hsp 27, 70 and 90 that have been studied in breast cancer

HSP	Size (kDa)	Localization	Putative Function	Effects in breast cancer cells
Hsp27	25–27	cytoplasm, nucleus	chaperone anti-apoptotic control of redox state of cells interaction with cytoskeleton stabilize actin filaments control actin polymerization	associated with: survival and drug resistance increased migration/metastasis shorter disease free survival
Hsp70 – Grp78 (BiP) – Hsp 70–2	72–78	cytoplasm, nucleus, or ER	chaperone function (protein folding) binding of mis-folded protein anti-apoptotic DNA replication protein retention in endoplasmic reticulum protein transport across membranes	associated with: increased proliferation lymph node metastasis poor differentiation poor outcome after treatment
Hsp90	90	cytoplasm	interactions with signalling proteins/nuclear receptors chaperone function interaction with cytoskeleton cell cycle control	associated with: increased proliferation lymph node involvement high Hsp90 associated with: early recurrence poor survival

THE SMALL HEAT SHOCK PROTEIN, HSP27

Hsp27 was first identified as a protein with high homology to the eye lens α-crystallin proteins and shares several physical characteristics with these proteins [reviewed in (Ciocca *et al*., 1993)]. For instance, analysis of the mouse homologue, Hsp25, showed that this protein has a compact two-domain structure, composed mainly of β-sheets that is similar to α-B crystallin (Merck *et al*., 1993a; Klemenz *et al*., 1994; Dillmann, 1999). A highly conserved region, (80–100 amino acids), is found in all family members and is referred to as the α-crystallin domain (because of high homology to α-crystallin protein) (Figure 1) (Merck *et al*., 1993b). This region conforms to IgG-like folds and is linked to a short flexible C-terminus. The amino terminus of the protein is less conserved but contains a short WDPF motif that is involved in oligomerization of the protein (Figure 1). Oligomerization of Hsp27 is largely determined by the phosphorylation state of the monomers as well as conditions such as the cellular environment e.g. temperature and pH (Lavoie *et al*., 1995; Rogalla *et al*., 1999; Benndorf *et al*., 2000). The phosphorylation of this protein on specific serine residues, (Landry *et al*., 1992) produce significant alterations in the quaternary structure of Hsp27 complexes and can profoundly change the functions of the protein in cells (see later).

Expression of Hsp-27

Hsp27 is constitutively expressed in several organs and tissues during embryonic development as well as in many adult tissues under normal conditions [reviewed in (Ciocca *et al*., 1993)]. For instance, Hsp27 has been detected in the eye, nervous system (NS), heart, blood and blood vessels, lung, bladder, colon and stomach, as well as in estrogen responsive organs such as uterus, vagina, cervix and placenta. Lower levels were detected in other tissues including epithelial cells of the breast, testes and striated muscle (Ciocca *et al*., 1983; Klemenz *et al*., 1993). Under normal physiological conditions, Hsp27 has been associated with reduced proliferation but increases cellular survival and differentiation, in many of these cell types (Ciocca *et al*., 1993).

In unstressed cells, Hsp27 levels are generally low and the unphosphorylated native protein can exist in complexes that could consist of up to 24 subunits

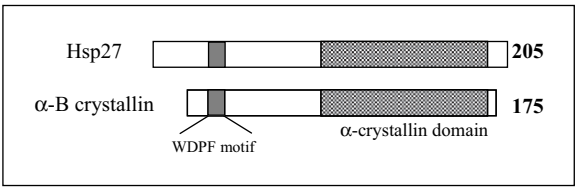

Figure 1. Schematic representation to show the regions of conservation within the domains of sHsps, Hsp27 and α-B crystallin. The short conserved region within the N' terminus is shown by the grey box whereas the α-crystallin domain in the C' terminus of the protein is shown by the stippled box

(6 tetramers) and can be 200-800kda in size (Merck *et al*., 1993a; Merck *et al*., 1993b). Under normal conditions, Hsp27 is found in detergent soluble cytoplasmic location within the perinuclear region near the Golgi complex (Arrigo *et al*., 1988).

However, under stressful conditions such as, for example, following heat stress injury, Hsp27 expression is increased at the transcriptional level and pre-existing and newly synthesized protein undergoes significant post-translational phosphorylation at specific amino acid residues, which alters its functions in these cells (see later). Such modifications result in the dissociation of larger Hsp27 complexes to form smaller complexes of the proteins (either as tetramers, dimers or monomers) which have distinct functions (Lambert *et al*., 1999). The phosphorylation of Hsp27 and subsequent conformational changes occur rapidly, since phosphorylation of Hsp27 can be detected within minutes following stress signals whereas the transcriptional regulation to increase its mRNA expression is seen after hours (Landry *et al*., 1991). Phosphorylation of Hsp27 occurs as a transient, reversible and highly dynamic process, which allows the protein to return to its unphosphorylated state that can form larger complexes and thus change its function (Zantema *et al*., 1992). Under many stressful conditions, Hsp27 also relocalizes within the nucleus and is resistant to detergent extraction and the extent of redistribution into the nucleus depends on the degree of stress (Arrigo *et al*., 1988) but whether this depends on phosphorylation is not clear (Kim *et al*., 1984).

Therefore the effects of Hsp27 depends on the levels of protein in cells as well as the phosphorylation state of Hsp27, which is in turn controlled by cellular signalling pathways that are stimulated by various stresses/growth conditions (discussed later).

Hsp27 Expression in Cancers

Elevated Hsp27 levels have been detected in a range of different tumours including breast cancer, prostate cancer, gastric tumours, head and neck cancers, uterine and ovarian cancers as well as in cancers arising from urinary system (bladder and kidney) and the nervous system (meningiomas, astrocytomas and neuroblastomas) (reviewed in (Ciocca and Calderwood, 2005)] (See: *Arrigo*, Chapter 4, *Brunet et al*, Chapter 12). In general, its expression in these types of cancers is associated with increased resistance to radiotherapy and to some chemotherapeutic drugs used for treatment of many cancers. Thus, over-expression of Hsp27 confers resistance to heat shock and to a range of cytotoxic agents such as cisplatin, staurosporine and H_2O_2 in cell lines derived from a number of different tumours e.g. Hep2 human laryngeal cells (Lee *et al*., 2007), breast cancer cells and prostate cancer cells. In contrast, loss of Hsp27 sensitizes human prostate cells to gamma radiation (Teimourian *et al*., 2006) and increases apoptosis in LNCaP prostate cancer cells (Rocchi *et al*., 2006). The significance of increased Hsp27 in terms of disease free survival, tumour recurrence and outcome after treatment, however, is much more variable and depends on the tumour type (see below). Therefore, Hsp27 appears to confer protection and drug resistance to different cancer cells and the mechanisms, by which these are achieved, will be discussed later.

Hsp27 in Breast Cancers

Hsp27 levels are elevated in a significant proportion of tumour biopsies and human breast cancer cell lines, compared with the lower levels found in normal human breast tissue and benign breast lesions [reviewed in (Ciocca *et al*., 1993; Ciocca and Calderwood, 2005) also see (Ciocca *et al*., 1982; Ciocca *et al*., 1983; Horne *et al*., 1988; Seymour *et al*., 1990a; Seymour *et al*., 1990b; Oesterreich *et al*., 1993; Tetu *et al*., 1995; Oesterreich *et al*., 1996b)]. Thus, over-expression of Hsp27 was detected in >60% of breast cancers. Further analysis based on tumour classification demonstrated elevated Hsp27 in ˜67% of ductal carcinomas in situ (DICS), ˜50% of cases of DICS associated with infiltrating ductal carcinoma and in 25-30% of infiltrating ductal carcinomas (Storm *et al*., 1995; Storm *et al*., 1996; Ioachim *et al*., 2003). Interestingly, analysis of biopsies taken from lymph node metastasis showed an even higher percentage with elevated Hsp27 (detected in >70% of patients with metastasis) (Storm *et al*., 1996). Furthermore, ∼62% of patients with infiltrating ductal carcinomas whose primary tumour were negative for this protein had Hsp27 over-expression in lymph node metastases (Storm *et al*., 1996). A number of independent studies undertaken to look at the clinical significance of high Hsp27 in breast cancers, demonstrated that elevated protein correlated with shorter disease free survival (DFS) and recurrence following treatment in patients with advanced breast cancer (Ciocca *et al*., 1993; Vargas-Roig *et al*., 1998). However, others did not see such strong correlation (Damstrup *et al*., 1992; Love and King, 1994; Tetu *et al*., 1995). A large study carried out by Oesterreich et al. (1996) (Oesterreich *et al*., 1996b) also concluded that although Hsp27 cannot be used as an independent prognostic marker in breast cancers, Hsp27 levels can predict poor outcome in a subset of estrogen receptor (ER) positive untreated breast cancer patients. Taken together, these studies strongly implicate elevated Hsp27 with metastatic spread and poor responses following treatment in a significant number of breast cancers.

Since Hsp27 was originally identified as an estrogen responsive gene in breast cancer cells, it was not surprising that subsequent studies showed significant correlation of this protein with the presence of the estrogen receptor (ER-α) in biopsies taken from patients with breast cancers (Ciocca *et al*., 1993; Martin *et al*., 2000; Ioachim *et al*., 2003). However, increased Hsp27 expression is associated with decreased proliferation in breast cancer cell lines and lower proliferative index in tumour samples (Vargas-Roig *et al*., 1997; Fanelli *et al*., 1998; Kindas-Mugge *et al*., 1998), indicating that Hsp27 does not enhance the proliferative effects of ER in these cells. Studies carried out by O'Neill *et al*. (O'Neill *et al*., 2004) suggests that elevated Hsp27 in ER-α positive benign neoplasia could promote the progression to more malignant phenotypes. This was supported by other studies which showed that elevated Hsp27 protein in breast cancer cells correlated well with enhanced anchorage independent growth (Rust *et al*., 1999), increased resistance to chemotherapeutic drugs such as doxorubicin and cisplatin and increased migration/metastasis of breast cancer cells in culture (Ciocca *et al*., 1992; Oesterreich *et al*., 1993; Yamamoto *et al*., 2001). In contrast, reducing Hsp27 in MCF-7 cells using antisense, inhibits growth and resulted in cellular hypertrophy (Mairesse *et al*.,

1996; Horman *et al*., 1999). Taken together with the observation of elevated Hsp27 in metastasis arising from breast cancers, these results predict that high levels of Hsp27 in breast cancers will give rise to aggressive diseases that are refractory to treatment and so have poor prognosis. Indeed many studies have shown that elevated Hsp27 in tumours correlated with shorter disease free survival and recurrence in node-negative breast cancer (Thor *et al*., 1991; Storm *et al*., 1996) whereas its induction following chemotherapy also predicts poor prognosis and shorter disease free survival (Vargas-Roig *et al*., 1998). However, this protein does not appear to be critical for resistance to treatment with the estrogen receptor antagonist, tamoxifen (Ciocca *et al*., 1998).

In terms of localization in breast cancer cells, Hsp27 is present in cytoplasmic granules in MCF7 grown in-vitro and in-vivo. If cells were grown in suspension (for example in ascites in nude mice), Hsp27 seems to have a more defined polarity to the apical cytoplasm (Ciocca *et al*., 1983; Ciocca *et al*., 1993) in line with its apparent role in cytoskeleton organization and cell migration (see below).

MECHANISMS THAT CONTROL EXPRESSION AND FUNCTION OF HSP27 IN BREAST CANCER CELLS

The elevated expression of Hsp27 in breast cancer cells and its association with survival, metastasis and drug resistance suggest that this protein plays an important role in determining the growth and behaviour of a subset of tumours in which it is expressed at high levels. A number of questions that arise are:

1. How does Hsp27 function to give rise to such distinct effects in cancer cells?
2. What are the conditions that can increase Hsp27 expression in breast cancer cells?
3. How are Hsp27 expression and function controlled at the molecular level?

If we can identify the conditions that lead to increased expression and alter the function of this protein in these cancer cells, it will help to better understand the molecular mechanisms that control its effects in breast cancer cells. This will also be useful to determine how its effects can be controlled or reversed in these cells.

Functions of Hsp27

Hsp27 protein can elicit diverse effects in different cell types since it demonstrates multiple functions that depend on the cell type and environment. Like other Hsps, Hsp27 can act as a molecular chaperone and participate in maintaining the integrity of cellular proteins particularly under stressful conditions (Ciocca *et al*., 1993; Rogalla *et al*., 1999; Calderwood *et al*., 2006). It can confer protection from apoptosis (Concannon *et al*., 2003), control the redox state of the cells (Arrigo, 2001) and contribute to cytoskeleton stabilization (Lavoie *et al*., 1995; Guay *et al*., 1997). Many of these functions are likely to be harnessed to give rise to the effects of Hsp27 in breast cancer cells. Before discussing the expression and implications

of elevated Hsp27 in breast cancers and highlighting how its expression levels are controlled in cancer cells, it is important to clarify how this protein acts in cells.

Chaperone Activity

The chaperone activity of Hsp27 allows it to participate in the assembly, folding, transport and degradation of cellular proteins under normal physiological conditions (Hartl, 1996; Richter-Landsberg and Goldbaum, 2003) but can also contribute to its effects such as survival and drug resistance in breast cancer cells. Following heat stress or under pathological conditions, increased Hsps will selectively bind non-native proteins and prevent aggregation (Fink, 1999). The accumulation of mis-folded protein in cancer cells (either as a consequence of mutations or adverse growth conditions) can trigger increased expression of Hsps (Hsp27 and 70) and under such conditions, the chaperone function of Hsp27 will prevent protein aggregation and enhance refolding and protein stabilization. It is also interesting to note that, in its chaperoning capacity, Hsp27 can act to inhibit protein translation following heat stress in 293T cells (Cuesta R. *et al*., 2006). To achieve this, Hsp27 binds to eIF4G, which is required as part of the cap-binding initiation complex and this association with Hsp27 results in its sequestration elF4G and so inhibition of translation under adverse conditions. Whether this function helps to determine the effects of Hsp27 in breast cancer cells remains to be established but it helps to demonstrate the complex effects by which this protein can give rise to quite diverse effects in cells, in response to different conditions.

The chaperone function of Hsp27 depends on large oligomeric complexes formed by the protein in its unphosphorylated state since these larger complexes appear to be required to bind tightly to unfolded proteins (Jakob *et al*., 1993; Rogalla *et al*., 1999). Upon phosphorylation, these large Hsp27 complexes dissociate and form smaller (tetrameric, dimeric or monomeric) complexes that are less effective chaperones but act in other capacities e.g. to protect cells from apoptosis and to associate with, and stabilize, cytoskeletal proteins (see later). It is interesting to note that unlike the larger Hsps which are ATP dependent, the small Hsps can act as chaperones in an ATP -dependent and -independent manner (Jakob *et al*., 1993; Ehrnsperger *et al*., 1997; Rogalla *et al*., 1999).

Stabilization/Polymerization of Cytoskeletal Proteins

The ability of Hsp27 to associate with structural proteins, such as actin, is important for maintaining the integrity of cytoskeleton and hence in conferring protection in a manner that is distinct from the classic chaperone functions. The association of Hsp27 with actin increases stability of F-actin microfilaments in response to insults such as heat stress and oxidants (Lavoie *et al*., 1993; Huot *et al*., 1995; Lavoie *et al*., 1995; Huot *et al*., 1996; Guay *et al*., 1997). Phosphorylated Hsp27 can stabilize actin filaments but inhibit actin polymerisation by acting as a barbed-end actin capping

protein. Thus Hsp27 seems to facilitate stabilization and remodelling of the actin cytoskeleton during stress (Miron *et al*., 1991; Lavoie *et al*., 1993; Benndorf *et al*., 1994; Lavoie *et al*., 1995; Guay *et al*., 1997; Benndorf *et al*., 2000). Interactions of Hsp27 with tubulin, tropomyosin and intermediate filaments have also been reported but the significance of these observations are yet to be determined. However, the ability of Hsp27 to protect and/ or stabilize the cytoskeleton under stressful conditions but also to alter the polymerization and stability of structural proteins might also contribute to the increased invasiveness and motility associated with elevated Hsp27 in cancer cells. This has been demonstrated experimentally by Rust *et. al*. (1999) who demonstrated that phosphorylation of Hsp27, following activation of MAP kinase pathways by anisomycin, lead to enhanced migration on the extra cellular matrix, laminin-5 (Rust *et al*., 1999). Phosphorylation of Hsp27 was also implicated in TGF-β induced MMP2 activation (Xu *et al*., 2006) whereas blocking Hsp27 phosphorylation inhibited migration in MDA-MB231 breast cancer cells (Shin *et al*., 2005).

Direct Regulation of Apoptotic Pathways

Inhibition of mitochondrial mediated apoptosis

Hsp27 can protect cells from apoptosis by directly regulating the apoptotic machinery [reviewed in (Concannon *et al*., 2003; Parcellier *et al*., 2003). For mitochondrial mediated apoptosis to be executed, formation of the apoptosome complex (which includes cytochrome C, caspase 9 and Apaf1) is a crucial intermediate step that is required for activation of downstream effector caspases such as caspase-3 (Samali *et al*., 2001; Schafer and Kornbluth, 2006). The release of cytochrome c from the mitochondria, in response to pro-apoptotic signals, is necessary for the formation of the apoptosome. It is thought that Hsp27 can disrupt the apoptosome possibly by binding to and sequestering cytochrome c and thus it indirectly inhibits the activation of the effector caspase-3 (Figure *2A*), (Bruey *et al*., 2000; Pandey *et al*., 2000 ; Samali *et al*., 2001; Paul *et al*., 2002; Concannon *et al*., 2003). Hsp27 may also inhibit apoptosis by directly interacting with pro-caspase-3 and thus prevent its cleavage to active caspase-3 (Figure *2A*) by delaying poly(ADP)polymerase cleavage by caspase-3 and by redistribution of pro-apoptotic BID (Garrido *et al*., 1999; Pandey *et al*., 2000; Concannon *et al*., 2001; Paul *et al*., 2002). These results suggest that Hsp27 can act at different levels to inhibit mitochondrial mediated apoptosis.

Inhibition of the Fas receptor mediated apoptosis

Hsp27 also inhibits apoptosis induced by activation of Fas cell surface death receptor (Fas R) (also called CD95) following binding of ligand, Fas-L (Mehlen *et al*., 1996b). The canonical pathway of activating apoptosis via Fas R activation, involves the recruitment of the Fas-associated death domain (FADD) adapter via the death domain motif and subsequent activation of caspase-8, which can then activate the

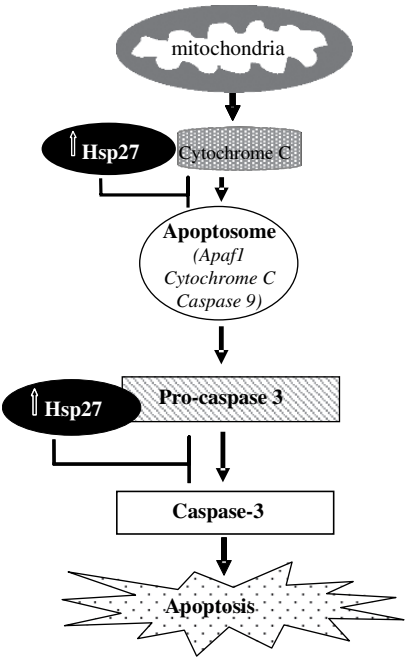

Figure 2A. Schematic representation to demonstrate the proposed mechanisms by which Hsp27 can protect cells from apoptosis via mitochondrial pathway when Hsp27 is proposed to (i) sequester cytochrome C and thus prevent apoptosome formation or (ii) by associating with and preventing activation of pro-caspase 3

downstream effector caspases such as caspase-3. It is possible that Hsp27 inhibits this pathway by its ability to interact with and inhibit pro-caspase 3 (Figure *2B*).

However, an alternative and independent pathway by which apoptosis can be induced by this pathway involves activation of the death-domain-associated protein (Daxx) by activated Fas R. Daxx was originally identified as a protein that binds to the death domain of Fas and potentiates Fas-induced apoptosis. Daxx seems to function as a pro-apoptotic protein by recruiting a mitogen-activated protein kinase kinase kinase (MAP3K), apoptosis signal-regulated kinase 1 (Ask1), which leads to the activation of c-Jun-N terminal kinase (SAPK1/JNK) pathway in a FADD-independent manner (Yang *et al*., 1997). Hsp27 can directly interact with Daxx and prevent its association with Fas R and Ask1, thus inhibiting apoptosis (Figure *2B*). This interaction is phosphorylation dependent since only smaller dimers of phosphorylated Hsp27 can associate with and inhibit Daxx (Charette *et al*., 2000; Charette and Landry, 2000). Immuno-cytochemical studies suggest that in order to induce apoptosis, Daxx translocates from the nucleus to the cytoplasm. It is possible that interaction of Hsp27 with Daxx protein prevents its translocation and may provide the mechanism by which Hsp27 can inhibit the apoptotic effects of Daxx (Charette and Landry, 2000).

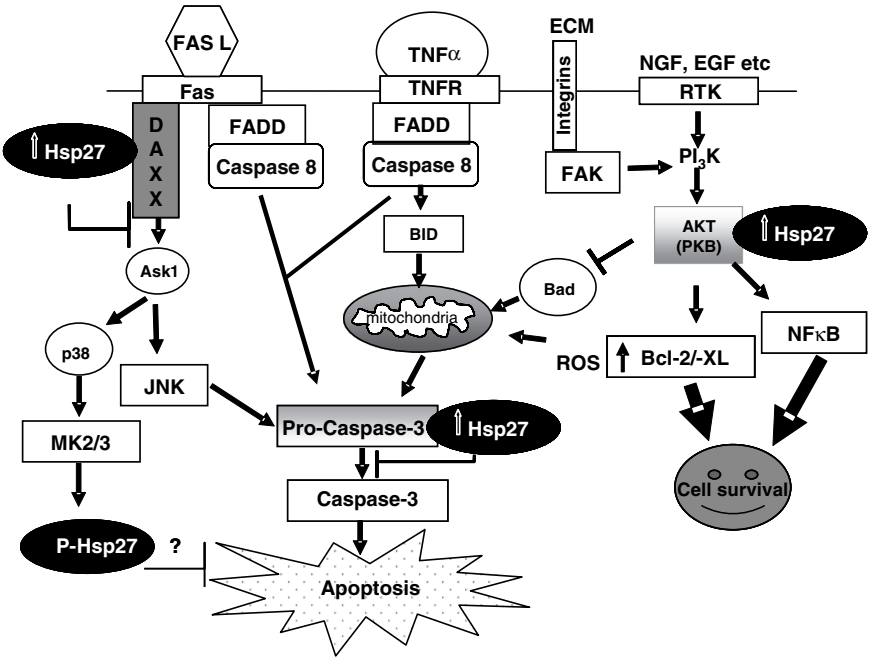

Figure 2B. Schematic representation to demonstrate the proposed mechanisms by which Hsp27 can protect cells from apoptosis via receptor mediated (FasR/TNFR) pathway by (i) interacting and inhibiting DAXX mediated activation of this pathway or (ii) associating with Akt and promoting survival. The receptor pathway can be mitochondria-dependent or independent

Akt activation

The regulation of Hsp27 function by phosphorylation has been widely studied and will be discussed in more detail later. However, interestingly, Hsp27 also seems to interact with and modify the effects of components of signalling pathways that converge on it (Figure *2B*). Thus, the unphosphorylated form of Hsp27 can directly associate with Akt (Mearow *et al*., 2002; Rane *et al*., 2003; Tanabe *et al*., 2005) and with p38 MAPK kinase (Zheng *et al*., 2006). The association of p38 with the Hsp27 complex appears to be dependent on its interaction with the upstream Hsp27 kinase MAPKAPK2 (MK2). MK2 shuttles p38 from the nucleus to the cytoplasm and is present with p38 within the Hsp27-Akt complex. p38 phosphorylates and activates MK2 which in turn phosphorylates Hsp27 in the canonical p38>MK2>Hsp27 pathway which is activated downstream of MKK3/6 in response to stress or pro-inflammatory cytokines such as TNFα. However, activation of this pathway results in release of both p38 and phosphorylated Hsp27 from the complex (Zheng *et al*., 2006) thus suggesting a feedback loop that controls the effects of Hsp27 in the cells. How this interaction alters survival is still not fully determined but Akt is an important survival pathway in many types of cancer cell and is an important therapeutic target. It is not clear, however, whether the pro-survival

effects of Akt activation are in any way related to the release of phosphorylated Hsp27 monomers/dimers from the complex or due to other ascribed functions of Akt activation, such as phosphorylation of Bad (Sen *et al*., 2003) or induction of Bcl-2/Bcl-X_L expression (Bai *et al*., 2005; van Nimwegen *et al*., 2006).

Modulation of Intracellular Redox Potential

Hsp27 also protects cells against the effects of accumulated reactive oxygen species (ROS) which are often found at high levels in cancer cells (see Figure *2C*) (Loft and Poulsen, 1996; Behrend *et al*., 2003). Reactive oxygen species (ROS) are highly reactive molecules that may accumulate as a consequence of alterations in metabolism and mitochondrial dysfunction, but could also arise as a result of changes in the cellular environment e.g. in response to hypoxia or exposure to tumour necrosis factor (TNF-α) [for review see (Waris and Ahsan, 2006)]. In cancer cells, ROS can also accumulate in response to treatments such as radio or chemotherapy, often used to kill tumour cells.

Although low levels of ROS can act in a signalling capacity (Nohl *et al*., 2003), significantly elevated levels can result in a stress response. This is because the highly reactive nature of ROS can cause damage to critical cellular components such as DNA, lipids and proteins. The effects of ROS depend on the nature and degree of damage. For instance, DNA damage caused by high levels of ROS is thought to contribute to the initiation or progression of carcinogenesis if the alterations occur on sequences of genes that encode for key regulatory proteins since this can result in the production of mutant proteins with altered function (Malins *et al*., 1996). An example of the importance of such changes in tumourigenesis can be demonstrated by findings that the tumour suppressor protein, p53 is mutated in >50% of cancers

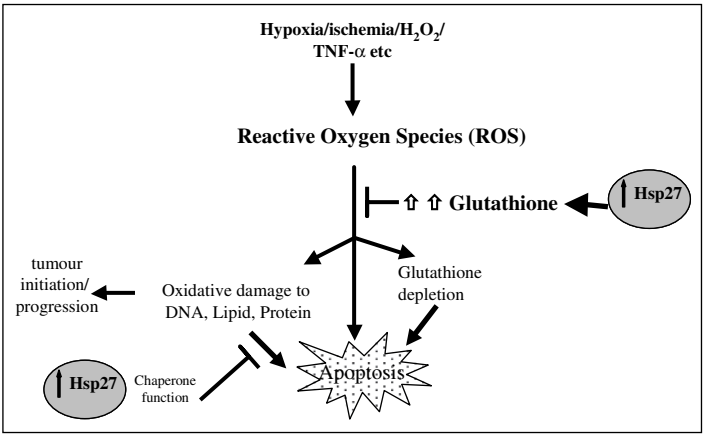

Figure 2C. Hsp27 can also protect cells from accumulation and damage caused by reactive oxygen species (ROS) by increasing the intracellular levels of glutathione and/or by preventing aggregation of damaged protein

and many p53 mutants cannot induce apoptosis or exercise checkpoint controls seen with the wild type protein. Therefore these mutants confer growth advantages to the cells [reviewed in (Schmitt *et al*., 2002)]. Changes within the regulatory promoter or enhancer DNA that controls the expression of specific proteins can also lead to altered gene expression (e.g. over-expression of oncogenes and/or repression of tumour suppressor genes) that contribute to initiation or progression of tumours (Wei, 1992). However, accumulated ROS can also trigger an apoptotic response by different mechanisms. For instance, damage to proteins by elevated ROS can cause mis-folded or damaged proteins to aggregate and trigger the stress response (Arrigo, 2001).

Induction of Hsp27 under these conditions can protect cells from the effects of ROS by increasing glutathione levels, which functions primarily to regulate the detoxification of ROS (see Figure *2C*) (Mehlen *et al*., 1995a; Mehlen *et al*., 1996a). In this capacity, Hsp27 has also been shown to protect cells from TNF-α induced apoptosis and from oxidative stress resulting from increased hydrogen peroxide (H_2O_2) or menadoine (Mehlen *et al*., 1995a; Mehlen *et al*., 1995b; Arrigo, 2001). The ability of Hsp27 to modulate glutathione activity requires the presence of the larger oligomers of Hsp27, which favour its chaperoning ability (Mehlen *et al*., 1995a). It has also been proposed that Hsp27, in this form, can also help to present oxidized proteins to the ubiquitin-independent 20S proteasome and thus prevent large aggregates of damaged proteins that can form lipofuscin that are damaging to the cell (Arrigo, 2001; Terman and Brunk, 2004).

CONDITIONS THAT COULD INCREASE HSP27 IN BREAST CANCER CELLS

Whilst Hsp27 can be induced in response to stress/heat shock, this pathway is not necessarily the mechanism by which it is elevated in breast cancer cells. In breast cancer, increased Hsp27 expression is sustained rather than the transient increases seen in the stress response. Previous studies showed that the hormone, estradiol, which is a key player in a significant percentage of breast tumours, could increase Hsp27 expression but it is not the only factor that contributes to increased sHsp in breast cancer cells. Whilst the precise mechanisms that lead to increased Hsp27 expression in breast cancer cells during either development or progression of this disease are still to be fully elucidated, it is clear that during the complex processes that underlie tumourigenesis, tumour cells are subjected to many stresses/conditions that are known to trigger the stress responses. We could speculate on conditions that might lead to the increase in Hsp27 in breast cancers (outlined below) but many of these remain to be clearly demonstrated.

For instance, genetic mutations that are common in cancer cells often result in the production of mutant proteins that may fail to fold appropriately or that demonstrated aberrant function. Accumulation or aggregations of mis-folded proteins are classic inducers of the stress response that results in increased expression of Hsps. This is exemplified by the association of Hsps with mutant tumour suppressor p53 protein, which is mutated in >50% of cancers. Many p53 mutant proteins fail to

fold appropriately and have been detected in complex with Hsp70 (Pinhasi-Kimhi *et al*., 1986). More recently, Hsp27 was also found in complex with mutant p53 and Hsp70 in squamous cell carcinoma cell line, A431 (Kindas-Mugge *et al*., 2002). Although the biological significance of complexes between different Hsps and p53 remains to be elucidated, it is thought that these Hsps act in their capacity as molecular chaperones to regulate p53 conformation.

Changes in cellular metabolism (e.g. in response to hypoxia and ischemia) that result in conditions such as low glucose, pH and oxygen, can also trigger the induction of Hsps in many cells [reviewed in (Ciocca *et al*., 1993; Ferns *et al*., 2006)]. Hypoxic and ischemic conditions can arise within the tumour environment as a result of inadequate angiogenesis or microvasculature that is required to support solid tumours, such as breast carcinomas, particularly when they expand rapidly. Such stresses can induce the accumulation of reactive oxygen species (ROS) but can also cause extensive damage to the cytoskeleton e.g. collapse of the intermediate filaments into large perinuclear aggregates, re-localisation of actin-containing fibres around the nucleus and disruption of the microtubules. Native Hsp27 can protect cells from such effects by stabilizing actin and microtubules (Lavoie *et al*., 1993; Benndorf *et al*., 1994; Lavoie *et al*., 1995; Loktionova and Kabakov, 1998).

REGULATION OF HSP27 EXPRESION AND FUNCTION IN BREAST CANCER CELLS

Since elevated Hsp27 is associated with a more aggressive subset of breast cancers that are resistant to treatment and thus have a poor outcome, there is considerable interest in understanding how levels of this small Hsp are regulated in breast cancer cells. In order to do this, it is essential to look at the molecular mechanisms that control the expression and function of this protein in breast cancer cells.

Hsp27 is regulated at the level of transcription, when factors that stimulate its expression converge to activate its promoter thus increasing the level of mRNA encoding this protein. Crucially, Hsp27 is also highly regulated post-transcriptionally by phosphorylation at specific residues of the protein. These changes, which modify its quaternary organization and hence change its functions, will be discussed later.

TRANSCRIPTIONAL REGULATION OF HSP27 EXPRESION

Both transcriptional and post-transcriptional changes in Hsp27 are influenced by the cell type and growth conditions. For instance, the signals that lead to the induction of Hsp27 expression following heat stress are distinct from those that stimulate its increased expression in tumour cells. Thus, following classic heat shock responses, there is a transient increase in the transcription of genes encoding for Hsps, which result in elevated expression and this depends on the action of specialized transcription factors termed, Heat Shock Factors (HSFs) (see later) (Hightower, 1991; Sorger, 1991; Morimoto, 1993). However, in breast cancer

cells, it is unlikely that this mode of transient expression observed following the classical stress responses will be responsible for the elevated and sustained levels of Hsp27 expression. It is evident that high levels of Hsp27 is achieved by a number of other transcriptional regulators which can also alter the growth and behaviour of breast cancer cells. In this regard, a number of transcriptional regulators have been identified as key players in influencing the levels of Hsp27 in cancer cells. The mechanisms by which these achieve their effects will be discussed below.

Regulatory Elements in Hsp27 Promoter

To identify the factors that contribute to increased transcription of Hsp27 mRNA and to understand their mechanism of action, it is important to analyze the regulatory promoter region that controls the expression of the human Hsp27 gene [for review of gene regulation see (Latchman, 2002a). The gene promoter is the region of DNA that facilitates the formation of the transcriptional initiation complex (TIC), which is required for RNA polymerase II (RNA pol II) to transcribe the gene. Since RNA pol II cannot bind to DNA, it is recruited to the promoter by its association with general transcription factors that form the TIC. Thus, the TIC comprises of a large complex of proteins that include a number of general transcription factors that are ubiquitously expressed. Many of these transcription factors recognise and bind to specific DNA sequences found in the gene promoter and play a critical role in the assembly of a competent TIC. For instance, the general TF, TFIID, contain the TATA binding protein that interacts with TA rich 'TATA box' in specific promoters and this initiates the assembly of the TIC and recruitment of the RNA pol II (TFIID) to the promoter (Latchman, 2002a).

Whereas the general transcription factors are ubiquitously expressed in all cells, the expression of genes in a cell-restricted or signal-specific manner requires the interaction of transcription factors (TF) that are expressed in a more restricted manner and so are only found in particular cells or under specific conditions. An example of a signal specific TF is the Heat Shock Factor-1 (HSF-1) which is activated in response to heat stress and can induce increased expression of Hsp27 under these conditions (Sorger, 1991). In contrast, hormone induced Hsp27 expression in response to estrogen is stimulated via the estrogen receptor, ER-α. Cell or signal specific transcription factors enhance transcription of the specific gene by binding to specific sites in the gene promoter and making contact with components in the TIC. Thus, the presence of DNA recognition sites for specific transcription factors in the promoter of a gene can highlight the transcription factors that could regulate the levels of that particular gene in a specific cells or under defined conditions (Latchman, 2002a).

Hickey and collegues (1986) (Hickey *et al*., 1986) were the first group to identify 5' sequences immediately upstream of the Hsp27 gene, which contained regulatory sequences including the transcriptional start site and heat shock elements (HSE). Subsequent studies by Oesterreich et al. (1996) (Oesterreich *et al*., 1996a) analyzed

a larger upstream region of the promoter (~1 kb) which contained many putative binding sites that could be recognized by a number of different transcriptional regulators. These include the HSE, recognized by HSFs (GAAnnnTTC at position −177 to −184), GC rich Sp1-binding sequences (GGGCGG at −102 to −107 and at position −145 and −153), CAAT box elements (GCAAT at −1048 and CCAAT at −372) and AP2 sequences at −145 and −153, which partially overlap with Sp1. There are also two palindromic sequences that resemble estrogen responsive element (ERE) half sites that are located at −921 (GGTCT-8bp spacer- TGACC) and at −68 (GGTCA- 13bp spacer- AGACC). Interestingly an AT rich sequence found within the 13 bp spacer of the ERE at position −68 is recognized by the Brn-3a and Brn-3b transcription factors and by the repressor, HET/SAF-B (see Figure 3). These proteins interact with the estrogen receptor (ER) and can modulate its transcriptional effects on the Hsp27 promoter and the implication of such regulation is discussed below. Interestingly, many of these regulatory regions are highly conserved between murine Hsp25 and human Hsp27, suggesting that these sequences are important for controlling the expression of this gene (Merck *et al*., 1993b).

The location of many of these regulatory elements were found within a short region of the promoter. Oesterreich *et al*., (1996a) used deletion constructs which contained different truncations of this regulatory region to show that the proximal 200 bp sequences within the Hsp27 promoter was sufficient to drive basal transcription of Hsp27 gene as well as cell specific enhanced transcription in breast cancer cells (Oesterreich *et al*., 1996a) (see Figure 3). Thus, this region contains functional regulatory domains that include the putative transcription initiation TATA box sequence, which facilitates the assembly of the transcriptional initiation complex. Although two A/T rich sequences were initially identified (Hickey et al), the sequences at position −25 represent the major transcriptional start site whereas the A/T sequence at position −75 position is recognised and bound by other transcription factors such as Brn-3b, Brn-3a and HET/ SAF-B (see also Figure 3) (Oesterreich *et al*., 1997; Farooqui-Kabir *et al*., 2004; Lee *et al*., 2005).

The TATA element together with the Sp1 elements is sufficient to drive basal level of transcription from Hsp promoter (Greene *et al*., 1987). However, the presence of other transcription factor binding sites identifies a number of other regulators that can modulate the activation of the promoter to either increase or reduce the rate of gene transcription. The relevance of these different regulators relates to the cell type and/or incoming signals that stimulate gene expression in distinct cells and implicate a complex mechanism for the control of Hsp 27 in different tissues or in response to different signals.

For instance, the heat shock element (HSE) is critical for responses to heat shock in many cell types and it is recognised by heat shock transcription factors (HSFs) which are induced and activated in response to this stress (Morimoto, 1993). The HSF1 transcription factor has been shown to be the primary mediator of the heat shock response in many cells and HSF-1 has been shown to be critical for the heat-inducible synthesis of Hsps (Pelham, 1982). Other family members, HSF2 and 4 have developmental roles. When activated, HSF-1 transcription factors

Proximal HSP27 promoter:

-211

HSE

GAATT CATTT GCTTT TCCTT AACGA GAGAA GGTTC CAGAT GAGGG CTGAA CCCTC TTCGG CCCGC CCACG GCCCC

SP1 ½ ERE Brn-3 ½ ERE

TGAAC GCTGG GGGAG GAGTG CATGG GGAGG GGCGG CCCTC AAACG *GGTCA* TTGC*C ATTAAT A*G*AGA CC*TCA AACAC

TATA

CGCCT GCATA AAATA CCCGA CTGGA GGAGC *ATAAA A*GCGC AGCCG AGCC -10

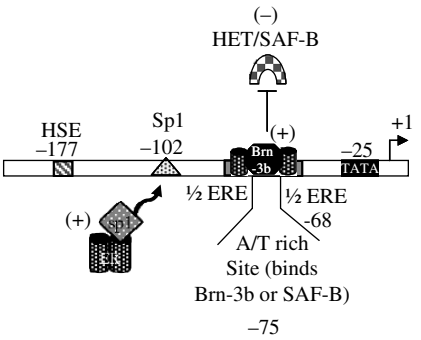

Figure 3. (A) Sequence of the proximal Hsp27 promoter highlighting the location of binding sites for different transcription factors in relation to the start site (+1); TATA- box, ERE (estrogen response element), Brn-3 site, Sp 1 and HSE (Heat Shock Element). (B) Schematic model to demonstrate how maximal transcription can be achieved on the proximal Hsp27 promoter in breast cancer cell. The estrogen receptor (ER) can bind to the two 1/2 ERE binding site. The A/T rich sequence is the between these two sites can be bound by the transactivator, Brn-3b which interacts with and enhances ER mediated transcription and appears to be important for maximal stimulation of this promoter. This site can also be bound by the repressor protein, HET/SAF-B which represses ER activation upon binding. Therefore the relative ratio of Brn-3b to HET/SAF-B protein is likely to be importantly in determining the effect of the ER on activating the Hsp27 promoter in breast cancer cells. The location of the Spl site is shown and binding of Spl to this site can recruit ER to the promoter without it binding to the ERE, indicating another mechanism for controlling Hsp27 expression. The position of the Heat shock element (HSE) is shown also. (+) indicated the enhanced activation of promoter activity by association of activator proteins with ER whereas (−) indicates a repressor that can prevent promoter activation

undergo conformational changes that result in the formation of trimeric complexes, which can translocate to the nucleus and bind to HSE DNA recognition sites found in the promoters of Hsps [reviewed in (Pirkkala *et al*., 2001; Tonkiss and Calderwood, 2005)]. In this capacity, HSFs can increase expression via the HSE found in the promoters of stress responsive genes. However, the Hsp27 proximal promoter contains a single HSE and is thus not as responsive to heat stress as although Hsp family members, such as Hsp70s, which contained several tandem HSE repeat in the promoter(s).

Regulation of Hsp27 Expression by Estrogen and ER

In consideration of the up-regulation of Hsp27 in breast cancer cells, it is noteworthy to recall that Hsp27 was identified as an estrogen responsive gene (Oesterreich *et al*., 1996) and that there is a striking correlation between the elevated levels of

this protein in breast tumours with ER positivity (see for instance, Lee *et al.*, 2005; Fanelli *et al*., 1998; Damstrup *et al*., 1992). These findings had suggested that the hormone, 17β-estradiol, (E-2) can stimulate the expression of Hsp27 and this was indeed shown to be the case since treatment of breast cancer cells with 10nM of E-2 was sufficient to increase Hsp27 mRNA levels (Adams *et al*., 1980; Ciocca *et al*., 1983; Dunn *et al*., 1993; Porter *et al*., 1996; Porter *et al*., 2001) (Edwards *et al*., 1981; Moretti-Rojas *et al*., 1988; Fuqua *et al*., 1989). E-2 classically acts to regulate the expression of responsive genes via estrogen receptors (ER) which recognises and binds to specific DNA sequences referred to as estrogen responsive elements (EREs) found in target gene promoters. The presence of two distinct ERE motifs within the Hsp27 gene promoter confirms that activation via ER is relevant (see Figure 3 also).

Two distinct isoforms of ER (ER-α and ER-β have been identified (Klinge, 2001; Hall *et al*., 2001; Sommer and Fuqua, 2001). However, a number of studies indicate that the ER-α isoform is over-expressed in breast cancer cells and is the isoform that is associated with changes in Hsp27 expression (Hall *et al*., 2001; Sommer and Fuqua, 2001). Therefore, reference to ER in the discussions below will relate specifically to studies undertaken with ER-α.

The canonical pathway for activation of the estrogen receptor (ER) occurs upon binding of the hormone, 17β-estradiol, to the ligand binding domain (LBD) of the receptor. This induces a conformational change in the receptor and causes displacement of inhibitory proteins that interact with the ER when it is inactive [for review see (Parker *et al*., 1993; Parker, 1998)]. Thus, in its inactive state, the ER is found in complex with a large number of inhibitory proteins such as HET/SAF-B (Hsp27ERETATA binding/protein/scaffold attachment factor), SMRT (silencing mediator of retinoid and thyroid receptors), RIP-140 (receptor-interacting protein-140), SHP (protein-tyrosine phosphatase), Sin3a (histone deacetylase complex subunit), DAX-1 (dosage-sensitive sex reversal/adrenal hypoplasia congenita critical region on the X chromosome), NCoR (nuclear receptor co-repressor) and larger Hsps, e.g. Hsp90. These repressors prevent gene transcription by the receptor, partly by sequestering it in the cytoplasm. However, following ligand binding, the receptor undergoes conformational changes that facilitate dissociation of the repressor proteins and receptor dimerization, a prerequisite for this receptor to bind DNA. The active ER also now associates with co-activators, such as SRC-1 (steroid receptor coactivator-1), NCoA(nuclear receptor co-activator), PCAF (p300/CBP-associated factor), CBP/p300 and POU transcription factor, Brn-3b, which enhances ER mediated transcription of its target genes.

Thus, the proteins that are co-expressed with ER in breast cancer cells will be critical for controlling the effect of this receptor on transactivation of target genes such as Hsp27 (McDonnell and Norris, 2002; Hart and Davie, 2002).

Regulation of Hsp27 Promoter Activity by Transcription Factors Brn-3b and HET/SAF1 upon Interaction with ER

The ability of ER to regulate Hsp27 at the transcriptional level is significantly modulated by other cellular transcription factors that are co-expressed with it in

specific cells. Such effects have been demonstrated for two cellular proteins, Brn-3b (Budhram-Mahadeo and Latchman, 2006) and SAF1/HET (Oesterreich *et al*., 1997), and because of their relevance to breast cancers, these will be described below.

The Brn-3b is a POU domain transcription factor (for review see Budhram-Mahadeo and Latchman, 2006) that binds directly to ER-α and this interaction occurs via the DNA binding domain (DBD) of the ER (Budhram-Mahadeo *et al*., 1998). Upon interaction, Brn-3b significantly increases transcription by ER on an ERE containing promoter in breast cancer cells. These results indicated that the Brn-3b transcription factor was a co-activator of ER that could modulate its effects in breast cancer cells.

It is noteworthy that Brn-3b is significantly elevated in >60% of breast cancers and when over-expressed in the ER positive breast cancer cell line, MCF7, it was sufficient to alter the growth and behaviour of these cells (Dennis *et al*., 2001; Budhram-Mahadeo and Latchman, 2006). Thus, stable over-expression of Brn-3b in MCF7 cells resulted in significantly increased in-vitro proliferation (in a monolayer) and anchorage independent growth (AIG). Significantly, high Brn-3b increased the number as well as the size of colonies formed in soft agar (AIG) compared with control cells (Dennis *et al*., 2001). Elevated Brn-3b also enhanced tumour growth *in-vivo* in xenograft models using another tumour derived cell line whilst decreased Brn-3b levels resulted in slower growing tumours in parallel studies (Irshad *et al*., 2004). In contrast, loss of Brn-3b using an antisense approach resulted in much slower growth in a monolayer as well as failure to form colonies in soft agar. These are all hallmarks of transformed cells suggesting that Brn-3b plays a critical role in controlling key processes that affect the fate of these cells. In addition, elevated Brn-3b confers resistance to anti-proliferative agents such as retinoic acid and increases the invasiveness of tumour cells, an effect that parallels the resistance conferred by high Hsp27 (Irshad *et al*., 2004). These studies suggest that Brn-3b may act at different levels to control genes associated with distinct cellular processes. Indeed this was shown to be the case since Brn-3b can activate genes which regulate cell proliferation, such as cyclin D1 and CDK4 which control G1 cell cycle progression (Budhram-Mahadeo *et al*., 2007; Samady *et al*., 2004) whilst it represses the gene promoter that controls the tumour suppressor, BRCA1 gene (Budhram-Mahadeo *et al*., 1999). Accordingly, tumours with high Brn-3b levels were found to express high cyclin D1 and CDK4 but low BRCA1 (Budhram-Mahadeo *et al*., 2007; Samady *et al*., 2004; Budhram-Mahadeo *et al*., 1999). In terms of its ability to confer resistance to some drugs, Brn-3b effects may be mediated by its ability to upregulate Hsp27 expression, since Hsp27 was identified as a Brn-3b target gene in breast cancer cells (Lee *et al*., 2005).

Interestingly, MCF7 cells over-expressing the Brn-3b protein showed significantly elevated Hsp27 protein levels whereas loss of Brn-3b resulted in decrease in this Hsp protein (Lee *et al*., 2005). This effect was unique to Hsp27 since Hsp70 and hsp90 remained unchanged under these conditions (Figure 4). Results of an analysis to look at the expression of Hsp27 in tumours that expressed different

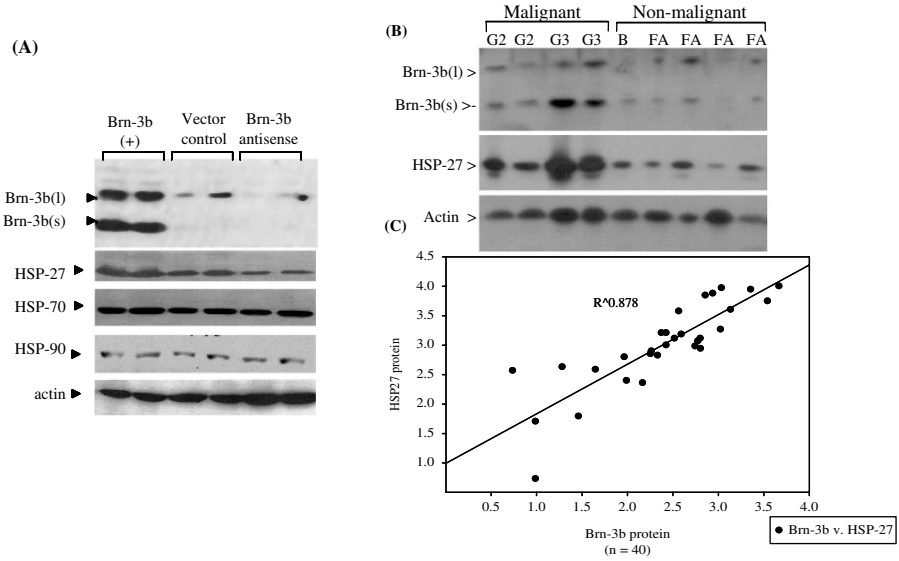

Figure 4. Correlation of levels of Brn-3b transcription factor with Hsp27 proteins in (A) MCF7 breast cancer cell line with high Brn-3b (Brn-3b(+) or reduced Brn-3b (Brn-3b antisense) compared with control cells (vector control). Hsp70 and Hsp90 are unchanged suggesting that Brn-3b specifically regulate Hsp27 expression. (B) shows that elevated levels of Brn-3b in breast cancer biopsies taken from grades 2 or 3 (G2 or G3) correlated well with increased Hsp27 protein compared with lower levels in non-malignant fibroadenomas (FA). (C) shows the significant correlation (R −0.88) following analysis of biopsies taken from 40 breast cancer patients

levels of Brn-3b protein demonstrated a significant and striking correlation between Brn-3b expression and Hsp27 (R = 0.879) in breast cancer biopsies (Figure 4) (Lee *et al*., 2005) and suggests that expression of Hsp27 maybe regulated by Brn-3b directly and/or indirectly upon interaction with ER.

This was tested in co-transfection assays, which demonstrated that increasing Brn-3b protein levels resulted in transactivation of the Hsp27 promoter, by 10–12 fold compared with controls (Lee *et al*., 2005). This was comparable to the effects of the ER alone which resulted in ∼15–20 fold induction of this promoter (Figure 5). However, co-expression of Brn-3b with ER resulted in an additive effect, causing ∼35–40 fold induction of this promoter (Figure 5) (Lee *et al*., 2005). This is particularly relevant since these two proteins are known to physically interact with each other to modulate transcription (Budhram-Mahadeo *et al*., 1998). In view of the cooperation between Brn-3b and ER to enhance Hsp27 promoter activity, it is interesting to note that RNA interference (RNAi) which specifically targeted Brn-3b mRNA could block transactivation of Hsp27 promoter by Brn-3b, but also reduce the transcriptional activity by the ER alone (suggesting that loss of endogenous Brn-3b can alter its effect) or the cooperation upon co-expression with Brn-3b (Figure 5) (Lee *et al*., 2005). Such results suggested that Brn-3b proteins

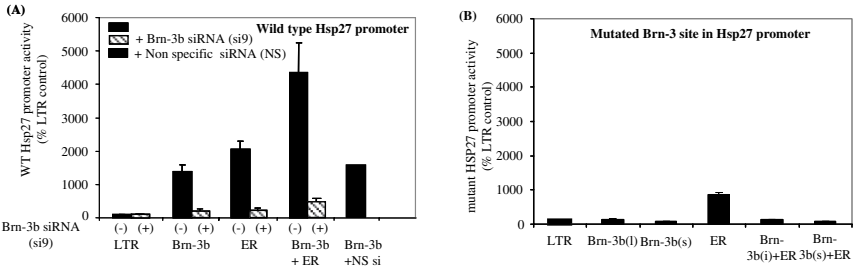

Figure 5. Reporter assays to show that Brn-3b is required for transactivation of Hsp27 promoter in MCF7 breast cancer cells. (A) Co-transfection was carried out using a Hsp27 promoter reporter construct (in which this promoter drives expression of the luciferase gene) and expression vectors for either Brn-3b transcription factor or the estrogen receptor (ER) alone or both together. These studies were carried out in the sbsence (−) or presemce (+) of short interfering RNA (siRNA) that specifically targeted Brn-3b(si9) and a non-specific siRNA (NS) was used to show that the effects were specifically related to losing Brn-3b

Results show that the promoter is strongly transactivated by Brn-3b alone (∼10–15 fold) or ER lone ((∼20 fold) compared with empty vector control (LTR). However, when co-transfected together, Brn-3b and ER cooperate to significantly enhance Hsp27 promoter activation. Decreasing Brn-3b by targeting with Si9 resulted in reduction of Brn-3b mediated effects but also reduced activation by ER. The non-specific siRNA (NS) did not affect the ability of Brn-3b to activate the promoter

(B) Similar co-transfections studies were undertaken using the Hsp27 promoter in which only the Brn-3b site is mutated. This promoter failed to be transactivated by Brn-3b but also showed attenuated activation by the ER (10 fold compared with ∼20 fold seen on the wild type promoter)

could regulate transcription of the Hsp27 promoter, directly and indirectly upon interaction with ER.

Chromatin immunoprecipitation assay demonstrated that Brn-3b does bind to the Hsp27 promoter *in-vivo* whilst electrophoretic mobility shift assays confirmed that Brn-3b binds to the A/T rich sequence, found between the two half ERE sites (at position −75) and failed to bind if this site was mutated (Lee *et al*., 2005). Furthermore, by mutating this site in an otherwise intact promoter (by site-directed mutagenesis) prevented transactivation of the promoter by Brn-3b protein although the basal promoter activity was unchanged. A critical finding in this study also revealed that if the Brn-3 site was lost in the Hsp27 promoter, then the effect of ER was attenuated (Figure 5B) (Lee *et al*., 2005). This decreased transactivation by the ER in the absence of Brn-3b, suggests that interaction of Brn-3b with the ER may prevent the receptor binding to the ERE and transactivating the promoter. This observation taken together with previous results using RNAi suggests that endogenous Brn-3b expressed in breast cancer cells, participates in stimulating ER mediated activation of Hsp27 promoter.

These findings are particularly interesting because Oesterrich et al. (1997) (Oesterreich *et al*., 1997) isolated the protein termed HET (Hsp27ERETATA binding) that could bind to this A/T rich sequence in the Hsp27 promoter. This protein demonstrated homology to the matrix/scaffold binding protein, SAF-B, which plays an important structural role and participates in the organization of

DNA within the nucleus. Similar to Brn-3b, HET/SAF-B also interacts with ER *in-vitro* and in-vivo and binds the ER DBD as well as the hinge region (Oesterreich *et al*., 2000). HET/SAF-B and ER can interact in the presence or absence of estradiol, but binding appeared to be increased in the presence of the estrogen antagonist, tamoxifen. Binding of HET/SAF-B to ER represses the effects of this receptor on transactivation so this protein is considered a co-repressor. The ability of HET/SAF-B to repress ER activity is dependent upon its interaction with the ER-DBD, which is also required for the association of the transactivator, Brn-3b, to the ER.

It is therefore interesting that Brn-3b appears to be necessary for maximal stimulation of the Hsp27 promoter by ER particularly in light of the fact that both Brn-3b and HET/SAF-B bind to the AT rich site between the two ½ EREs in the proximal Hsp27 promoter (see Figure 3). Since HET/SAF-B is a repressor of this receptor, it is possible that when Brn3b is bound to this site, its interaction with the ER facilitates the strong binding of the ER complex to the promoter and enhances transcriptional activation. Since Brn-3b can bind to ER, off as well as on DNA, mutation of the Brn-3 site will result in loss of Brn-3b binding to this site but will not affect its interaction with ER and remove it from the ERE in the promoter. This will compromise ER complex formation on the promoter with consequent loss of transactivation. However, it is also possible that the binding of Brn-3b to this site prevents the binding of HET/SAF-B repressor protein, which also recognizes and binds to this sequence (Figure 3B). Loss of Brn-3b binding to this site may facilitate the binding of HET factor, which can then interact with and inhibit ER mediated transcription. Irrespective, of which of the hypotheses applies to these cells, it is evident that the balance of proteins such as Brn-3b or HET/ SAF-B, which are co-expressed with the ER in breast cells, will serve to regulate the transcriptional effects on target genes such as Hsp27, which in turn, can act to modify cellular behaviour.

It is interesting that many of the effects of over-expressing Hsp27 in these cells were also observed when Brn-3b protein is increased. Thus independent studies have shown that increasing levels Hsp27 result in increased anchorage independent colony formation (Lemieux *et al*., 1997) similar to the effect seen upon over-expression of Brn-3b (Dennis *et al*., 2001) whilst the ability of Hsp27 to confer resistance to chemotherapeutic drugs (Whelan and Hill, 1993; Mehlen *et al*., 1995c; Garrido *et al*., 1997; Mairesse *et al*., 1998; Hansen *et al*., 1999) in cancer cells is paralleled by observation that high levels of Brn-3b protein in neuroblastoma cells results in resistance to growth inhibitory stimuli (Irshad *et al*., 2004). Both Brn-3b and Hsp27 also increase the invasiveness of cancer cells (Irshad *et al*., 2004; Lemieux *et al*., 1997). Thus, many of the effects associated with high Hsp27 are also seen with elevated Brn-3b suggesting a similar pathway by which the cells are affected. The correlation of Brn-3b with levels of Hsp27 in breast cancer cell lines and breast biopsies together with the transcriptional activation of the Hsp27 promoter by Brn-3b either alone or when co-expressed or interacting with ER would suggest that the regulation of Hsp27 by Brn-3b is important in determining

the downstream effects observed in cancer cells. Its regulation of Hsp27 may thus represent an important mechanism by which this transcription factor may contribute to changes observed in breast cancer cells.

Interaction of ER with Sp1 on the Hsp27 Promoter

The regulation of gene expression by ER, upon association with other cellular proteins, is quite complex since it can also mediate its effects independently of its own binding site, the ERE, when the receptor is recruited to gene promoters by its association with other transcription factors. This has been demonstrated with the Sp1 transcription factor and AP1 transcription complex (fos/jun heterodimers) which can interact with the ER and modulate transcriptional regulation of target genes (Porter *et al*., 1997; Safe, 2001). The effects of such interactions are dependent on the cell type and specific gene promoter. For example, upon interaction of Sp1 with ER, these proteins are recruited to GC rich Sp1 sites where they can regulate gene expression. The presence of Sp1 sites in the proximal Hsp27 promoter (see Figure 3B) suggests that this is likely to provide additional control of Hsp-27 expression (Porter *et al*., 1996; Oesterreich *et al*., 1996a; Porter *et al*., 1997).

The Sp1 transcription factor is a ubiquitously expressed gene and so is also found in breast epithelium. It regulates the expression of many target genes that control cell growth or are involved in key developmental processes in many tissues (Black *et al*., 2001) (black et al 2001) since mice lacking this protein display gross morphological defects very early in development (Marin *et al*., 1997) (marin 1997 – cell). The interaction of Sp1 with ER was demonstrated in live MCF7 cells by Kim et al (2005) (Kim *et al*., 2005) using fluorescence resonance energy transfer (FRET). This interaction required the C-terminal DBD of Sp1 and occurred in the presence of the hormone E2 but interestingly, can also occur in the presence of hormone antagonists, 4-hydroxytamoxifen (4-OHT), and ICI 182. Further analysis of the Hsp27 promoter region showed that sequences between -105 to -84 (containing Spl and two half ERE sites) were sufficient to facilitate binding of both proteins in complex in electrophoretic gel mobility shift assay (Porter *et al*., 1997; Porter *et al*., 2001). Furthermore, a truncated promoter containing this region was sufficient for E2 to induce promoter activition by 2-3 fold.

Interestingly, a number of studies showed that although the Hsp27 promoter is activated by the hormone, E2, the anti-estrogen, ICI164 384, appears to induce Hsp27 mRNA expression by ~2.5 fold compared with the 3.2 fold induction by E2 (Porter *et al*., 2001). In this study, it was proposed that the association of the ER with Sp1 on the GC rich Sp1 site was required for these effects by both estrogens and anti-estrogens and may contribute to responses to anti-hormone treatment in some patients (Porter *et al*., 2001). Similar interactions have been described between AP1 (Fos/Jun) and ER, but the relevance in terms of Hsp27 remains to be determined.

These different studies demonstrate that the control of Hsp27 transcription in breast cancer cells is controlled by many different factors with the estrogen receptor

being a key player. However, a complex mechanism that involves other transcriptional regulators appears to be key in determining the effects of ER on this promoter, with the ER cofactor Brn-3b enhancing Hsp27 expression via the ERE/Brn-3 site, whereas Sp1/ER complexes can activate the promoter via the Sp1 site. However, the latter complex with Sp1 is responsive to estrogen hormone or its antagonists and thus may give rise to distinct effects e.g. in response to treatment.

Other Factors that Modify Hsp27 Expression in Breast Cancer Cells

Given that Hsp27 can give rise to diverse effects in different cells, it is not surprising that other important pathways seem to converge on this factor to control its expression. For instance, recent studies have shown a correlation of increased Stat-3 in breast cancer cell-lines with elevated Hsp27 levels whereas reduction of Stat3 using siRNA resulted in decreased Hsp27 (Song *et al*., 2004). Although there is evidence for a transcriptional increase of Hsp27 mRNA in the presence of high Stat-3, it is not clear whether this transcription factor has a direct effect in modulating the Hsp27 promoter. Furthermore, the Stat 3 associated increase in Hsp27 levels is also accompanied by increased phosphorylation at Ser-78, which alters the function of the protein (Song *et al*., 2004). Thus Stat-3 may provide an alternative mechanism for increasing both the levels and function of Hsp27. Stat 3 is a member of the signal transducers and activators of transcription (STAT) family of proteins (Zhong *et al*., 1994). Stat3 is elevated in many breast cancers and is activated in response to cytokines and growth factors (Darnell, Jr. *et al*., 1994; Schaefer *et al*., 1995). When activated by phosphorylation, Stat3 proteins dimerize and are translocated into the nucleus where they can transactivate target genes containing its binding site. Increased Stat-3 is associated with tumourigenesis as it stimulates cell proliferation, promotes angiogenesis and confers resistance to some chemotherapeutic agents. Interestingly, a physical association of Hsp27 with Stat-3 was also identified (Song *et al*., 2004) and taken together with the findings of Stat-3 regulating Hsp27 expression levels, these findings point to a complex mechanism for controlling gene expression in breast cancer cells. Whilst the functional significance of the association of Stat-3 with Hsp27 remains to be fully elucidated, its ability to control the expression of Hsp27 is interesting and suggests that the expression of this Hsp in breast cancers can be controlled via divergent pathways.

Thus the activity of the Hsp27 promoter and hence the level of Hsp27 expression is regulated by a complex number of factors and underlies the diverse functions that are regulated by this factor in breast cancer cells.

POST-TRANSCRIPTIONAL REGULATION OF HSP27

Although transcriptional regulation is important in controlling Hsp-27 levels and thus function in breast cancer cells, its diverse effects in cells are also profoundly altered by post-transcriptional modification (see Figure 6). In particular, the effects

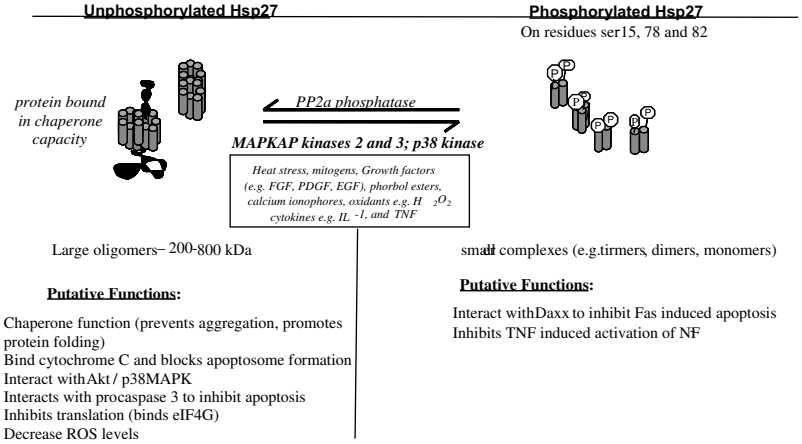

Figure 6. Summary of the changes on Hsp27 confered by different phosphorylation state. In the unphosphorylated states, the larger oligomers have distince functions compared with the smaller Hsp27 complexes formed upon phosphorylation and the proposed functions of the distinct complexes are shown. The signals that can give rise to the different states are also summarized

of phosphorylation on Hsp-27's ability to act in different capacities have been widely studied [reviewed in (Ciocca *et al*., 1993; Garrido, 2002)]. In its native, unphosphorylated form, Hsp27 form very large complexes that are very important for some modes of action (Rogalla *et al*., 1999; Garrido *et al*., 2003). This unphosphorylated form of Hsp27 confers protection against inhibitors of specific intracellular signalling pathways (e.g. pertussis toxin) and other cytotoxic agents perhaps by acting in its capacity as a molecular chaperone. Unphosphorylated Hsp27 is also thought to contribute to the inhibition of apoptosis by association with pro-caspases, since recombinant Hsp27 which is unable to form large oligomers cannot prevent pro-caspase-3 cleavage and activation by cytochrome c (Garrido *et al*., 2003).

Hsp27 undergoes rapid phosphorylation in response to a number of extra cellular factors such as heat shock; mitogen activated protein kinases (MAPKs); growth factors (e.g. FGF, PDGF, EGF), phorbol esters, calcium ionophores, agonists such as bradykinin, lymphotoxin, ATP and cytokines such as interleukin (IL)-1, and tumour necrosis factor (TNF)-α (Figure 6) [reviewed in (Ciocca *et al*., 1993)]. However, Hsp27 phosphorylation can also be induced in unstressed cells, in response to stimuli such as serum, mitogens, cytokines and differentiation signals that are relevant during development. Upon phosphorylation, the larger Hsp27 complexes dissociate to form smaller oligomers which demonstrate distinct functions (Rogalla et al. 1999) (Lavoie *et al*., 1993; Lavoie *et al*., 1995; Guay *et al*., 1997). For example, Hsp27 monomers inhibit actin polymerization and associate with Daxx to inhibit apoptosis. Phosphorylated Hsp27 also appears to enhance migration of cells on matrices such as laminin-5 and such effects would contribute to its ability to increase on migration and invasiveness of cancer cells (Rust *et al*., 1999).

Three main phosphorylation sites that have been characterized in human Hsp27 are located at serine residues (ser-15, 78 and 83) (Landry et al. 1992) and two in mouse Hsp25 (Ser-15 and 86) (Gaestel *et al*., 1991). Ser-15 is found in the conserved N-terminal domain whilst conserved site Ser-82/86 is found at the beginning of the second β-strand (Rogalla et al. 1999). These serine residues are phosphorylated by a variety of kinases including MAPKAPK-2 (Mitogen activated protein kinase – activated protein kinase 2 or MK2), MAPKAPK-3 (MK3) and PRAK. MK2 and MK3 are activated downstream of the stress activated protein kinase-2 (SAPK2), p38 (Rogalla *et al*. 1999; Huot et al. 1995; New et al. 1998). Phorbol esters can also induce Hsp27 phosphorylation and this is though to act via the delta isoform of protein kinase C (Regazzi *et al*., 1988). The activating transcription factor 3 (ATF3) and c-Jun can induce phosphorylation and increase transcription of Hsp27 (Nakagomi *et al*., 2003). Therefore, a number of signalling pathways converge on Hsp27 to determine its phosphorylation state.

Phosphorylation of Hsp27 occurs rapidly after stimulation and is detectable within minutes of exposure to stress (Landry *et al*., 1992; Ahlers *et al*., 1994; Engel *et al*., 1995; Guay *et al*., 1997) and this is often followed by increases in the expression levels of the protein within several hours (Landry *et al*., 1991; Landry *et al*., 1992). However, this is a transient and reversible process since the protein returns to basal levels after removal of the stress event. Although the mechanism by which the protein is dephosphorylated is still to be fully elucidated, the protein phosphatase 2A (PP2A) has been shown to be critical for this event. Thus Cairns et al demonstrated that although both PP2A and PP2B can dephosphorylate Hsp27 *in-vitro*, *in vivo* data suggest that the PP2A enzyme is primarily responsible for the dephosphorylation of Hsp27 in MRC-5 cells (Cairns *et al*., 1994). For Hsp-27 phosphorylation to occur, the balance of kinase activation over phosphatase in activation must be shifted. This seems to acquire in activation of PP2A phosphatase as well as activation of p38>MK2. This can be achieved in a number of ways: for instance PP2A activity is, in turn, regulated by phosphorylation so that phosphorylation of PP2A at tyrosine residues results in its inactivation so it can no longer dephosphorylate Hsp27. Furthermore, oxidation of critical cysteine residues in PP2A under conditions of oxidative stress can also inactivate it (Guy *et al*., 1993; Cairns *et al*., 1994). Thus, the signals that leads to phosphorylation of Hsp27 act at different levels to control its function in cells.

Thus, this mode of post-transcriptional regulation of Hsp27 presents dynamic and robust mechanisms, which can regulate the diverse effects of Hsp27 in different cell types. Interestingly, there is significant overlap in signalling pathways that control the phosphorylation of Hsp27 and alter the expression of regulators that can, themselves, increase Hsp27 gene transcription. For instance, pathways activated by phorbol esters, growth factors and mitogen activated pathways can also induce increases in the levels of transcription factors such as Stat-3 (Zhong *et al*., 1994) and Brn-3b (Budhram-Mahadeo and Latchman, 2006), which are known activators of Hsp27 expression. Thus, the activation of such signalling pathways may alter

the expression as well as effects of Hsp27 in breast cancer cells. Questions remain as to how such events relate to the cytoprotective properties of Hsp27 and cell survival. Although many studies have associated Hsp27 phosphorylation with protection against stress by stabilising actin stress fibres and/or other cytoskeletal components (Lavoie *et al*., 1995; Loktionova and Kabakov, 1998; Charette *et al*., 2000), in some circumstances, such as in response to mild stresses, phosphorylated Hsp27 monomers/dimers can appear in insoluble aggregates and thus appear to be in a conformation/compartment unavailable for functional protection (Vertii *et al*., 2006). Therefore, phosphorylated Hsp27 does not appear to be necessary for protection, at least in some cell types or under certain circumstances, such as against oxidative stress ((Mehlen *et al*., 1996b). Furthermore, it seems that severe proteotoxic stresses override any function of Hsp27 phosphorylation by causing Hsp27 aggregation irrespective of its phosphorylation state (Vertii *et al*., 2006). Therefore, it is still far from clear what precise functions require phosphorylation of Hsp27 at its major sites. A summary of the possible regulation of Hsp27 function by phosphorylation is shown schematically in Figure 6.

PERSPECTIVE

During tumourigenesis, changes in genes that are expressed in cancer cells lead to alterations in many proteins that can confer growth and survival advantages but also change behavioural properties such as increased migratory potential. The elevated Hsp27 in a significant proportion of breast cancers points to an important function for this protein and this is reflected in the large volume of literature that reported on studies that have attempted to analyse the expression, function and control of this small Hsp. In this chapter, we have highlighted findings pertaining to Hsp27 in breast cancers with specific focus on the regulation of expression and function in breast cancer cells.

The high degree of homology in structure and function of Hsp27 in diverse organisms suggest important and conserved functions for this protein. Indeed, many studies have demonstrated that Hsp27 can protect cells following heat stress as well as in response to a variety of other stimuli including cytokines, cytotoxic drugs, oxidative stress and ionising radiation. In addition to its protective role, Hsp27 can also participate in maintaining cytoskeletal integrity. These effects arise from the ability of Hsp27 to act in different capacities. For example, as a molecular chaperone, Hsp27 participates in protein folding and prevent protein aggregation and degradation. Its anti-apoptotic effects arise from the ability of Hsp27 to directly interact with different components of the apoptotic machinery or by increasing glutathione levels, thereby prevent damage by ROS accumulation. However, Hsp27 can also associate with and stabilize cytoskeletal proteins and depending on its phosphorylation state, it can affect actin polymerization.

The ability of Hsp27 to act at these different levels can lead to diverse effects depending on the cell type or signal. For instance, in normal cells, Hsp27 expression is associated with decreased proliferation and cellular differentiation whereas in

breast cancer cells, elevated Hsp27 reduces proliferation but confers resistance to treatment (chemotherapy or radiotherapy) and increases migratory potential and invasiveness in many cancers. Thus, an integration of Hsp27 ability to act in a protective capacity and to regulate the cytoskeleton is likely to contribute its effects in breast cancer cells. Because it is associated with poor prognosis and response to treatment in a subset of tumours, it is necessary to understand how Hsp27 is increased in breast cancer, as this increase is unlikely to be due to transient induction of the heat shock response.

In this regard, a number of factors have been identified, that controls the transcription of Hsp27 in breast cancer cells and thus has clinical relevance to breast cancer in terms of the mechanisms by which sustained elevation of Hsp27 occurs in the absence of heat stress. Since its identification as an estrogen response gene, high levels of Hsp27 have been shown to correlate well with the estrogen receptor, ER-α which can activate the promoter via imperfect ERE recognition sequences. This receptor seems to play a pivotal role in the activation of the Hsp27 promoter and its effects can be modulated by a number of other transcriptional activators, such as Brn-3b into, Sp1 and Stat-3, as well as the repressor, HET/SAF-B (see Figure 6). Brn-3b and Sp1 are of particular interest since they interact with the ER and can modify its ability to activate the Hsp27 promoter. Thus, Brn-3b is required for the maximal activity of the Hsp27 promoter by ER on its cognate ERE DNA recognition site, since abolishing endogenous Brn-3b resulted in attenuated Hsp27 expression even when ER is increased. Brn-3b binds to the same site as the repressor HET/ SAF-B, so it is likely that levels of these opposing factors will contribute to the effects of ER on Hsp27 expression. In contrast, Sp1 interacts with and recruits ER to the promoter independently of the ERE suggesting a distinct mechanism by which this factor can alter Hsp27 expression.

The ability of Brn-3b to stimulate expression of Hsp27 on its own and to cooperate with the ER is significant because Brn-3b is, itself, elevated in >60% of breast cancers. When increased in breast cancer cells, Brn-3b increases proliferation in-vitro and the rate and size of tumour growth in-vivo (with invasion into surrounding tissues). It is interesting that although Brn-3b promotes proliferation in breast cancer cells, Hsp27 is anti-proliferative. However, like Hsp27, Brn-3b also enhances anchorage independent growth whilst conferring resistance to growth inhibitory drugs and increasing migratory potential of the cells. Thus, it is likely that the increase of Hsp27 expression by Brn-3b can give rise to distinct effects in these cancer cells. Transcription factors, such as Brn-3b, are likely to act by regulating different subsets of genes depending on the cell stimuli. For instance, in addition to transactivating Hsp27, Brn-3b can also stimulate the increase of other genes associated with increased proliferation e.g. cyclinD1 and CDK4 whilst inhibiting the tumour suppressor gene, BRCA1. Thus, it is possible that during tumour initiation, regulators such as Brn-3b will increase genes that favour proliferation and tumour expansion. As the tumour grows, cells are likely to encounter stresses associated with changes in the tumour microenvironment and the presence of Brn-3b and ER

will enhance the expression of Hsp27, which protects from such stresses whilst conferring migratory potential and drug resistance.

Given its diverse effects in these cells and its association with poor prognosis and short-disease free survival in a subset of breast cancers, there is great deal of interest in attempting to control Hsp27 expression and/or reverse the effects of this protein in breast cancer cells. Understanding the mechanism/pathways that control the expression and function of in breast cancer cells is vital for identifying strategies that can be used to target this protein or its regulators for therapeutic intervention with view to reducing Hsp27 and/or reversing its effects in the cells.

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CHAPTER 6

ROLE OF HEAT SHOCK PROTEIN HSP25/27 IN THE METASTATIC SPREAD OF CANCER CELLS

MARÍA A. BAUSERO[1] AND ALEXZANDER ASEA[2,*]

[1]*Cell Biology, Institut Pasteur de Montevideo, Calle Mataojo 2020 CP 11400 Montevideo, Uruguay*
[2]*Division of Investigative Pathology, Scott & White Memorial Clinic and Hospital and the Texas A&M University System Health Science Center College of Medicine, 1901 South 1st Street, Temple, Texas 76504, USA*

Abstract: An estimated 1.15 million new cases of invasive breast cancer occurred in women worldwide in the year 2002 accounting with 410,712 new deaths. The 5-year relative survival rate for localized breast cancer (cancer that has not spread to lymph nodes or other locations outside the breast) has increased from 72% in the 1940s to 97% today. However, the survival rate is lower (79%) in patients with regional spread while for women with distant metastases, the rate is 23% (cancer expected to occur in women in the United States in the year 2004). In this ultimate stage of malignant transformation, the tumors become resistant to most therapies, and the disease is almost incurable. Therefore, therapeutic interventions designed to inhibit metastatic spread of cancer cells are critical. This chapter will briefly cover recent advances in the study of tumor metastasis and the important role played by heat shock protein 25/27

Keywords: Cancer, chaperokine, heat shock proteins, inflammation, metastasis, migration, tumors

Abbreviations: dsRNA, double-stranded RNA; ECM, extracellular matrix; HSP, heat shock proteins; Hsp25, twenty-five kilo-Dalton HSP; Hsp72, seventy kilo-Dalton HSP; HSF-1, heat shock factor-1; IFN-γ, interferon-gamma; IL, interleukin; NK cell, natural killer cell; MT-MMP, membrane type-matrix metalloproteinases; RNAi, RNA interference; siRNA, short interference RNA; TGF-β1, transforming growth factor-beta1

*Corresponding author: Chief, Division of Investigative Pathology, Scott & White Clinic and The Texas A&M University System Health Science Center College of Medicine, 2401 South 31st Street, Temple, TX 76508 USA, Tel: +1 (254) 743-0201, Fax: +1 (254) 743-0247, e-mail: aasea@swmail.sw.org or asea@medicine.tamhsc.edu

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INTRODUCTION

Tumor-host interactions play an important role in determining tumor progression, especially in cases that involve metastasis. Biological response modifiers including cytokines, chemokines, growth factors and heat shock proteins (HSP) have been shown to orchestrate some of these events. HSP are highly conserved proteins found in all prokaryotes and eukaryotes. Under normal physiological conditions, HSP are expressed at low levels (Craig and Gross, 1991). However, a wide variety of stressful stimuli, including environmental stimuli (UV radiation, heat shock, heavy metals and amino acids), pathological stimuli (viral, bacterial and parasitic infections or fever, inflammation, malignancy or autoimmunity) and physiological stimuli (growth factors, cell differentiation, hormonal stimulation or tissue development), induce a marked increase in intracellular HSP synthesis, a process known as the stress response (Lindquist and Craig, 1988). Hsp25, the murine homologue of human Hsp27, is a ubiquitously expressed member of the small HSP family that has been implicated in various biological functions including its association with estrogen responsive malignancies and is expressed at high levels in biopsies as well as circulating in the serum of breast cancer patients (Ciocca and Roig, 1995; Ciocca et al., 1998; Ciocca and Vargas-Roig, 2002). Activation of the heat shock responsive element on the *hsp25/27* gene results in biological functions associated with cytoprotection from a variety of stressful stimuli as mentioned above. The upregulation of intracellular Hsp25/27 protects cells from tumor necrosis factor-alpha (TNF-α)-mediated apoptosis by a mechanism that involves the down regulation of reactive oxygen species (Lavoie et al., 1995; Mehlen et al., 1996; Rogalla et al., 1999). (Also See *Arrigo*, Chapter 4, *Budram-Mahadeo*, Chapter 5, *Brunet et al*, Chapter 11, this volume). It is thought that Hsp25/27 confers resistance to chemotherapeutic agents through its anti-apoptotic actions and has become a therapeutic target of profound interest. It has been hypothesized that the expression level of Hsp25/27 can determine the fate of the cell in response to a death stimulus, and apoptosis-inhibitory HSP, in particular Hsp27 and Hsp70, may participate in carcinogenesis (Didelot et al., 2006).

THE METASTATIC PROCESS

Organs are protected and sustained by several types of tissue barriers including the basement membrane, the extracellular matrix (ECM) and interstitial connective tissue. For metastasis to occur, these barriers have to be breeched by tumor cells that have been released from a primary tumor. This is the first step in the progression of metastasis and involves proteolytic breakdown of the ECM. Several enzymes are involved on these processes and studies have shown that metalloproteinases are required to invade tissues, enter the bloodstream and metastasize to other organs (Kim et al., 1998). Matrix metalloproteinases together with membrane type (MT)-MMP and serine proteases are the major enzymes responsible for ECM degradation (for review see (Egeblad and Werb, 2002)). Recent studies have shown that a high

tumorigenic potential in a murine breast cancer model is characterized by a high secretion of MMP together with IL-6 and MCP-1 whereas in cells with a low tumorigenic potential the secretion of these factors is significantly lower (Neumark et al., 2003). Studies by the group of Saiki and colleagues further demonstrate the association of MMP-9 on the progression of breast cancer. When mice were given vanillin orally, the number of metastasis to the lungs after inoculation of 4T1 breast adenocarcinoma cells was significantly reduced (Lirdprapamongkol et al., 2005). These authors concluded that the mechanism by which metastasis was abrogated was via vanillin-induced inhibition of MMP-9 expression by 4T1 tumor cells (Lirdprapamongkol et al., 2005). The MMP-9 antagonist TIMP-1 is able to activate Ras. A possible reason could lie in the intrinsic differences between human and murine sequences when over expressed in a tumor mouse model. Studies looking at the effect of over expression of Hsp25/27 have shown that it plays an important role on cycle by a mechanism that involves cycle arrest in G1, S, and G/M (Park et al., 2002; Lee et al., 2004). In our studies, we demonstrate that 4T1 cells transfected with siRNA-Hsp25 induces suppression of cell cycle arrest at G2/M phase and that MMP-9 is down regulated with a concomitant increase of TIMP-1 after silencing the /hsp 25/ gene shedding light to some of the possible mechanisms by which Hsp25 increases de metastatic potential of 4T1 cells (Bausero et al., 2006).

TARGETING HSP25/27: ANTI-CANCER THERAPEUTIC TARGET

Hsp25/27 Increases the Metastatic Potential of Tumor Cells

Most cancers are almost incurable after they have spread out from the primary site and have invaded other organs. We demonstrated that a percentage of 4T1 breast adenocarcinoma cells express Hsp25 on their surface, and this particular phenotype increases their metastatic potential (Bausero et al., 2004). Since Hsp25 has been shown to be highly expressed in biopsies of breast cancer patients (Ciocca and Vargas-Roig, 2002) and has been implicated as a regulator of metastasis, this protein has become of therapeutic interest.

Hsp25/27 Expression Increases Tumor Cell Resistance to Chemotherapeutic Agents via Anti-apoptotic Mechanisms

Studies designed to determine the role of Hsp25/27 and Hsp70 during tumor progression (Oesterreich et al., 1996; Ciocca et al., 2003) and in response to current anti-cancer drugs (Gross et al., 2003b) attribute a modest prognostic value of Hsp27 in breast cancer and show it to be implicated in drug resistance (Gross et al., 2003c). Several studies have been performed to determine the role of Hsp25/27 and Hsp70 during tumor progression (Ciocca and Vargas-Roig, 2002) and in response to current anticancer drugs (Ciocca et al., 1998; Ciocca et al., 2003). Several studies report that enhanced or maintained expression of Hsp27 is an independent and accurate predictor of poor clinical outcome

for individuals with breast (Ciocca and Vargas-Roig, 2002) or prostate cancer (Cornford et al., 2000). The inhibition of apoptosis and the resistance to chemotherapeutic agents have an impact on tumor progression, metastasis, and therapy outcome (Soldes et al., 1999). Small heat shock protein expression is essential to protect differentiating cells against apoptosis (Soldes et al., 1999).

Hsp25 belongs to the family of small heat shock proteins and is the murine homologue of human Hsp27. In contrast to large heat shock proteins, Hsp25/27 act through ATP-independent mechanisms, and *in vivo*, and acts in concert with other chaperones by creating a reservoir of misfolded proteins (Egeblad and Werb, 2002). Activation of the heat shock-responsive element on the *hsp25/27* gene results in biological functions associated with cytoprotection from a variety of stressful stimuli.

Hsp25/27 Induces Anti-inflammatory Responses and Favors Tumor growth and Oncogenesis

The expression of Hsp25/27 within the tumor micro environment has been shown to induce anti-inflammatory responses by immunocompetent cells and enhance tumor growth (De et al., 2000). In this study, De and coworkers demonstrated that exogenous Hsp27 admixed with human monocytes induced the release of IL-10 and TNF-α (De et al., 2000). It was shown that the Hsp27-induced IL-10 production occurred via the p38-dependent pathway, which proved to be independent of TNF-activation, thereby suggesting that this signals a predominantly anti-inflammatory signal within the tumor micro-environment (De et al., 2000). Studies from the Multhoff laboratory on the expression of Hsp72 on the surface of tumors have contributed greatly to furthering our knowledge of NK cell-susceptible tumor targets. In comparison with immunocompetent cells, malignant tumor cells, including biopsies from colorectal, lung, neuronal and pancreas carcinomas, liver metastases and leukemic blasts of patients with acute myelogenous leukemia, express high levels of surface Hsp72 (Botzler et al., 1996; Multhoff and Hightower, 1996; Multhoff et al., 1997; Botzler et al., 1998; Hantschel et al., 2000).

ANTI-METASTATIC THERAPIES: HEAT SHOCK PROTEIN 25/27

Our group demonstrated that silencing the *hsp25* gene in the highly metastatic murine 4T1 breast cancer inhibits cell migration (Bausero et al., 2006). Previous studies clearly demonstrated that RNA interference (RNAi) technology can be used to study the function of different genes and their role on formation of metastasis in breast cancer (Lee et al., 2004). RNAi is a form of post-transcriptional control in which the introduction of a double-stranded RNA (dsRNA) into a cell leads to the homology-dependent degradation of its cognate mRNA (Fire et al., 1998). In mammalian cells, short dsRNA (21–23 mer) is specific and is referred as short interference RNA (siRNA). The siRNA then hybridizes into their cognate mRNA which induces specific degradation of the target mRNA (Elbashir et al., 2001). Our

group further demonstrates that silencing the *hsp25* gene of the highly metastatic 4T1 breast adenocarcinoma cell line suppresses proliferation and cell migration and for the first time, that silencing of the *hsp25* gene downregulates MMP-9 and a concomitantly upregulation of its antagonist, TMIP-1 (Bausero et al. 2005). We propose the use of siRNA to target the down-regulation of Hsp25/27 renders tumor cells more sensitive to commonly used cytotoxic drugs.

It is important to note that although targeting Hsp25/27 is critical in inhibiting the metastatic spread of tumor cells, Hsp72 surface expression makes tumor cells sensitive to natural killer (NK) cell-mediated killing (Gross et al., 2003a; Gross et al., 2003b; Gross et al., 2003c). This implies that therapies that can upregulate Hsp72 surface expression and concomitantly suppress Hsp25/27 surface expression on tumors might result in the suppression of tumor growth and abrogate the tumors' metastatic potential.

Enhanced expression of intracellular Hsp70 has clearly been shown to be anti-inflammatory (Cahill et al., 1996), anti-apoptotic (Jaattela et al., 1998) induce cell cycle arrest (Calderwood and Asea, 2002) and protect cells from stressful stimuli (for review see (Jaattela, 2002)). In contrast, enhanced extracellular expression of Hsp70 either on the surface of tumors or in the extracellular milieu either enhances NK cell-mediated lysis (Farkas et al., 2003) or upregulates APC-mediated acute phase responses (Asea et al., 2002), respectively. Using this logic, it might be expected that Hsp25 would exhibit a similar differential pattern of activation depending on its cellular location. Contrary to these predictions, tumor development and metastatic potential of 4T1 breast adenocarcinoma cells was demonstrated to favor tumors that over express high levels of Hsp25 on their plasma surface, while tumors that express Hsp70 on their plasma surface are sensitive to anti-tumor effector cells (Bausero et al., 2004). We hypothesized that therapies that can upregulate Hsp70 surface expression and concomitantly suppress Hsp25/27 surface expression on tumors, might result in the suppression of tumor growth and abrogate the tumors metastatic potential (Bausero et al., 2004).

Our results do not exclude the possibility that other genes are important in metastasis of tumors. Indeed, it has recently been demonstrated that silencing the chemokine receptor *CXCR4* gene using siRNA in 4T1 cells prevents primary tumor formation in some mice and surviving mice did not develop macroscopic metastases (Smith et al., 2004). In a second study, Liang and colleagues demonstrate that silencing the chemokine receptor CXCR4, using siRNA effectively blocks metastasis of human breast carcinoma cell line MDA-MB-231 (Liang et al., 2005). In invasion studies, these authors show that each siRNA-CXCR4 constructed (siRNA1 and siRNA2) suppress migration by approximately 60%. However, a combination of both siRNA1+2 blocks migration by 90% (Liang et al., 2005). In our studies, we demonstrate that a single siRNA-Hsp25 effectively blocks migration by >90% (Bausero et al., 2006). *In vivo* studies are currently underway to test the efficacy of the siRNA-Hsp25 in a tumor animal model (Bausero et al, *in preparation*).

CONCLUSIONS

The working model that we propose is that tumors which express high levels of surface-bound Hsp25/27 and low Hsp72 creates a tumor mircoenvironment that induces an anti-inflammatory by invading immunocompetent cells (Figure 1; top panel). This supports the growth of large primary tumors and favors the metastic spread of tumors to distant organs. However, therapies which target Hsp25/27 including RNAi abrogate metastatic spread of tumors (Figure 1; top panel). In addition, induction of the stress response by hyperthermia, pro-inflammatory cytokines stimulates the expression of surface bound Hsp72 and its subsequent release into the tumor microenvironment (Figure 1; bottom panel). This favors a pro-inflammatory immune response by immunocompetent cells infiltrating the

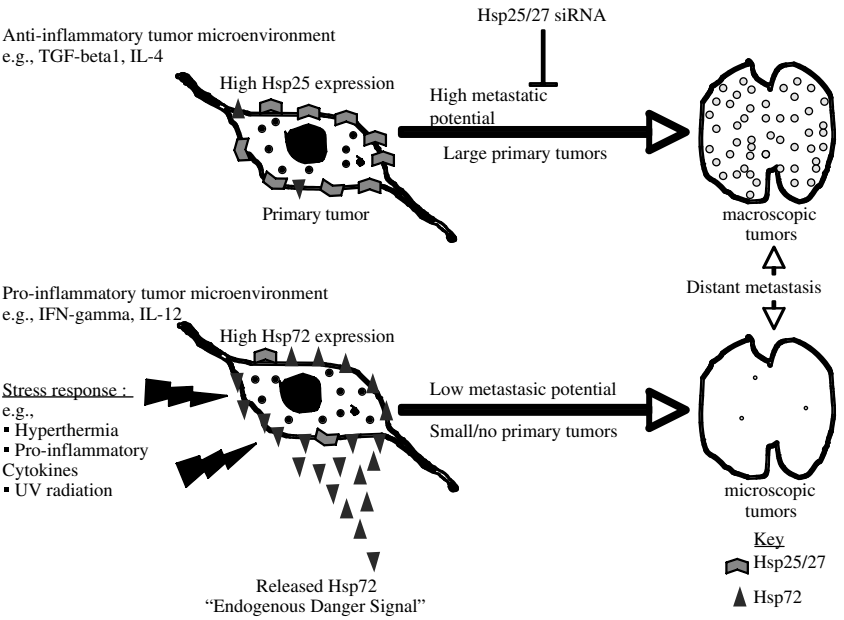

Figure 1. Schematics representation for role of Hsp25/27 in metastatic spread of tumor cells. Primary tumors which express high levels of surface bound Hsp25/27 (red hexagon) and low Hsp72 (blue triangle) helps to create a tumor mircoenvironment that induces an anti-inflammatory by infiltrating immunocompetent cells (top panel). This supports the growth of large primary tumors and favors the metastic spread of tumors to distant organs. However, therapies which target Hsp25/27 including RNAi abrogate metastatic spread of tumors (top panel). In addition, induction of the stress response (filled lightning blots) including hyperthermia, pro-inflammatory cytokines stimulates the expression of surface bound Hsp72 and its subsequent release into the tumor microenvironment (bottom panel). This favors a pro-inflammatory immune response by immunocompetent cells infiltrating the tumor microenvironment, known as the chaperokine effect of circulating Hsp72. The result is a small or no primary tumor and tumors that do survive will exhibit low or no metastatic potential (bottom panel)

tumor microenvironment, known as the chaperokine effect of circulating Hsp72. The result is a small or no primary tumor and tumors that do survive will exhibit low or no metastatic potential (Figure 1; bottom panel).

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CHAPTER 7

HEAT SHOCK CHAPERONE MORTALIN AND CARCINOGENESIS

CUSTER C. DEOCARIS[1], SUNIL C. KAUL[2] AND RENU WADHWA[2,*]

[1]*Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki, Japan*
[2]*National Institute of Advanced Industrial Science & Technology (AIST), Central 4, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan*

Abstract: Overexpression of mortalin in a wide variety of malignancies is generally associated with highly advanced and aggressive cancers. In the past decade, since its discovery, the classic understanding linking the role of this protein to cancer has relied on: (a) its mitochondrial functions (as the main import protein and mitochondrial housekeeper) consonant to the increased energy demand by cancer cells, and/or (b) its pro-proliferative, cytoplasmic sequestration/degradation of wild-type p53. Recent studies have ascribed new tumorigenic functions for mortalin expanding its roles in signal transduction, mitochondrial and extra-mitochondrial chaperonization, and even maintenance of genome stability. Recently, we have proposed a "molecular sponge" hypothesis based on chaperone-drug interactions and observed mortalin-dependent response of tumor cells to some anti-cancer agents. These new paradigms on mortalin biology are expected to yield unique promises in biomedicine

Keywords: Mortalin/mtHsp70/Grp75, neoplasm, immortality, senescence, mitochondria, aging

INTRODUCTION

Fifteen years ago, arduous cell fusions of mortal (MEF) and immortal (MN48-1, derivative of NIH 3T3) cells, and tracking of serially-passaged parental and hybrid cell populations showed segregation of a protein with mortal phenotype. After the cloning, the protein was named as mortalin, due its apparent association with cellular aging (senescence). Immunostaining using rabbit polyclonal antibody raised against mortalin showed its pancytoplasmic staining (Wadhwa et al., 1993a). Subsequent experiments revealed that the anti-mortalin antibody cross-reacted with an unknown protein of perinuclear distribution, this time, in immortalized mouse cells. It was

*Corresponding author: Research Institute for Cell Engineering, National Institute of Advanced Industrial Science & Technology (AIST), Central 4, 1-1-1 Higashi, Tsukuba, Ibaraki 305 8562, Japan, Tel: +81 29 861 9464, Fax: +81 29 861 2900, e-mail: renu-wadhwa@aist.go.jp

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later confirmed that this protein is a variant of mortalin and was labeled as the perinuclear mortalin (or mot-2). This, henceforth, prompted that the first discovered mortalin be distinguished as the pancytoplasmic mortalin, or mot-1 (Wadhwa et al., 1993b). cDNA cloning and homology search placed it in the heat shock protein 70 (Hsp70) family (Wadhwa et al., 1993a) and assigned it to chromosomes 18 and X in mouse, and chromosome 5q31.1 in human by fluorescence *in situ* hybridization (Kaul et al., 1995). Breeding experiments with mice confirmed that mot-1 and mot-2, which segregated in F1 and F2 progenies, are proteins encoded by two separate alleles in mouse (Kaul et al., 2000a; Wadhwa et al., 1996).

The two forms of mouse mortalin, the pancytoplasmic (mot-1) and perinuclear (mot-2) differ by only two nucleotides in the carboxy-terminus of their open reading frame, resulting in two amino acid changes. Expression of the cytoplasmic form by transfection of mot-1 cDNA (isolated from CD1-ICR mouse embryonic fibroblasts) to NIH 3T3 cells induced cellular senescence. However, the perinuclear form expressed by mot-2 cDNA (isolated from NIH 3T3 cells) did not yield an equivalent effect. The data suggested that the senescence-inductive function is exclusive for cytosolic mortalin or mot-1 (Wadhwa et al., 1993c). Incidentally, mortalin was independently cloned as the peptide binding protein, PBP74 (Dahlseid et al., 1994; Domanico et al., 1993); mitochondrial heat shock protein, mtHsp70 (Bhattacharyya et al., 1995); and glucose-regulated protein, Grp75 (Webster et al., 1994) implying multi-functionality of this protein.

THE MORTALIN MACHINE

At the heart of its bio-functions is the crystal structure of mortalin that could be predicted based on homology modeling with evolutionary conserved Hsp70 family members (Mayer and Bukau, 1998). Similar to its close relatives in Hsp70 family of proteins, it contains the two inter-connected principal domains: the N-terminal ATPase and C-terminal regions. The structure of mortalin's N-terminus was deduced based on the X-ray data of bovine heat shock cognate protein (Hsc70) (Deocaris et al., 2007). As shown, its 44-kDa structure contains four sub-domains, I-IIA and I-IIB, that folds as a deep catalytic pocket stabilized by metal-binding (Sriram et al., 1997). Allosteric changes within the ATPase domain are communicated to the peptide-binding domain (PBD) through the chaperone's highly evolutionary conserved interdomain linker/interface (Jiang et al., 2005; Mayer et al., 2000). This 18-KDa PBD is composed of two sets of four-stranded anti-parallel β-sheets that form a twisted sandwich. When compared to the ATPase domain, there is a wider sequence variation in the PBD with other Hsp70s. This variation is believed to underlie the diversification of Hsp70 interacting clients (Rudiger et al., 2000). Flanking the PBD is a 10-kDa long C-terminal helix made-up of five helical domains (A-E). This interesting structure, called the "substrate lid", does not contact the peptide substrate directly and has the ability to flip-flop. This lid also functions as

a molecular "latch" that locks-in substrates during an ADP-bound state (Zhu et al., 1996).

Given the opposing functions of mortalin: mot-1 being associated with limited lifespan and senescence (Wadhwa et al., 1993c), while mot-2 with lifespan extension, immortalization, malignant transformation, and late-stages of cancers (Kaul et al., 1998; 2003), it is remarkable that such subtle differences in two amino acids (V618M and R624G, for mot-1 and mot-2, respectively) result to these polemic phenotypes. To appreciate how these changes alter mortalin functions, the structure of the PBD was understood in the context of how mortalin facilitates entry/exit of the protein substrates. Apart from the flexible lid structure that 'flip-flops' above the substrate cleft, electrostatic "latches" between the lid and the cleft appear to be critical. One "latch" consisting of Asp477, Arg513, Glu586, and His590 is common with other Hsp70s (Mayer et al., 2000), while an additional "latch" that we identified on the opposite end, consisting of Arg574, Arg578, and Glu628, appeared to be mortalin-specific. A replacement of Gly624 (in mot-2), located at the C-terminus of α-helix C, by Arg (in mot-1) is likely to extend the α-helix C. In contrast, Gly, a strong helix breaker, shortens the L3 (C-D) loop. The latter should perturb the structure of the "mortalin-specific latch", presumably pulling apart the electrostatic attractions that could result in the loss of the chaperone action of the molecule. Additionally, the altered secondary structure of mot-1 and mot-2 proteins was also evident from their different mobility on SDS-PAGE (Kaul et al., 2000b). This electrophoretic behavior reflected the subtle changes in the flexibility of the proteins and was consistent with different chaperone and biological activities of the two proteins (Kaul et al., 1998; 2003; Wadhwa et al., 1993c).

Studies on *E. coli* Hsp70 chaperone, DnaK, have shown that upon binding of ATP, its ATPase domain communicates the conformational changes to the PBD. Co-chaperones DnaJ and GrpE nucleotide-exchange factor also bind to the ATPase region and facilitate the release of ADP (Harrison, 2003). After binding of ATP to Hsp70, DnaJ permits the hydrolysis of DnaK-bound ATP allowing the ADP-bound DnaK to interact more strongly with unfolded proteins. The nucleotide exchange factor GrpE then enables the recycling of DnaK back into an ATP-bound state and permits an efficient release of the substrate (Harrison et al., 1997). With the discovery of multiple classes of GrpE-like proteins in mammals (Naylor et al., 1998), a unique human GrpE homologue restricted to the mitochondria, HMGE, was reported to form chaperone/co-chaperone pairing with mortalin (Choglay et al., 2001). Meanwhile, the mitochondrial localization and interaction of mammalian cytosolic DnaJs (DJ2 and DJ3) with Hsc70 (Terada and Mori, 2000) make them suitable candidate co-chaperones of mortalin. Although binding of DJ2 has not yet been proven, this co-chaperone facilitates mitochondrial protein import and can functionally replace bacterial DnaJ (Terada et al., 1997). Deletion of MDJ1p, a functional partner of mitochondrial Hsp70 in prevention of irreversible protein aggregation in the matrix, but not in protein import into mitochondria, caused aggregation of mitochondria (Kawai et al., 2001).

MITOCHONDRIA, MORTALIN AND THE CANCER ENERGY ECONOMY

More than 80 years ago, Nobel laureate Otto Warburg of the Kaiser Wilhelm Institute, now known as the Max Planck Institute, suggested that the impaired oxidative metabolism (anaerobiosis) maybe the cause of malignant growth. Later termed as Warburg's hypothesis, this provocative assumption, unrecognized by many scientists at the time, has been experimentally addressed for many decades. Indeed, as a hallmark of cancer growth, increased glycolysis and lactate production in tumors, corroborated with a wide spectrum of oxidative phosphorylation deficits and decreased availability of ATP associated in malignancies (Ristow, 2006). Although there is no universal metabolic alteration for malignancies, there are several differences between the mitochondria of transformed versus normal cells that have been noted with regards to: (a) substrate preference for oxidation reactions, (b) degree of acceptor control ratios, (c) rates of electron and anion transport, (d) control of calcium holding and efflux, (e) mtDNA status, and (f) rates of mitochondrial biogenesis (Modica-Napolitano and Singh, 2002; Ristow, 2006; Wallace, 2005).

As the mitochondrion produces ATP for almost all cellular processes, it consumes more than 98% of total cellular oxygen. In addition, the mitochondria have also been implicated in the carcinogenic process because of their role in apoptosis, and critical in the proper regulation of calcium fluxes and redox state of cells (Don and Hogg, 2004; Hanahan and Weinberg, 2000). A role for mitochondrial reactive oxygen species (ROS) in cancer is supported from observations with mice heterozygous for the Mn-superoxide dismutase. These transgenic animals exhibit increased levels of 8-oxo-2-deoxyguanosine (8oxodG) in nuclear DNA and have 100% increase in tumor incidence compared with the wild-type mice (Van Remmen et al., 2003). Many types of human malignancy such as, breast, colorectal, liver, pancreatic, lung, prostate, bladder and skin cancer have been shown to harbor somatic mtDNA mutations (Chatterjee et al., 2006; Ohta, 2006). It has been observed that tumor mtDNA somatic mutations range from severe insertion-deletion and chain termination mutations to mild missense mutations. Interestingly, of the 190 tumor-specific mtDNA mutations, more than two-thirds are mtDNA variants found in the general population. These similarities are hypothesized to reflect the two possible functional consequences of mtDNA mutations in tumors: First, severe mutations could inhibit oxidative phosphorylation, increase ROS production, and promote genomic instability as well as tumor cell proliferation; and second, milder mutations may be adaptive responses of tumors to new environments (Brandon et al., 2006). Mitochondrial ROS production is also associated with inactivation of some tumor suppressors, i.e., $p16^{INK4a}$ and p53 (Arbiser, 2004; Arbiser et al., 2004). Thus, we envision that the mitochondrial mortalin is being pushed to work harder to meet increasing energy demands, and to regulate mitochondrial stress associated with mitochondrial biogenesis and the chaperonization of mutated mitochondrial proteins.

Mortalin as a mitochondrial import motor. With only a limited number of genes encoded by the mitochondrial genome, most proteins within this organelle are

nuclear-encoded and are imported from the cytosol. A specialized and elaborate translocation system in this powerhouse-organelle has thus evolved which ensures that the proteins destined for the mitochondrion are properly recognized, delivered and sorted to their proper compartments (Koehler, 2004; Rehling et al., 2004; Wiedemann et al., 2004). During the import of mitochondrial-targeted proteins, the proteins pass through the membranes via the 'Translocase of the Outer Membrane' (TOM). After crossing the outer membrane, precursor proteins segregate to two structurally distinct 'Translocases of the Inner Membrane' (TIM), TIM23 and TIM22. Transport of bulky proteins requires unfolding during shuttling and refolding to native conformers to regain function. The mitochondrial molecular chaperone, mortalin, plays key role in these processes. The fact that no cell can survive without mortalin (or when harboring deletion mutations in Ssc1, as in the case of yeast cells) underscores the importance of mortalin to life (Craig et al., 1987).

Energy required to translocate precursor proteins across TIM channels in cells is derived from two sources. First, it comes from the membrane potential generated in the matrix that generates an "electrophoretic" effect for proteins. The pulling action, as a consequence, assists in the unfolding of the rest of precursor domains that remain outside the mitochondrion, and also directly stimulates the channel protein TIM23 (Geissler et al., 2000; 2001; Grigoriev et al., 2003). As the electrostatic potential is being considered insufficient for the bulk transport of entire proteins, the second source of energy is extracted from matrix ATP. Mortalin is the only ATPase component of this pre-protein mitochondrial import complex. TIM44, an adaptor protein tethered at the TIM23 translocase, manipulates chaperone function of mortalin essential for protein translocation (Ostermann et al., 1989; 1990; Schneider et al., 1994; Truscott et al., 2001). It is still debatable as to the manner the mortalin machinery performs its protein translocation function across the two mitochondrial membranes. The two pre-protein transport models involving mortalin, i.e., the "trapping" and the "motor" model hypotheses have been reviewed elsewhere (Deocaris et al., 2006).

Mortalin as a post-entry chaperone. While an entry into the mitochondrion is only a part of the mitochondrial biogenesis story, the next ordeal is for the pre-proteins to refold back, assemble, sort and then perform their proper duties as components of the bio-energetics network. Initial yeast-interactive screen for mortalin-binding proteins isolated the mitochondrial proteins Hsp60 and later confirmed the functionality of their interactions (Wadhwa et al., 2005). Newly imported mitochondrial pre-proteins interact with Hsp60 as soon as they reach the matrix compartment (Hartl et al., 1992; Langer and Neupert, 1991; Mahlke et al., 1990). Together with mortalin, Hsp60 constitute the most important housekeeping system for mitochondrial proteome integrity. Hsp60 is also an essential gene in yeast (Cheng et al., 1989).

Mortalin as a mitochondrial house-keeper. Within the ROS-bathed cellular environment, genetic mutations stochastically accumulate with time and propel the hallmark genomic instability in cancers. Chaperones function to neutralize the conformational changes in proteins to potentially avoid lethal phenotypes (Soti and Csermely, 2002; Soti et al., 2003). Consistent with the major involvement of

ROS-related mutational events in cancers, Bert Vogelstein's group found that the majority of mutations in 10 human colorectal cancer cell lines were (i) somatically acquired mtDNA mutations involving the transitions at purines, (ii) the detected mutations, however, were not associated with major perturbations of mitochondrial functions, as oxygen consumption and the respiratory chain enzymatic activities of several of the lines were not significantly changed (Polyak et al., 1998). Because cancer cells generally have incurred nuclear gene mutations that attenuate apoptotic signaling, it is suggestive that chaperone buffering within the mitochondrion itself could be a plausible way of how cancer cells tolerate high mitochondrial mutational loads.

On the other hand, mitochondrial proteins "age" rapidly with progressive acquisition of oxidative-damage and are diverted to a chaperone-protease triage. The fidelity of proteolytic degradation within the mitochondria is critical for cell survival. Inhibiting the proteasome, for example, has been shown to dramatically alter mitochondrial homeostasis that manifests a reduction in complex I and complex II activities, increase in mitochondrial ROS production and decrease in intra-mitochondrial protein translation. This is further exacerbated by an impairment of lysosomal-mediated degradation of mitochondria or autophagy (Sullivan et al., 2004). The fate of the polypeptide is mainly determined by the decision to either associate with proteases for degradation or to be repaired by the mitochondrial Hsps. In contrast to the cytosol that relies on poly-ubiquitination of proteolytic substrates to ensure targeting to the 26S proteasome, there is no evidence for the existence of a similar system in the mitochondria. The matrix-localized PIM1 and membrane-bound *m*-AAA proteases have overlapping substrate specificities and they assign a central role to the mortalin chaperoning system during the degradation of misfolded polypeptides (Savel'ev et al., 1998; 1999). Alternatively, *i*-AAA protease of the inner mitochondrial matrix is similarly capable of sensing the folding state of their substrates and exerts its own chaperone-like properties (Leonhard et al., 1999). Several studies have shown the involvement of mortalin in ROS management and apoptotic response of cells (Liu et al., 2005; Kim et al., 2006; Orlov and Hamet, 2006; Jin et al., 2007; Czarnecka et al., 2006; Pshezhetsky, 2006; Pellegrini et al., 2005). Up-regulation of mortalin suppresseed the engagement of apoptosis from various stressors, e.g. arsenite in rat lung epithelial cells (Lau et al., 2004), mercury in renal cells (Stacchiotti et al., 2006, differentiation agent 1,25-dihydroxyvitamin D3 in rat gliomas (Baudet et al., 1989), and glucose starvation and ischemia reperfusion in Chinese Hamster Lung [CHL] cells (Gao et al., 2003).

THE PRODIGAL MITOCHONDRIAL CHAPERONE

Several groups have reported that mortalin can be found in extra-mitochondrial sites. Confocal laser microscopy of the native protein with protein-specific antibodies in a variety of cell lines revealed its presence in the endoplasmic reticulum, cytoplasmic vesicles and cytosol (Domanico et al., 1993; Poindexter, 2002; Ran et al., 2000; Singh et al., 1997; Soltys and Gupta, 1999; 2000; Wadhwa et al., 1995). The problem

of how such mitochondrial matrix macromolecules arrive at its extra-mitochondrial locations still remains a mystery. There is evidence to support that after their residence in the mitochondrion, some proteins are actually exported to different intracellular compartments (Soltys and Gupta, 1999; 2000). Binding of mortalin to residents of different organelles may assist in its relocation to multiple subcellular sites. Far-western screening identified glucose regulated ER chaperone (Grp94) as one of its binding partners. Mortalin-Grp94 interaction has been confirmed by mammalian two-hybrid assays, *in vitro* and *in vivo* co-immunoprecipitations (Takano et al., 2001). In addition, mortalin is also found to bind to membrane-associated proteins such as fibroblasts growth factor -1 (FGF-1) (Mizukoshi et al., 1999) and IL receptor type-1 (Sacht et al., 1999), and to the peroxisomal protein mevalonate pyrophosphate decarboxylase (MPD) (Wadhwa et al., 2003b). In a manner re-enacting its role in mitochondrial import, mortalin also functions in trafficking of non-mitochondrial proteins. Interestingly, its interaction with FGF-1 was coupled to its cell-cycle dependent tyrosine phosphorylation (Mizukoshi et al., 2001). Similarly, ATP-sensitive association of mortalin was shown to be involved in the internalization of IL receptor type-1, critical in the signaling pathway of the pro-inflammatory cytokine IL-1 (Sacht et al., 1999). More recently, serine/threonine kinase Akt, a key mediator of cell survival and cell growth, is recently found to be a binding partner of mortalin as well, however, functional significance of this interaction, as well as many others, has not yet been investigated (Vandermoere et al., 2007). Mortalin viewed as a prodigal mitochondrial chaperone is poised to assume more expansive roles in signal transduction, cell communication and neoplastic development primarily by its ectopic interactions with its protein clients.

MORTALIN AND IMMORTALITY, A GATEWAY TO CARCINOGENESIS

Somatic cells have evolved mechanisms to put restrains on proliferation. In the face of chronic life stress, oxidation reactions accumulating to genetic and epigenetic errors, senescence (or cellular aging) could provide a fail-safe mechanism preventing cells from turning into cancers. In corollary to this, what characterizes the large majority of tumors is their ability to overcome the aging roadblock and acquisition of unlimited potential to divide.

Numerous studies in the recent past have established the critical role of tumor suppressor pathways (Rb and/or p53, and their regulators, such as $p16^{INK4a}$, $p21^{WAF1}$ and ARF) in cellular senescence (Strano et al., 2007; Campisi, 2005a). There are several reviews on the involvement of Hsps in aging at the cellular level (Soti and Csermely, 2000; Soti et al., 2003). Overall, cells derived from aged animals and those that have become senescent in the culture dish share the common features of a dysfunctional chaperone system (Nardai et al., 2002), baseline depression of stress and anti-oxidative protein levels (Kregel et al., 1995), weaker activation of inducible chaperones (Liu et al., 1989) and poorer binding of HSF to the heat shock element (Choi et al., 1990).

While chaperones have been consistently on the frontline counteracting senescence, mortalin, on the other hand, plays "*Dr Jekyll-Mr. Hyde"* with immortality (Kaul et al., 2007). Depending on its subcellular localization, mortalin assumes functional distinctions; pancytoplasmic mortalin mediated senescence and the perinuclear mortalin drive the cells to immortality. Malignant transformation of NIH 3T3, life span extension of MRC-5 and attenuation of differentiation of HL-60 cells by overexpression of mot-2 have been explained, at least in part, by its p53 inactivation function. Mortalin and p53 interact in the cytoplasm resulting in the nuclear exclusion and transcriptional inactivation of the latter (Merrick et al., 1997; Wadhwa et al., 1998; Walker et al., 2006). Notably, mortalin and p53 co-localize in transformed, but not in normal human cells. Based on the studies using deletion mutants of mot-2 and p53, their binding regions were assigned to an amino-terminus region of mot-2 and the carboxy-terminus region of p53, the cytoplasmic sequestration domain (Kaul et al., 2001; Ostermeyer et al., 1996; Wadhwa et al., 2002b). Indeed, small p53 peptides that bind to mortalin were able to act as binding antagonists resulting in translocation and reactivation of wild type p53 (Kaul et al., 2005). In addition to an inactivation of p53 function (Kaul et al., 2005), mortalin was shown to abrogate the control of p53 on centrosomal duplication (a hallmark of cancers) (Ma et al., 2006). Mortalin was shown to preferentially associate with duplicated centrosomes and its overexpression overrode the p53-dependent suppression of centrosome duplication (Ma et al., 2006).

As a native of the mitochondrion, in principle, mortalin is envisioned to play an even more expansive role in modulating apoptosis. Treatment of cells with ultraviolet radiation induces the release of monomeric p66Shc which then travels to mitochondrion, dissociates from an inhibitory complex with mortalin and finally triggers the collapse of the mitochondrial trans-membrane potential (Orsini et al., 2004). Similarly, after Fas receptor/Fas ligand interaction, N-terminal portion of cyclin dependent kinase 11 (CDK11 p60) is translocated from the nucleus to the mitochondria most likely by its association with the import motor protein, mortalin (Feng et al., 2005). p53 has also been reported to translocate to the mitochondria and induce apoptosis (Marchenko et al., 2000). Mitochondrial-localized p53 during apoptosis relies on the direct interaction of Bcl2 and Bcl-xL with p53 as detected by co-immunoprecipitation experiments (Mihara et al., 2003). In addition, p53 can also abstract Bcl-xL and Bcl2 from tBid, Bak and Bax creating apoptotic homodimers (Chipuk et al., 2004).

MORTALIN UP-REGULATION IN CLINICAL SAMPLES

With the concept of stress-adapted malignant tumors, the stress-protein mortalin is recently being appreciated to play an important role during carcinogenesis. Implicated in its etiology are the changes in expression levels, post-translational modifications and extra-mitochondrial localizations. In our panel of more than 100 immortalized and transformed human cell lines, all cell lines that have been analyzed so far exhibited only the perinuclear mortalin staining pattern. Furthermore, its

expression levels indicate apparent bi-phasic behavior in terms of mortalin (protein) expression: an initial elevation in mortalin levels occur during immortalization (relative to a down-regulation during replicative senescence in human fibroblasts) followed by an up-regulation at a later stage that coincides with the acquisition of an invasive phenotype (unpublished observation). Consistently, by analyzing the proteomics in tissue arrays, Dundas et al. have identified mortalin as a new prognostic marker for colorectal cancers. Its overexpression correlated with poor patient survival supporting its role in colorectal neoplasia and its importance as a tumor marker for disease management (Dundas et al., 2004). Global profiling of the cell surface proteome applied to cancer cells, including the SH-SY5Y neuroblastoma, the A549 lung adenocarcinoma, the LoVo colon adenocarcinoma, and the Sup-B15 acute lymphoblastic leukemia (B cell) cell lines and ovarian tumor cells, revealed the remarkable abundance of several chaperone proteins, including Grp78, mortalin, Hsp70, Hsp60, Hsp54, Hsp27, and protein disulfide isomerase (Shin et al., 2003).

Immunohistochemical studies of mortalin in normal and tumor human brain sections revealed that in normal brain sections, the mortalin expression was seen mainly confined to neurons. Normal astrocytes showed undetectable expression of mortalin. Contrastingly, there were an increased number of mortalin-immunopositive cells in the three grades of astrocyte tumors, i.e., low-grade astrocytoma, anaplastic astrocytoma, and glioblastoma. Other types of brain tumors, such as meningiomas, neurinomas, pituitary adenomas, and metastases, also showed relatively elevated levels of mortalin expression as well. The observed abundance of mortalin-immunopositive cells with malignant progression of brain tumors and its correlation with the expression of Ki-67, a cell proliferation marker, suggested the involvement of perinuclear mortalin during malignant transformation of brain tissues *in vivo* (Takano et al., 1997).

In a proteomic analysis of chronic myeloid leukemia (CML) cells, a hematopoietic cancer with a hyper-activated Bcr-Abl protein tyrosine kinase, mortalin was identified as one of the four major proteins that were abnormally expressed. Surprisingly, there was no correlation between the protein expression changes and mRNA levels (Smith et al., 2002) supporting the likelihood that post-translational changes in mortalin may be responsible for the malignant behavior. Of note, mortalin was shown to be tyrosine-phosphorylated in breast tumor cells along with cytoskeletal molecules (actin, tubulin, and vimentin) and other molecular chaperones (heat-inducible Hsp70, and the Hsc71) (Lim et al., 2004). Associated with its phosphorylation, mortalin is known to show enhanced binding with FGF-1 and is considered as among the determining factors that regulates its client interactions. Mortalin-FGF-1 interaction is significant during the late G1 phase of the cell cycle and is thought to affect the mitogenic activity of FGF-1 (Mizukoshi et al., 1999; 2001). Phosphorylated form of mortalin is associated with the prevention of apoptosis in CCL39 lung fibroblasts by pervanadate, a stress-inducing agent. In this model, however, the universality of mortalin phosphorylation is questioned as cytokines and growth factors were ineffective (Bhat et al., 2004). p75, similar to mortalin, is

tyrosine phosphorylated in response to hydrogen peroxide- and vanadate-treatment (Hadari et al., 1997). For a non-human model for leukemia, Walker et al used the soft shell clam *Mya arenaria* that readily develops a fatal neoplasm. Interestingly, in this novel molluscan tumor model, the cancer-associated behavior of mortalin and p53 were also noted. Mortalin and p53 proteins form complex in the cytoplasm of leukemic clam hemocytes, but not in normal hemocytes. Treatment of the leukemic clam hemocytes with MKT-077, a cationic inhibitor of mortalin, revealed the abrogation of mortalin and p53 interaction, resulting in the translocation of p53 to the nucleus. Thus, the data from the clam further implies that mortalin-based phenotype is an evolutionarily conserved feature of human cancers (Walker et al., 2006). In addition, we have recently observed that the tumor cells expressing higher endogenous levels of mortalin are more sensitive to MKT-077, a mitochondriotropic agent. Overexpression of mortalin enhanced sensitivity to mortalin, whereas knockdown by mortalin-shRNAs made the cells more resistant to MKT-077 toxic effects (Deocaris et al., 2007). While it has been previously proposed that the mitochondria could serve not only as a 'drug depot' but also as a slow-release actuator for delocalized lipophilic cations (DLCs), such as MKT-077 or Rhodamine 123 (Bernal et al., 1982; Lampidis et al., 1983), in the light of our recent data, mortalin may be considered as a "molecular sponge" for DLCs in this organelle.

MORTALIN-BASED CANCER THERAPIES ON THE HORIZON

During the therapy of malignant tumors it is desired that the agents attack preferentially tumor cells without exerting adverse effects on normal cellular compartments. But this is rarely achieved with the currently available drugs. It has recently been revealed that the induction of the phenotypes associated with replicative senescence in tumors, which is also called premature/accelerated senescence or "stress-induced premature senescence", qualitatively equals the impact of programmed cell death by forcing cells to exit the cell cycle irreversibly. This form of senescence can be induced after treatment with low-doses of DNA-damaging drugs, histone deacetylase, oxidative stress, retinoids, ultraviolet (UV) light, or γ-irradiation. The fact that the induction of senescence occurs at fairly low drug levels, it is yet to be clinically realized the impact of senescence-based therapies as an approach that can control tumors with minimal toxicity to normal cells (Schmitt et al., 2002). te Poele et al have proposed that the premature senescence is a highly relevant factor for determining treatment outcome and such phenomenon may be of value in choosing the appropriate chemotherapeutic strategy (te Poele et al., 2002). A common feature behind many of these different types of growth arrest states observed in normal, immortalized and tumor cells is the wild type activity of tumor suppressor genes (Dai and Wang, 2006; Campisi, 2005b; Kahlem et al., 2004; Roninson et al., 2001).

The present review has revealed aspects of the multi-functionality of mortalin in various cancer processes. Starting with the system of differential protein screening in naturally aging (in primary fibroblasts), escaped aging (in immortal fibroblasts

and tumor cells) and conditionally aging (in mortal hybrids derived from cell fusion) cells, we first identified mortalin as associated with mortal phenotype. Subsequent analysis, showing multi-functionality of the protein, has acquired in-depth insights about its unique biology and involvement in aging and cancerous phenotype of cells. When the expression of mortalin was suppressed in genetically defined immortal cells, produced by functional inactivation of p53 and pRB tumor suppressor proteins and simultaneous activation of telomerase function in normal human cells, they underwent a permanent growth arrest (Wadhwa et al., 2004). Regulation of immortalization by promoting molecular pathways by other genes is still a poorly understood phenomenon. Since senescence has always been dominant over immortality, a feature shared by most of the cancers, and given the mitochondria play a central role in the control of aging processes and cell survival, it is expected that the drugs targeting the major immortality-associated mitochondrial chaperone, mortalin, may someday be an additional armamentaria against cancer.

Functional small RNAs. The use of mortalin-specific RNA-helicase-linked hybrid ribozymes with a linked unwinding activity is an approach to catalytically suppress expression of mortalin. When delivered to transformed human cells, these novel RNA drugs were able to induce growth arrest (Wadhwa et al., 2003a).

Small molecules. MKT-077, a cationic rhodacyanine dye analogue (because of its selective toxicity to cancer cells, it has been under preclinical cancer therapeutical trials) binds to mortalin (mot-2), and abrogates its interactions with the tumor suppressor protein, p53. Using MKT-077-conjugated Sepharose beads, V5-tagged mortalins expressed in COS7 cells were pulled down from cellular lysates, and MKT-077 binding site on mortalin was assigned to amino acid residues 252–310 (Wadhwa et al., 1998). MKT-077 was shown to cause nuclear translocation and reactivation of p53 function in cancer cells and thus could be one of the candidates to be used for therapy of cancers with wild-type p53 (Wadhwa et al., 2000; 2002a). Induction of senescence like growth arrest by bromodeoxyuridine (Michishita et al., 1999) and 5-aza-2' deoxycytidine (Widodo et al., 2007) also caused shift in sub-cellular distribution of mortalin from perinuclear to pancytoplasmic type suggesting that these stress-induced premature senescence caused by these drugs involve mortalin. In retrospect, shift in mortalin distribution from perinuclear to the cytoplasmic type can be used as a reliable assay for screening of senescence inducing drugs.

Synthetic peptides. It was found that mortalin binds to a carboxyl terminus region of the tumor suppressor protein p53 (Kaul et al., 2001). By *in vivo* co-immunoprecipitation of mot-2 with p53 and its deletion mutants, the mot-2-binding site of p53 was mapped to its carboxyl terminus 312–352 amino acid residues. We attempted to disrupt mot-2-p53 interactions by overexpression of short p53 carboxyl-terminal peptides and found that p53 carboxyl-terminal peptides (amino acid residues 312–390, 312–352, 323–390, and 323–352) localize in the cytoplasm, whereas 312–322, 337–390, 337–352, and 352–390 locate mostly in the nucleus. Most interestingly, the cytoplasmically localizing p53 peptides harboring the residues 323–337 activated the endogenous p53 function by displacing it from

p53-mortalin complexes and relocating it to the nucleus. Such activation of p53 function was sufficient to cause growth arrest of human osteosarcoma and breast carcinoma cells (Kaul et al., 2005).

Immunotherapy. A hypothesis "mimotope-hormesis", which shows evidence for epitope mimicry (mimotopy) between tumor-up-regulated mortalin and various Hsp70s of infectious agents, seem to bolster the efficacy of mortalin-based immunotherapy. From such mimotope phenomenon, it has been proposed that the assaults of infection during early adulthood could fortify the immune system to evoke more potent defenses against late-onset diseases, such as cancer, via autoimmunity. Interestingly, both experimental and clinical data support the beneficial role of autoimmunity in long-term cancer survivors (Deocaris et al., 2005). Thus, among the clinical applications, mortalin-based vaccine or antibody treatment will certainly be a powerful tool in our fight against cancer.

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CHAPTER 8

ROLE OF MOLECULAR CHAPERONES IN CELL SENESCENCE

MICHAEL Y. SHERMAN* AND JULIA YAGLOM
Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118, USA

Abstract: Many types of cancer cells constitutively express major molecular chaperones at high levels. Recent findings demonstrate that specific depletion of individual chaperones leads to activation of p53 pathway and subsequently triggers cellular senescence. Here we discuss a possibility that in cancer cells high levels of chaperones serve to keep the p53 signaling under control, thus allowing cancer cells to evade the default senescence and form tumors

Originally, at the height of excitement about apoptosis, it was generally accepted that apoptosis plays the major role in control of cancer development. It was established that cells encounter multiple apoptotic stimuli during cancer progression, including activation of pro-apoptotic myc oncogene (Pelengaris et al., 2002, 2000; Prendergast, 1999), anoikis, i.e. substrate detachment-induced apoptosis upon invasion and metastases (Valentijn et al., 2004), apoptotic stimuli of tumor milieu, like TNF, FAS, hypoxia, nutrient deprivation, etc. (Leist and Jaattela, 2001; Zhang et al., 1998). Accordingly, it was suggested that the well-documented anti-apoptotic potential of the heat shock proteins (See *Brunet et al* Chapter 11) may play a critical role in suppression of apoptosis in cancer cells (Jolly and Morimoto, 2000; Mosser and Morimoto, 2004). In line with this suggestion, recent research from many labs dissected multiple effects of the major heat shock proteins, Hsp72 and Hsp27, on various components of multiple apoptotic pathways (Beere, 2005, 2004; Gabai and Sherman, 2002), including Bax, JNK, FAS receptor, Smad, lysosome stability, and others. However, direct involvement of Hsps in suppression of cancer-related apoptosis has not been clearly confirmed. More recently, the attention of the

*Corresponding author: Department of Biochemistry, Boston University School of Medicine, 715 Albany St., K323, Boston, MA 02118, Tel: (617) 638-5971, Fax: (617) 638-5339, e-mail:sherma1@bu.edu

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cancer community has shifted towards a novel role of cell senescence in control of cancer development, and with this shift our view on the role of Hsps in cancer has also evolved towards appreciation of the major role of Hsps in regulation of the senescence program.

Cellular senescence was described as a limit to a number of divisions that a normal cell can undergo. For example, normal fibroblasts can undergo about 60 divisions in culture before acquiring a specific "flat" morphology and becoming permanently growth arrested (Hahn, 2005; Stewart and Weinberg, 2006). Epithelial or hematopoetic cells also have a limit on the number of divisions, which is usually below twenty (Chen and Goligorsky, 2006; Brandes et al., 2005; Fehrer and Lepperdinger, 2005; Sherr et al., 2005; Piacibello et al., 2005; Chen, 2005). Originally it was thought that the replicative senescence is an ultimate result of the telomers shortening, but at present it is commonly accepted that senescence could be triggered by various types of DNA damage that results in accumulation of cell cycle inhibitors p16 and p21 (Jacobs and de Lange, 2005; Ben-Porath and Weinberg, 2005; Sharpless, 2004; Roninson, 2002, 2003). Senescence is a very complex program with multiple end points that include not only growth arrest, but also enlargement of cells, extensive vacuolization, repression and de-repression of certain sets of genes, secretion of various signaling molecules, inhibition of the heat shock response, and other manifestations.

The senescence program seems to represent one of the major breaks on cancer emergence at the cellular level. Indeed, limiting cell divisions seems to be a perfect way of preventing tumor growth (Parkinson et al., 2000). Another alternative to achieve the same goal is through activation of apoptosis. Mammalian cells appear to utilize both programs to counteract action of the major oncogenes. In fact, as counterintuitive as it sounds overexpression of major oncogenes could either activate apoptosis, as seen with myc, or trigger senescence as seen with Ras, Her-2, PTEN, Raf, and others oncogenes of the Ras pathway (Sebastian et al., 2006; Benanti and Galloway, 2004; Mason et al., 2004; Peeper et al., 2001; Chen et al., 2005; Olsen et al., 2002; Zhu et al., 1998).

The discovery that cancer cells could become senescent was quite unexpected since tumor cells by definition divide indefinitely. Nevertheless, being unrestrained by the replicative senescence, many cancer cells still could undergo senescence (so-called premature senescence) in response to activation of oncogenes (Figure 1), e.g. Ras or Her-2, or to various DNA-damaging drugs, like doxorubicin (Chang et al., 1999a,b).

Oncogenes of the Ras pathway activate two parallel pathways, including the proliferation response and p53. Normally p53 induces p21 and causes growth arrest and senescence, thus preventing cell proliferation. However, high levels of Hsps inhibit p53 and allow cells to proliferate.

In fact, the major mechanism of action of many anti-cancer drugs seems to be activation of the premature senescence program (Lowe et al., 2004; Narita and Lowe, 2004). These data demonstrate that the senescence program remains functional even after cancer transformation. Premature senescence in cancer cells

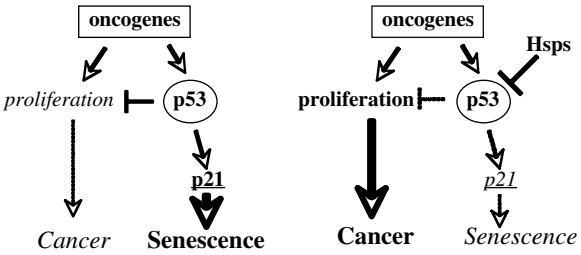

Figure 1. Role of Hsps in suppression of the oncogene-induced senescence

depends upon activation of p21, while p16 seems to be involved only in senescence of normal cells.

In general oncogenes of Ras pathway activate the senescence program via several mechanisms that involve activation of p53, the transcriptional activator of p21. For example, these oncogenes can activate the DNA damage response kinase ATM because of excessive activation of DNA replication forks, and ATM phosphorylates and stabilizes p53 (Di Micco et al., 2006). Another mechanism involves induction of p19ARF that inhibits the p53 ubiquitin ligase Hdm2, also leading to stabilization of p53 (Bihani et al., 2004). In turn stabilization of p53 leads to its accumulation and activation. Since cancer cells have active oncogenes that can trigger cell senescence, in order to proliferate they have to acquire mechanisms that completely or partially suppress p21. Indeed, it is well known that mutations that result in disabling of p21 transcriptional regulator, p53, are extremely common in human cancers. However, in many cancer types the p53 pathway remains functional, and therefore alternative mechanisms of suppression of p21 must operate in these tumors.

How cancer cells with activated oncogenes and intact p53 pathway escape senescence? Cancer cells constitutively overexpress several major chaperones (Calderwood et al., 2006). It appears that these chaperones play an important role in suppressing the senescent program, thus allowing cancer cells to proliferate. A critical observation on the role of chaperones in suppression of cellular senescence was the finding that a specific downregulation of Hsp70-2, a member of Hsp70 family, leads to rapid senescence of various cancer cell lines (Rohde et al., 2005; Daugaard et al., 2005). Hsp70-2 was originally described as a testis-specific chaperone, but more recently it became clear that it is widely expressed in a variety of cancer cell types (Rohde et al., 2005; Daugaard et al., 2005). Depletion of this protein using siRNA approach caused permanent G1 arrest, cell enlargement and flattening typical for senescent cells. These alterations were associated with upregulation of p53. Gene array analysis showed that expression of a large number of genes was significantly altered (up- or downregulated) upon Hsp70-2 depletion. The pattern of the gene expression alterations had a lot of similarities to that found in senescence caused by doxorubicin (Rohde et al., 2005; Chang et al., 2002, 2000). One of the genes upregulated at both conditions was MIC-1, a cytokine of TGFβ family, which appeared to be critical for senescence. In fact, expression of siRNA

against MIC-1 prevented development of flat morphology and other hallmarks of senescence after depletion of Hsp70-2. Overproduction of MIC-1 is controlled by p53. Therefore the primary effect of Hsp70-2 depletion seems to involve p53 activation, which in turn triggers the senescence program. It is likely that this process involves induction of p21, because it is a direct p53 transcriptional target. On the other hand, MIC-1 appears to serve as an additional critical p53-dependent co-stimulator of cell senescence. It is likely that MIC-1 is secreted from cells and further promotes the senescence program, thus providing both autocrine and, possibly, paracrine stimulation. Thus Hsp70-2 seems to play a role in keeping p53 pathway suppressed, and Hsp70-2 depletion leads to the abrogation of this control and reactivation of the default senescence program.

A distinct member of the Hsp70 family, the major inducible heat shock protein Hsp72 plays a similar function in cancer cells, though the ways of controlling the p53 pathway by Hsp70-2 and Hsp72 appear to be different (see below). Depletion of Hsp72 led to a strong activation of p53, induction of p21, cell cycle arrest at both G1 and G2 phases, appearance of acidic β-gal activity and other hallmarks of senescence (Yaglom et al., 1993). Previously, Hsp72 was shown to bind p53 (Nihei et al., 1993; Ehrhart et al., 1988) and retain it in cytoplasm, implying that depletion of Hsp72 would release p53 and allow its nuclear translocation and activation of the transcription targets. In addition, p53 was stabilized upon depletion of Hsp72, due to reduction of activity of the p53 ubiquitin ligase Hdm2. Defining the primary effect of Hsp72 depletion on p53 pathway is rather tricky since p53 and Hdm2 represent a negative feedback loop, where p53 induces Hdm2, while Hdm2 facilitate degradation of p53. However, it was shown that suppression of Hdm2 upon Hsp72 depletion is the primary event, since under these conditions inhibition of Hdm2 was seen even in p53 knockout cells (Yaglom et al., 1993).

Interestingly, though p53 and p21 clearly contributed to development of senescence in Hsp72-depleted cells, other pathways also appear to be involved since a significant senescence was seen after Hsp72 depletion in p53 knockout cells. The p53-independent senescence pathway under these conditions was associated with the reduction of expression and increase in the inhibitory phosphorylation of the cdc2 kinase (Yaglom et al., 1993). In line with this finding, partial depletion of cdc2 also caused senescence in p53 knockout cells.

There are reports that yet another member of the Hsp70 family, i.e. mitochondrial chaperone Grp75 also could activate senescence pathway through the control of p53 (See: *Wadhwa*, Chapter 7). Being the major mitochondrial chaperone, Grp75 shows a pan-cytosolic localization in normal cells. Upon immortalization, however, it changes its localization and moves into the perinuclear zone (Wadhwa et al., 1996, 1999). Association of this protein with cell immortalization is reflected in its alternative name, mot-2 (mortalin). Interestingly, overexpression of mot-2 protein in *C.elegans* significantly extended the worm's life span (Yokoyama et al., 2002). Importantly, overexpression of mot-2 in normal human lung fibroblasts led to a temporal escape of fibroblasts from the replicative senescence, allowing extra 12-18 doublings (Kaul et al., 2003). Effects of mot-2 on temporal suppression of

senescence were associated with inhibition of p53. Mot-2 directly interacts with p53 and suppresses its activity by sequestering p53 in cytosol (Wadhwa et al., 1998, 2002; Kaul et al., 2005). Accordingly, disruption of mot-2 –binding to p53 by specific peptides leads to p53 translocation to nucleus and concurrent activation (Wadhwa et al., 2000). Therefore, several major Hsp70 family members appear to be involved in the control of p53.

Members of the Hsp70 family are not unique in their ability to interfere with senescence, as depletion of Hsp27 in transformed cells, similarly led to activation of the senescence program. In fact, upon siRNA-mediated depletion of Hsp27 human colon tumor cells, HCT 116 acquired all major hallmarks of senescence associated with activation of p53 and induction of p21 (O'Callahan et al., 2007). Similar effects were seen upon depletion in HeLa cells of a distinct chaperone vcp/p97 that controls delivery of ubiquitinated substrates to proteasome. Vcp/p97 downregulation led to activation of p53 and subsequent p21 induction and cell cycle arrest accompanied by cell flattening, vacuolization, and enlargement (Wojcik et al., 2004).

As discussed previously, some chaperons could directly interact with p53 and suppress its activity. In addition, the fact that depletion of different chaperones results in activation of p53 and leads to senescence suggests that these effects could be mediated through the chaperones' general involvement in protein folding and degradation. In fact, there is a possibility that depletion of individual chaperones may reduce a refolding/degradation capacity of cells, thus causing a build-up of abnormal polypeptides, proteotoxicity and subsequent activation of p53. In line with this suggestion, certain protein damaging stresses that cause a build-up of abnormal polypeptides, like heat shock or oxidative stress, lead to activation of p53 (Miyakoda et al., 2002; McNeill-Blue et al., 2006; Das and Dashnamoorthy, 2004). However, surprisingly, targeted depletion of individual chaperones Hsp72 or Hsp27 in HCT-116 cells did not result in accumulation of abnormal protein species. Accordingly, no accumulation of oxidized or ubiquitinated proteins were detected (O'Callahan et al., 2007). Ubiquitin-proteasome degradation and refolding of model substrates were normal, indicating that UPS and the chaperone machinery were not overwhelmed. Moreover, there was no activation of the heat shock response, suggesting the lack of the proteotoxic stress (O'Callahan et al., 2007). Furthermore, depletion of either Hsp72 or Hsp27 had only minor effects on heat shock sensitivity of these cells, as judged by activation of apoptosis. Therefore, effects of depletion of individual chaperones on the p53 pathway appear to control this pathway by specific mechanisms, rather than through evoking general proteotoxic stress The findings that distinct chaperones are involved in control of the p53 and possibly other senescence pathways may help to explain why chaperones are often overexpressed in a variety of cancers. In fact, as discussed in Chapter 2 in this book, high expression of Hsp72 and Hsp27 often correlates with the grade of tumors, drug resistance and overall poor prognosis. Similarly, Hsp70-2, mot-2 and vcp/p97 were reported to be highly expressed in various tumors, and with some tumors they could be used as prognostic factors (Calderwood et al., 2006). Therefore cancer cells in contrast to normal cells selectively overproduce these chaperones individually or together.

The overproduction of chaperones in tumors could be achieved through various mechanisms, including overexpression of the major heat shock transcription factor Hsf1 (Tang et al., 2005; Hoang et al., 2000), phosphorylation of Hsf1 by Akt kinase activated by certain oncogenes (Khaleque et al., 2005), expression of a splicing isoform of p63 (Wu et al., 2005) and probably other mechanisms.

What could be the specific physiological significance of increased chaperone expression in cancers? As mentioned above, activation of certain oncogenes (e.g. Ras, Her-2 or PTEN mutation) in normal cells stimulate p53 and forces cells to senesce, which serves as an important defense mechanism against cancer transformation. Depletion of various chaperones triggers senescence suggesting that chaperones serve as endogenous suppressors of a latent senescence program. In other words, in many cancers various chaperones keep p53 under control, thus allowing cancer transformation to occur (Figure 1). It is possible that tumors accumulate chaperones as a means to avoid the oncogene-induced senescence. This mechanism of evading activation of the senescence program in tumors could be an alternative to acquiring mutations in p53 pathway, which takes place in a large fraction of tumors. All together these data imply that heat shock proteins could serve as new important targets for anticancer therapies.

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CHAPTER 9

INVOLVEMENT OF HEAT SHOCK PROTEINS IN PROTECTION OF TUMOR CELLS FROM GENOTOXIC STRESSES

CORNELIA O'CALLAGHAN-SUNOL AND VLADIMIR L. GABAI*
Boston University Medical School, Boston, MA 02118, USA

Abstract: Anti-apoptotic functions of heat shock proteins Hsp70 and Hsp27 are well established. However, radiation and genotoxic antineoplastic drugs at clinically relevant doses induce apoptosis mostly in lymphoid cells, while in epithelial tumors they evoke different type of response, mainly senescence and mitotic catastrophe, which leads to loss of clonogenic potential of cells. Here we review old and new data showing that upregulation of Hsp27 or Hsp70 levels protect various tumor cell lines from gamma- and UV-radiation and genotoxic anti-neoplastic drugs. Accordingly, downregulation of Hsp27 or Hsp70 levels by antisense or siRNA sensitizes tumor cells to these agents. Importantly, protection and sensitization by modulation of Hsp27 or Hsp70 levels were manifested not only by modulation of apoptosis, but by clonogenic survival as well, and recent data indicate that these Hsps can suppress also drug-induced senescence. Several studies demonstrated that intrinsic and acquired chemo- and radioresistance in tumor cell lines and in patients with certain forms of cancers can be associated with upregulation of Hsp27 and/or Hsp70. Possible mechanisms of Hsp-induced protection, in particular, modulation of p53-dependent and p53-independent DNA-damaging signaling pathways, are discussed

Keywords: Hsp27, Hsp70, radio- and chemoresistance, senescence, clonogenic survival, apoptosis, p53

INTRODUCTION

Radiation and genotoxic drugs are still the most common agents in treatment of various forms of cancer. However, despite the progress in chemo-and radiotherapy, resistance of tumor cells to the treatment is the main obstacle in cancer cure. Therefore, elucidation of mechanisms of tumor cell resistance to DNA-damaging agents will help to find new drugs or their combination. Among various endogenous factors of tumor radio-and

*Corresponding author: Boston University Medical School, Boston, MA 02118, USA, K-323, Department Biochemistry; Boston University School of Medicine, 715 Albany St, Boston, MA 02118, USA, Tel: 1-617-638-5970, Fax: 1-617-638-5339, e-mail: gabai@biochem.bumc.bu.edu

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chemoresistance, heat shock proteins (Hsps) apparently play an important role (See *Ciocca et al*, Chapter 2, this volume). As discussed elsewhere in this book, various tumors express higher levels of Hsps comparing to normal tissues, and overexpression of Hsps is regarded as important anti-apoptotic mechanism during tumor development. Hsps may also protect cancer cells from harmful factors of tumor microenviroment like hypoxia, or immune attack (TNF, FAS). Anti-apoptotic function of Hsps, especially Hsp70, is often considered as a major factor in chemoresistance of human cancers (See: *Brunet*, Chapter 11). However, no clear correlation was found between pro- or anti-apoptotic markers and chemo- radioresistance of most common solid tumors. Furthermore, despite numerous efforts, only a few drugs directly modifying apoptotic pathway are currently in clinical trials. Such disappointing results apparently lie in overestimation of role of apoptosis in cancer cell death upon conventional drug and radiation treatment. It is now becoming obvious that apoptosis is the major form of cell demise only in lymphoid cells, whereas in most common epithelial tumors (e.g., breast or prostate) clinically relevant doses of radiation or genotoxic drugs do not cause any apoptosis. Instead, as it was demonstrated during last years, they may cause growth arrest leading to DNA repair and cell survival, or, if DNA repair is unsuccessful, it led to premature senescence, or mitotic catastrophe (see ref (Roninson, 2003; Roninson et al., 2001; Schmitt, 2007) for review) (Figure 1). Numerous *in vitro* studies clearly demonstrated that senescence and/or mitotic catastrophe rather than apoptosis is the main cause of elimination of epithelial tumor cells (Schmitt, 2007). Although mitotic catastrophe is difficult to assess *in situ*, markers of senescence (beta-galactosidase staining) was indeed found in patients undergoing chemotherapy (Roninson, 2003; Schmitt, 2007). As a combined measure of different modes of cell death, the most reliable method is apparently clonogenic assay, since it measures ability of a cell to divide and form a colony. Therefore, this assay is basically independent on the way how

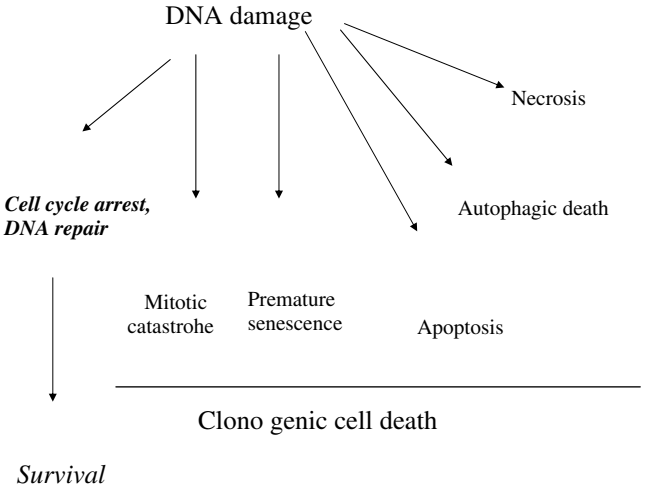

Figure 1. Responses of a cell to DNA damage (see text for explanations)

a cell is killed, e.g., by apoptosis, necrosis, autophagic cell death, mitotic catastrophe, or some unknown at present time mechanism, and senescent cell can not form colonies as well (Figure 1). It is not surprising that in many cases inhibition of apoptosis of tumor cells does not preserve their clonogenic ability since irreversibly damaged cells die by other mechanisms (see, e.g. (Zhang et al., 2006)). Invented more than five decades ago, this assay is still a gold standard for evaluation of cell sensitivity to radiation and various drugs, and there is a good correlation between clonogenic ability of cells *in vitro* and tumor response *in vivo*. In this chapter we discuss some old and new data regarding role of Hsp27 and Hsp70 in chemo- and radioresistance of tumor cells.

HSP27 AND RESISTANCE OF CELLS TO GENOTOXIC STRESSES

The first works addressing the role of Hsp27 in resistance to genotoxic drugs were performed during the early 90s (See: *Arrigo*, Chapter 4). Hout et al found that overexpression of Hsp27 conferred resistance to doxorubicin, as well as other drugs such as colchicine, vincristine, actinomycin D, hydrogen peroxide and sodium arsenite (Huot et al., 1991). The extent of doxorubicin resistance was proportional to the levels of Hsp27 in different clones. As a result of this work, Hsp27 was proposed as a determinant of clinical resistance to antineoplastic drugs. Other works from several groups have confirmed these findings and it is now clear that Hsp27 plays a role in acquisition of resistance to doxorubicin. This drug exerts its cytotoxic action via inhibition on topoisomerase II and subsequent generation of lethal double strand breaks in the DNA. Other inhibitors of topoisomerases are used in the clinics are etoposide (inhibitor of topoisomerase II) and camptothecin (inhibitor of toposiomerase I), and Hsp27 has been highlighted as a molecule conferring chemoresistance in both cases (Table 1). In addition to chemotherapeutic drugs, radiation is the most common agent in treatment of various forms of cancer, and several reports indicate that increased levels of Hsp27 can confer radioresistance to various cell types (Table 1). Below we will discuss available data as well as some new insights regarding the mechanisms of protection of Hsp27 from most studied agents, topoisomerase II inhibitors and ionizing radiation.

The cellular response to genotoxic stressors, either to die or not to die, may largely depend on the extent of the damage in the DNA. It is therefore logical to suggest that the protective role of Hsp27 could be due to an ability to reduce the genotoxic burden in the cells. This could be achieved either by decreasing the damage per se or increasing the repair capability of cells. In this sense, it was showed that thermotolerant cells accumulated less DNA damage after doxorubicin and repaired DNA aberrations more efficiently than non-thermotolerant counterparts as assessed by alkaline comet assay (Nadin et al., 2003), a method to determine overall DNA damage (single and double strand breaks etc). It is important to stress, however, that thermotolerant cells overexpress a cohort of Hsps, not only Hsp27, and therefore it is difficult to draw conclusions regarding the role of particular Hsps in DNA repair mechanisms from this experiment.

Table 1. Effects of modulation of Hsp27 levels on response to DNA damaging agents

Treatment	Cell line	Hsp27 levels modulation	Effect/assay	Reference
Doxorubicin (topoisomerase II inhibitor)	MCF-7 human breast carcinoma	Heat shock	Increase in colony forming ability in soft-agar	(Ciocca et al., 1992)
		Antisense	Decrease in colony forming ability in soft-agar	(Oesterreich et al., 1993)
	MDA-MB-231 human breast carcinoma	Heat shock	Increase in colony forming ability in soft-agar	(Ciocca et al., 1992)
		Overexpression	Increase in colony forming ability in soft-agar	(Oesterreich et al., 1993)
		Overexpression	Decrease in apoptosis (DNA laddering, cell morphology)	(Hansen et al., 1999)
	HCT116 human colon carcinoma	siRNA	Decrease in clonogenic survival, increase in senescence	O'Callaghan-Sunol et al, submitted
	Chinese hamster ovary	Overexpression	Increase in clonogenic survival	(Huot et al., 1991)
Etoposide (topoisomerase II inhibitor)	U937 human leukemia	Overexpression	Decrease in apoptosis (Hoescht staining)	(Parcellier et al., 2003)
	TF-1 human erythroleukemic cell line	siRNA	Increase in apoptosis (anexin V/PI staining)	(Schepers et al., 2005)
Camptothecin (topoisomerase I inhibitor)	Wehi-s murine fibrosarcoma	Overexpression	Decrease in apoptosis (MTT)	(Samali and Cotter, 1996)
Cisplatin(crosslinker)	HT-29 human colon carcinoma	Overexpression	Decrease in apoptosis	(Garrido et al., 1997)
	Chinese hamster ovary	Overexpression	Increase in clonogenic survival	(Fortin et al., 2000)
Ionizing radiation	L929 murine fibroblast	Overexpression	Increase in clonogenic survival; decrease in apoptosis (DNA fragmentation)	(Park et al., 2000a)

(*continued*)

Table 1. (*continued*)

Treatment	Cell line	Hsp27 levels modulation	Effect/assay	Reference
			Decrease in apoptosis	(Cho et al., 2001)
			Increase in clonogenic survival	(Yi et al., 2002)
			Increase in clonogenic survival; decrease inapoptosis (DNA fragmentation)	(Cho et al., 2002)
	Mouse RIF (radiation induced fibrosarcoma)	Overexpression	Clonogenic survival Apoptosis (chromatin condensation)	(Lee et al., 2002)
	MEF wt and Hsf1-/-	Overexpresion	Decrease in apoptosis (PARP cleavage TUNEL assay), necrosis (PI staining); increase in clonogenic survival.	(Kabakov et al., 2006)
	Jurkat T-cell leukemia cells	Overexpression	Decrease in apoptosis (DNA fragmentation and caspase activation)	(Lee et al., 2005)
	DU145 human prostate cancer	Antisense	Decrease in clonogenic survival	(Teimourian et al., 2006)
UVC	HCT116 colon carcinoma cells	siRNA	Decrease in clonogenic survival	Gabai et al, manuscript in preparation
	human embryo-derived fibroblastic cell line	Antisense	Decrease in clonogenic survival	(Wano et al., 2004)

Note: No protection by Hsp27 upregulation from gamma-radiation: (Ekedahl et al., 2003; Fortin et al., 2000)

Wano et al (Wano et al., 2004) established a clear protective role of hsp27 to UVC induced damage. They demonstrate that overexpression of Hsp27 resulted in more efficient clearance of toxic motifs –thymidine dimers and (6-4)photoproducts- in response to damage induced by UV light. Finally, Kabakov et al showed by alkaline and neutral comet assays that overexpresion of Hsp27 lead to decreased initial DNA damage upon ionizing radiation (Kabakov et al., 2006). It is important to note that whereas alkaline comet assay measures all types of breaks in the DNA, neutral comet measures only double strand breaks. In this work, the protective effect of Hsp27 was accompanied with suppression of p53 accumulation after exposure to radiation, suggesting that reduction in p53 activation may be a consequence of attenuation of its upstream activator, DNA damage (Kabakov et al., 2006).

Data in our lab (Callaghan-Sunol et al, submitted) has shown that downregulation of Hsp27 by siRNA in human colon carcinoma sensitized cells to low doses of doxorubicine. This phenomenon was associated with exacerbated senescence upon doxorubicin treatment in Hsp27-depleted cells (Figure 2A). Moreover, downregulation of Hsp27 on its own lead to appearance of senescent phenotype, indicating that Hsp27 can protect cancer cell lines from endogenous as well as drug-induced senescence. It is now well established that DNA damage can trigger the activation senescence programs, in particular, through p53/p21 pathway. We did not observe

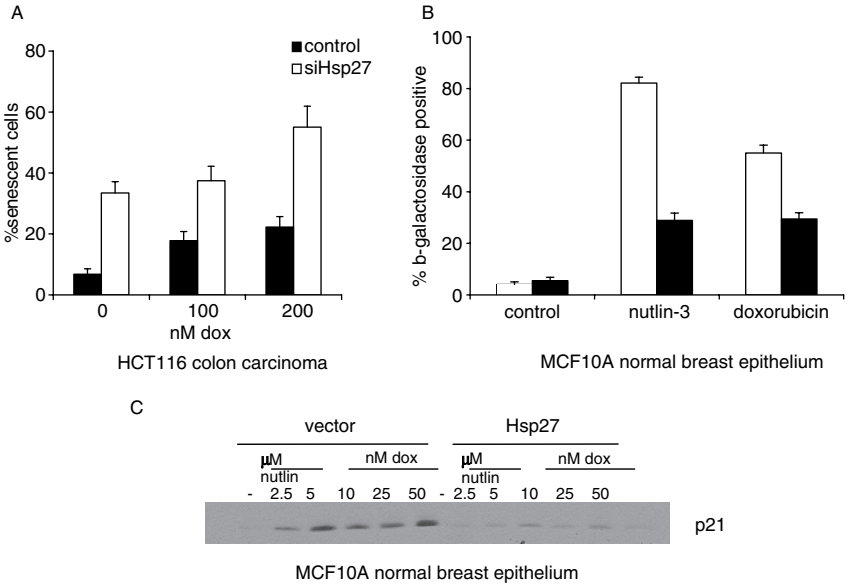

Figure 2. Hsp27 inhibits senescence induced by doxorubicin (colon carcinoma HCT116 cells and normal breast epithelial cells MCF10A) and nutlin-3 (MCF10A) by suppression of p53-p21 cascade. **A.** Percentage of senescent cells (enlarged flat cells) upon treatment with doxorubicin in control and Hsp27-depleted HCT116 cells. **B.** Percentage of senescent cells (as b-galactosidase positive cells) upon doxorubicin and nutlin-3 treatment in MCF10A control and overexpressing cells. **C.** Accumulation of p21 upon doxorubicin and nutlin-3 treatment in MCF10A control and Hsp27 overexpressing cells

either appearance of DNA damage or activation of upstream components of the DNA damage response upon depletion of Hsp27, indicating that depletion of Hsp27 per se does not lead to genotoxic stress. We did observe, however, activation of p53 and accumulation of p21, suggesting that high levels of Hsp27 in cancer cells suppress p53 activation. Moreover, we found that overexpression of Hsp27 in human normal breast epithelial cells protected cells from senescence induced by doxorubicin (Figure 2B). This effect of hsp27 was accompanied by suppression of p53 activation (accumulation of p21) upon treatment with doxorubicin (Figure 2C). Interestingly, similar effects were found when cells were treated with nutlin-3 (Figure 2B, C), a small molecule that inhibits Mdm2 and therefore activates p53 without generating DNA damage, indicating that in our cellular systems Hsp27 contributes to survival of cells by suppression DNA damage response at the level of p53, independently of DNA damage.

In addition to decrease in the genotoxic burden and suppression of p53 response, Hsp27 cellular levels have been shown to modulate several survival pathways. Here we will focus on the role of Hsp27 in the regulation of NF-κB, PKCδ and p38 and c-jun kinases and what consequences it might have for the survival of cells.

Works from two independent labs have shown that overexpression of Hsp27 results in downregulation of IκB and therefore, higher activity of the transcription factor NF-κB, a well-known pro-survival factor. According to Parcellier et al. this effect is the result of enhanced activation of the proteasomal activity in Hsp27 overexpressing cells when treated with etoposide or TNFα (Parcellier et al., 2003). Hsp27 mediates the degradation of IκB by facilitating the delivery of phosphorylated form of IκB to the proteasome when cells are treated with the above mentioned stressors, accelerating its degradation. This effect appears to be specific for Hsp27, since other chaperones that confer resistance to TNFα or etoposide, like Hsp70, do not seem to bind to phospho-IκB (Parcellier et al., 2003). In another work, Yi et al. address role of NF-κB hyperactivation by Hsp27 in cellular protection from ionizing radiation (Yi et al., 2002). They showed that overexpression of Hsp27 increased transcriptional activity of NF-κB that, in turn, lead to accumulation of MnSOD, a superoxide scavenger. Since ionizing radiation exerts its detrimental effects on cell viability partially via the generation of reactive oxygen species (ROS), it is natural to suggest that increased levels of MnSOD can contribute to the protective effects of Hsp27 (Figure 3).

PKCδ is a member of the protein kinase C family, and it is associated with suppression of cell cycle progression and activation of apoptosis. Modulation of this protein, therefore, may be crucial to dictate the fate of cells, especially lymphoid, upon treatment with genotoxic agents. In Jurkat cells, overexpression of Hsp27 lead to suppression of PKCδ activation and reduced generation of ROS upon treatment with ionizing radiation (Lee et al., 2004) (Lee et al., 2005). In another work by the same authors, reduced activation of PCKδ upon Hsp27 overexpression was attributed to decreased ROS content in murine fibroblasts, most likely due to increased levels of MnSOD (see above) in the cell. Suppressed activation of PKCδ resulted in reduced activation of ERKs, and this suppressed activation of ERKs was suggested to be responsible for protective effects of Hsp27 (Cho et al., 2001; Cho et al., 2002). Although it is generally understood that activation of ERKs leads to cell survival,

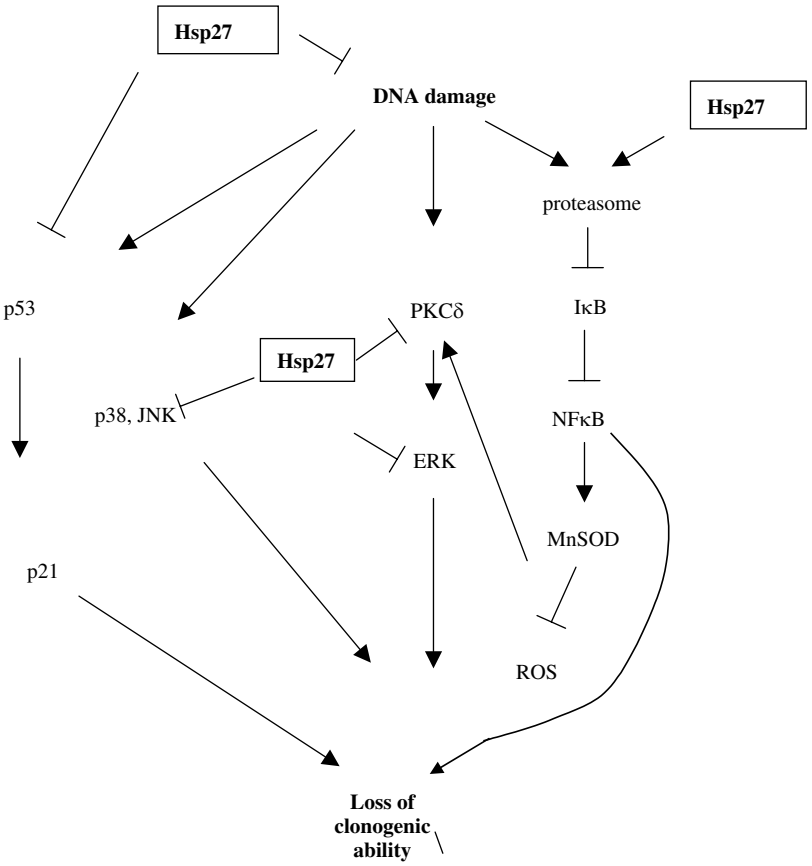

Figure 3. Suggested mechanisms of protection from genotoxic stresses by Hsp27 (see text for details)

it now accepted that in some scenarios hyperactivation of this MAP kinase cascade can be detrimental for cell viability. The ability of Hsp27 to modulate MAP kinase signaling has also been demonstrated for other two kinases in the same family: p38 and JNK. It was shown that treatment of leukemic cells with etoposide lead to apoptotic cell death characterized by cytochrome c release from mitochondria, which was preceded by activation of p38 and JNK kinases. Interestingly, cells where Hsp27 was downregulated were more sensitive to etoposide and p38 and JNK activation as well as cytochrome release from mitochondria was exacerbated (Schepers et al., 2005).

HSP70 AND RESISTANCE OF CELLS TO GENOTOXIC STRESSES

Historically, the effect of Hsps on tumor cell resistance to genotoxic stresses was first studied by radiobiologists in early 80-s when hyperthermia in combination with radiation was introduced in clinic. The main conclusion of these studies was that

thermotolerant cells (i.e. cells with high levels of Hsps) generally do not demonstrate higher radioresistance (see, e.g. (Harston-Eaton et al., 1984)). In early 90-s, however, it was shown that thermotolerant breast tumor cells do demonstrate resistance to some DNA-damaging drugs, e.g., doxorubicine, but not others (e.g. cisplatin, 5-fluorouracil) (Ciocca et al., 1992). Later, besides doxorubicine, resistance to other clinically used anti-cancer drugs such as campothecin, topotecan, etoposide, cisplatin and others was shown (see Table 2). As one can see from the Table 2, protection from various types of DNA-damaging agents (topo I and II inhibitors, crosslinkers, inhibitors of DNA synthesis, UV and gamma-radiation) was found in different normal and transformed tumor cell lines, although in some cells no protection was seen (Table 2). Of note, Hsp70 (also called Hsp72) can protects from almost the same treatments as Hsp27 (cf Table 1). Unfortunately, however, in most of these studies, especially in 90s, high doses of drugs and only short-term viability assay (apoptosis) were used. As we discussed in Introduction, apoptosis is not the main mode of epithelial tumor cell death upon low doses of drugs, so the relevance of most of these studies to clinics is unclear. But for lymphoid cells where protection from drug-induced apoptosis by Hsp70 was also seen, apoptosis usually represent the main mode of their death. Since mechanisms of anti-apoptotic function of Hsps are discussed elsewhere in this book, here we will describe only that is related to genotoxic part of apoptotic pathway.

But first lets consider data regarding effects of Hsp70 on DNA damage itself since decreased damage and/or increased repair obviously should decrease apoptosis and increase overall cell survival. In 2001 R. Bases and collegues found that Hsp70 can associate in vivo and in vitro with HAP-1 endonuclease, a key enzyme in base-excision repair (BER), and stimulates its activity (Kenny et al., 2001). Interestingly, N-terminal ATPase fragment of Hsp70, but not C-terminal substrate-binding fragment was sufficient for activity (Mendez et al., 2003). In recent studies in human leukemic cells R. Bases demonstrated that treatment with siRNA to Hsp70 inhibited repair of abasic sites and sensitized cells to gamma-radiation; however, no evidence of Hsp70 depletion in these cells was presented (Bases, 2005, 2006). In mice with knockout of major inducible Hsp70, Hsp70.1 and Hsp70.3, Hunt et al. found increased levels of spontaneous or radiation-induced chromosomal aberrations and suggested that Hsp70 may be involved in maintaining genomic stability (Hunt et al., 2004). In Hsp70.1/3 knockout fibroblast the authors also found decreased telomerase activity and less inhibition of radiation-induced of DNA-synthesis (apparently indicating impairment of cell cycle checkpoints), but mechanisms of these effect were not elucidated (Hunt et al., 2004). Recent study of Kabakov et al. demonstrated that Hsp70 may directly protect DNA from damage. In MEF expressing Hsp70, radiation-induced DNA damage (assessed by alkaline and neutral comet assays) was significantly reduced (Kabakov et al., 2006). Accordingly, in human lung carcinoma A549, Niu et al (Niu et al., 2006) found that Hsp70 overexpression decreased UVC-induced DNA damage (by alkaline comet assay). Therefore, Hsp70 may be directly involved in protection/repair of DNA, however, further studies are necessary to elucidate the mechanisms.

Table 2. Effects of modulation of Hsp70 levels on response to DNA damaging agents

Treatment	Cell line	Hsp70 level modulation	Effect/assay	References
Campothecin(topo I inhibitor)	U937 human lymphoid	Heat shock	Decrease in apoptosis (morphology, DNA ladder)	(Samali and Cotter, 1996)
	Jurkat human lymphoid	Heat shock, Overexpression	Decrease in apoptosis (Morphology, Tunel)	(Creagh and Cotter, 1999)
	Wehi murine fibrosarcoma	Overexpression	Decrease in apoptosis (MTT, Tunel, DNA Ladder)	(Samali and Cotter, 1996)
Topotecan(topo I inhibitor)	Wehi murine fibrosarcoma	Overexpression	Decrease in apoptosis (MTT)	(Sliutz et al., 1996)
Etoposide(topo II inhibitor)	U937 human lymphoid	Heat shock	Decrease in apoptosis (morphology, DNA ladder)	(Samali and Cotter, 1996)
	MEF	Overexpression	Decrease in apoptosis (MTT)	(Nylandsted et al., 2004)
		ADD70 binding[1]	Increase in apoptosis	(Schmitt et al., 2003)
Doxorubicin (topo II inhibitor)	HL-60	Geldanamycin treatment, overexpression	Decrease in apoptosis (PARP, chromatin condensation, MTT)	(Demidenko et al., 2005)
	MCF-7, MDA-MB231 breast carcinoma	Heat shock	Increase in clonogenic survival	(Ciocca et al., 1992)
	Wehi murine fibrosarcoma	Overexpression	Decrease in apoptosis (MTT assay)	(Karlseder et al., 1996) (Jaattela et al., 1998)
	ME-180 human cervix carcinoma	Antisense	Increase in apoptosis (MTT assay)	(Jaattela et al., 1998)
	HeLa cervix, HCT116 colon carcinoma	siRNA	Decrease in clonogenic survival, increase in senescence	Gabai et al (submitted)
Cisplatin (crosslinker)	MEF, U937, MCF7, HeLa, SW480, HT29, B16	ADD70 binding[1]	Increase in apoptosis (PI, crystal violet)	(Schmitt et al., 2003)
	B16 mouse melanoma, Prob rat colon carcinoma			(Schmitt et al., 2006)

	PC-3 human prostate carcinoma	Antisense	Decrease in clonogenic survival	(Gabai et al., 2005)
Gemcitabine(DNA synthesis)	Wehi murine fibrosarcoma	Overexpression	Decrease in apoptosis (MTT assay)	(Sliutz et al., 1996)
γ–radiation	3T3 mouse fibroblasts	Overexpression	Decrease in apoptosis(Hoehst staining)	(Lee et al., 2001)
	3T3 mouse fibroblasts, RIF mouse fibrosarcoma	Overexpression	Increase in clonogenic survival	(Park et al., 2000b)
	MEF	Overexpression	Increase in clonogenic survival, apoptosis (PARP cleavage, TUNEL assay), necrosis (PI staining)	(Kabakov et al., 2006)
		Hsp70 k/o; overexpression	Increase in clonogenic survival	(Hunt et al., 2004)
	PC-3 human prostate	Antisense	Decrease in clonogenic survival	(Gabai et al., 2005)
	THP-1 human leukemia	siRNA	Decrease in clonogenic survival	(Bases, 2005)
	HCT116 colon carcinoma	siRNA	Decrease in clonogenic survival, increase in senescence	Gabai et al (submitted)
UVB	Wehi murine fibrosarcoma	Overexpression	Decrease in apoptosis (MTT)	(Simon et al., 1995)
UVC	MEF	Overexpression	Decrease in apoptosis	(Steel et al., 2004)
	HCT116	siRNA	Decrease in clonogenic survival	Gabai et al, submitted

[1]ADD70 binding – neutralization of Hsp70 by expression of peptide derived from apoptosis-inducing factor (AIF)

Note: No protection from apoptosis: In MEF: Hsp70-overexpression: etoposide (Steel et al., 2004); γ-radiation (Buzzard et al., 1998) Jurkat, lung carcinoma by HS: γ-radiation (Ekedahl et al., 2003).

No sensitization to apoptosis: In lung carcinoma: by siHsp70: γ-radiation, cisplatin, etoposide (Ekedahl et al., 2003). In PC-3 cells: by antisense - to doxorubicine, UVC, etoposide (Gabai et al., 2005). In colon adenocarcinoma: by antisense - to doxorubicine (Musch et al., 2001).

Two major components are now considered as upstream mediators of DNA-damage-induced apoptosis: p53 and caspase-2 (see ref (Norbury and Zhivotovsky, 2004; Roos and Kaina, 2006) (Zhivotovsky and Orrenius, 2005) for review). Surprisingly, though, there is no data regarding effect of Hsp70 on caspase-2 activity, although Hsp70 apparently inhibits apoptosis upstream mitochondria thus suppressing caspases-9 and 3 (Steel et al., 2004). There are few publications where effect of Hsp70 on p53 was studied. Lee et al found that overexpression of Hsp70 in 3T3 cells reduced accumulation of p53 and its downstream target p21 after gamma-radiation (Lee et al., 2001). Accordingly, overexpression of Hsp70 in MEF also diminished p53 accumulation (Kabakov et al., 2006). We have recently found that modulation of p53 pathway by Hsp70 may be critical for its protective effect. In HCT116 human colon carcinoma cells decrease in Hsp70 expression by siRNA sensitized them to three diverse types of genotoxic agents: UV-radiation, gamma-radiation and doxorubicin (Gabai et al, submitted). Of note, these treatments did not cause apoptosis; instead, they provoke mainly mitotic catastrophe (under UVC radiation), senescence (upon doxorubicine treatment) or combination of both (upon gamma-radiation). Apparently, sensitizing effect of Hsp70 in these cells was dependent on p53, since it disappeared in p53 knockout derivate of this cell line. Using alkaline and neutral comet assays, we assessed DNA damage in Hsp70-depleted cells but did not find significant difference. However, Hsp70 depletion stabilized p53 upon gamma-radiation and doxorubicin and caused higher p21 accumulation (Figure 4A, B). Interestingly, Hsp70 downregulation, similar to Hsp27 downregulation (see above), in HCT116 cells and several other tumor cell lines by itself causes activation of

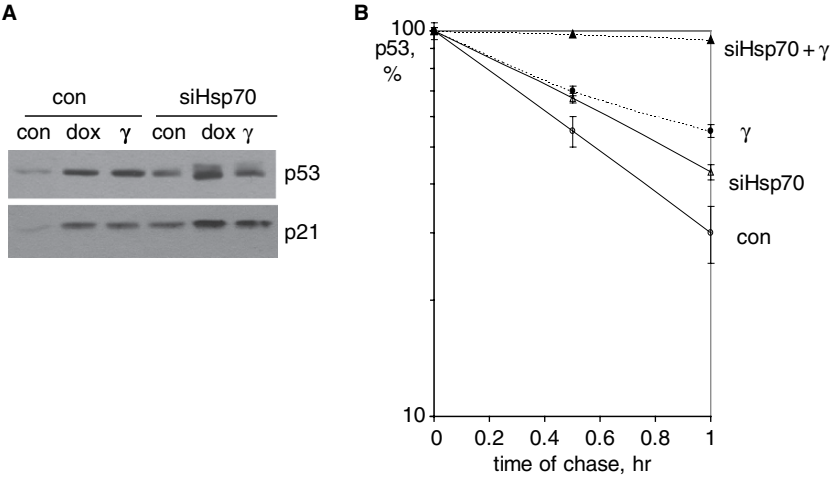

Figure 4. Downregulation of Hsp70 in HCT116 colon carcinoma activates p53/p21 pathway upon genotoxic stresses. **A**. Increase accumulation of p53 and p21 in Hsp70-depleted cells after treatment with doxorubicin (100 nM) or γ-radiation (5 Gy) after 24 h. **B**. Increased stabilization of p53 after radiation of Hsp70-depleted cells. Two hours after γ-radiation (5 Gy) cells were treated with protein synthesis inhibitor emetine (10 μM) for times indicated. Note that Hsp70 depletion itself stabilizes p53 (B) and causes p21 accumulation (A)

p53 pathway and senescence, an effect obviously independent on DNA-damage (Yaglom et al, 2007, see also chapter by M. Sherman in this book). From our and above described data from literature we suggest that Hsp70 may modulate p53 pathway thus protecting cells from apoptosis, senescence, and mitotic catastrophe.

Although p53 is the major effector of genotoxic stresses, and its modulation by Hsp70 may be critical for protection of normal and tumor cells expressing wild-type p53, Hsp70 apparently can protect lymphoid tumor cells which lack functional p53, (e.g., U937, or HL60, see Table 2). As demonstrated in HL-60 cells, Hsp70 expression inhibited etoposide-induced Bax conformation change, its translocation to mitochondria, and downstream events (release of cytochrome c and cytosol, activation of caspase-9 and caspase-3 (Guo et al., 2005). Thus, inhibition of Bax translocation by Hsp70 seems to be common effect for both genotoxic and non-genotoxic stresses (Guo et al., 2005; Ruchalski et al., 2006; Stankiewicz et al., 2005).

Activation of Bax by DNA damage may occur via p53-dependent or p53-independent pathways; in latter case, caspase-2 cleaves Bid thus promoting Bax translocation (Roos and Kaina, 2006). On the other hand, etoposide and UV radiation can cause Bax phosphorylation and translocation via JNK/p38 kinase activation (Kim et al., 2006). Interestingly, both inhibition of Bid cleavage and stress-kinase activity are well-documented effects of Hsp70 for non-genotoxic stimuli such as heat shock, or TNF (e.g., (Gabai, 1997; Gabai et al., 2000; Stankiewicz et al., 2005)). Therefore, it is tempting to speculate that in case of p53-independent apoptosis, Hsp70 exerts its protective effect by inhibiting JNK/p38 and/or Bid cleavage and Bax translocation (Figure 5). However, further research is obviously necessary to clarify this problem.

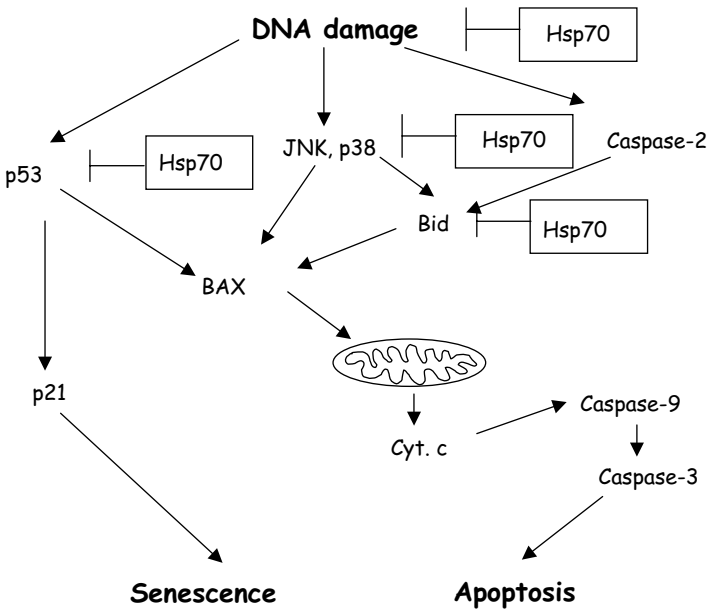

Figure 5. Suggested mechanisms of protection from genotoxic stresses by Hsp70 (see text for details)

HSP90 INHIBITION AND SENSITIZATION OF TUMOR CELLS TO RADIATION AND GENOTOXIC DRUGS

Hsp90 is regarded as a promising target for antineoplastic drug, and currently there are several clinical trials of geldanamicin analogues, a highly specific Hsp90 inhibitors, for treatment of solid tumors. In 2003, several independent groups reported that treatment of various tumor cell lines with geldanamycin or its analogue, 17-AAG, significantly enhance their radiosensitivity as assessed by clonogenic assay (Bisht et al., 2003; Russell et al., 2003) (Machida et al., 2003). Among tumor cell lines where sensitization was seen were gliomas, prostate, lung, colon and cervix carcinomas (Bisht et al., 2003; Russell et al., 2003) (Machida et al., 2003); importantly, there was no radiosensitization by 17-AAG in normal fibroblasts (Russell et al., 2003), but their transformation with E6/E7 oncogenes led to marked sensitization (Bisht et al., 2003). Furthermore, 17-AAG demonstrated significant radiosensitizing effect in vivo (with xenografts in nude mice) (Bisht et al., 2003). There are several possible signaling components affected by Hsp90 inhibition which may be responsible for radiosensitizing effect such as Her-2, Raf-1, ERK and Akt, and treatment with geldanamycin caused degradation of these components. Interestingly, in normal fibroblasts geldanamycin induced same degradation of Her-2, Raf-1 and Akt as in tumor cells, but does not cause radiosensitization (Russell et al., 2003). The authors suggested that tumor cells, in contrast to normal, are more dependent on survival pathways. However, there is another possibility. Along with degradation of components of pro-survival pathways, geldanamycin also activates heat shock response leading to accumulation of Hsp70 and Hsp27, which, as we described above, potentially can protect both normal and tumor cells from DNA-damaging agents, including radiation. Indeed, if tumor lympoid cells were treated first with geldanamycin and then with doxorubicin, they demonstrate resistance to apoptosis, apparently due to Hsp70 accumulation (Demidenko et al., 2005) (Robles et al., 2006), whereas treatment with doxorubicin first and then with geldanamycin had significant synergistic effect (Robles et al., 2006). Since normal cells usually express lower levels of Hsps than carcinoma cells, geldanamycin may induce marked increase of Hsp70/27 in these cells, which may compensate for sensitizing effect of geldanamycin-induced degradation of signaling components. In most carcinoma cells, however, increase in Hsps levels upon geldanamycin treatment may not be significant, and the sensitizing effect prevails. Anyway, for better radio- and chemo-sensitization of tumor cells Hsp90 inhibitors which do not activate heat shock response seems preferable. Of note, both radio-and chemosensitizing effects of geldanamycin were apparently independent on p53 status (Russell et al., 2003) (Robles et al., 2006).

HEAT SHOCK PROTEINS AND CHEMO-RADIORESISTANCE IN CANCER PATIENTS

Intrinsic and acquired chemo- and radioresistance are the major obstacles for effective treatment of cancer. As described above, numerous in vitro studies indicate

that modulation of Hsp27 and Hsp70 levels in various tumor cell cultures affects their sensitivity to radiation and other DNA-damaging agents (Tables 1, 2). The question arises, however, whether expression of Hsps in real human cancers correlates with their chemo- and radiosensitivity. But first lets consider some data indicating that acquired chemoresistance in vitro can be associated with overexpression of Hsps.

This is important since DNA-damaging drugs, in contrast to heat shock, proteasome inhibitors, or geldanamycin, do not usually activate HSF-1 and induce Hsps. Furthermore, several mechanisms of acquired chemoresistance are described; the most common is associated with expression of transporters (MDRs) which facilitates drug efflux. Despite these reservations, however, several studies clearly demonstrate that selection of human tumor cells in vitro with increasing concentrations of chemotherapeutic drugs leads to accumulation of several Hsps. For instance, selection of head and neck carcinoma cell line to cisplatin leads to increased levels of heat shock cognate protein Hsc70 (Johnsson et al., 2000). Accordingly, the same protein was overexpressed in cisplatin-resistant cervix carcinoma (Annalisa Castagna, 2004), or fibrosarcoma with pleotropic resistance (for doxorubicine, etoposide etc) (Davidovich and Roninson, 2000). HCW-2 derivative of HL-60 promyelocytic leukemia cells resistant to daunorubicin, radiation and other treatments demonstrated 5-fold increase in levels of inducible Hsp70 (Salvioli et al., 2003). In melanoma cell line selected for etoposide and cisplatin resistance, overexpression of both Hsp70 and Hsp27 was found (Pranav Sinha, 2003). In radiosensitive subclone of bladder carcinoma, downregulation of Hsp27 was found (Kassem et al., 2002), while radioresistant, but not radiosensitive glioma cells accumulated Hsp70 after radiation (Brondani Da Rocha et al., 2004). Finally, resistance of breast cancer cells to doxorubicine was associated with accumulation of Hsp27 (Liu et al., 2006), while Hsp27-related protein aB-crystalin was overexpressed in melanoma resistant to cisplatin and etoposide (Wittig et al., 2002). Thus, emergence of tumor cells resistant to DNA-damaging drugs can be associated with expression of Hsp70/Hsc70 and/or Hsp27. Although the mechanism by which chemoresistant tumor cells overexpress Hsps has not been elucidated, it may not involve overexpression of HSF-1, at least in some cases. Indeed, expression of HSF-1 did not increase levels of Hsps in PC-3 human prostate carcinoma (Hoang et al., 2000) or U2OS human osteosarcoma cells (Tchenio et al., 2006), while knockout of HSF-1 did not decreased Hsp levels in several tumor cell lines, except HCT116 colon carcinoma (Zaarur, 2006) (Gabai et al, unpublished data). Since promoters of Hsp genes contain many regulatory elements, it is not easy to evaluate which of these elements are responsible for Hsp accumulation during acquired chemoresistance. Among possible candidates is, for instance, CCAAT box located in Hsp70 promoter and activated by p53 analogue, DNp63a; interestingly, wt p53 antagonizes activity of DNp63a and suppress Hsp70 (Wu et al., 2005). Furthermore, topotecan (inhibitor of Topo I, see Table 2) can induce Hsp70 expression in p53 knockout, but not parental HCT116 colon carcinoma, which represents a potential mechanism for Hsp70 activation by genotoxic drugs (Daoud et al., 2003).

There are several clinical studies indicating that Hsp expression in tumors can be associated with resistance to chemo-radiotherapy (see also ref (Ciocca and Calderwood, 2005) for review). In 1993 Ciocca et al found that in breast cancer patients receiving adjuvant chemotherapy, Hsp70 expression was the only independent factor of disease recurrence (Ciocca et al., 1993). In breast cancer patients treated with radiotherapy with or without hyperthermia, expression of Hsp70 after treatment correlated with low probability to attain a complete response (Liu et al., 1996). In locally advanced breast cancer treated with induction chemotherapy, Vargas-Rois observed that high nuclear expression of Hsc70/Hsp70 and total expression of Hsp27 correlated with a shorter disease-free survival (Vargas-Roig et al., 1998). In ovarian tumors resistant to cisplatin or chlorambucil, levels of Hsp27 (by ELISA) were around four-times higher than in sensitive tumors (Langdon et al., 1995). However, in another study, response of ovary carcinomas to chemotherapy did not correlate with levels of Hsp27 (assessed by immunostaining), although patients with Hsp27-negative tumors of stage III-IV had much better progression-free and overall survival (Henriette J.G., Arts, 1999). Local control after radiation in head and neck squamous cell carcinoma was independent on Hsp27 levels (Fortin et al., 2000). However, in patients with esophageal squamous cell carcinoma, better overall survival after radio-and chemotherapy correlates with lower expression of Hsp27 and Hsp70; at the same time, no correlation with modulators of apoptosis, bax and bcl-2, was found (Miyazaki et al., 2005) . Interestingly, better prognosis also correlates with a high expression of p21, demonstrating, obviously, that p53/p21 pathway was activated in the patients with lower expression of Hsp27 and Hsp70. These clinical data are in concordance with in vitro studies indicating that downregulation of Hsp27 or Hsp70 can activate p53/p21 pathway (see above).

CONCLUSION

Available data indicate that both Hsp27 and Hsp70, besides being molecular chaperones, are also implicated in protection of tumor cells from DNA-damaging antineoplastic agents. Most studies demonstrated that artificial modulation of their levels affects cell's sensitivity to radiation and diverse drugs, including inhibitors of topoisomerases I and II, cross-linkers and some others. Besides protection from apoptosis, higher levels of Hsp27 or Hsp70 can also increase clonogenic ability of cells, apparently decreasing senescence and mitotic catastrophe. There are several possible mechanisms of Hsp-mediated protection from DNA damage; they include modulation of p53-dependent pathways (p21, Bax) and p53-independent pathways (stress kinases JNK and p38, ERK, Bid, NF-kB), but their significance needs further elucidation. Intrinsic and acquired resistance to DNA-damaging agents in various tumor cells lines can be associated with overexpression of Hsp27 and/or Hsp70. In patients with certain forms of cancers treated with chemo- or radiotherapy, higher levels of Hsp27 and/or Hsp70 expression correlated with resistance to therapy and worse prognosis.

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CHAPTER 10

HSP70 IN TUMORS: FRIEND OR FOE?

CRISTINA BONORINO* AND ANA PAULA SOUZA

Faculdade de Biociências e Instituto de Pesquisas Biomédicas, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil

Abstract: Hsp70, the most highly conserved and abundantly induced of the stress proteins, appears to play dual and opposing roles in cancer. On the one hand, Hsp70 promotes growth and survival of tumor cells by engaging misfolded or aggregated proteins and proteins involved in cell proliferation. As such, it endows tumor cells with stress resistance. However, Hsp70 can also promote tumor immunity by stimulating innate immune mechanisms and enhancing cross-presentation of tumor antigens to lymphocytes. In this chapter, we review these opposing functions of HSP70 in the context of potential strategies for its use as a tool in cancer biology and therapy

Keywords: HSP70, tumors, immunotherapy, vaccination, apoptosis, necrosis

HHSP70 IN TUMORS70 AS TUMOR PROTECTOR: FOE?

Tumors become established when cells are able to multiply in an abnormal, uncontrolled fashion due to decisive genetic modifications. In order to grow, the resulting cell mass must successfully compete with normal surrounding tissues for nutrients and oxygen. The accumulation of genetic modifications results in the expression of damaged proteins, which aggregate and interfere with established intracellular signaling pathways, thus posing an intrinsic threat to cell survival. Moreover, damaged self-constituents are potential targets for the immune system, which can use a variety of extrinsic cytotoxic mechanisms to eliminate the tumor. By definition, tumor cells growing *in vivo* are under stress.

*Corresponding author: Department of Cellular and Molecular Biology and Institute for Biomedical Research. Av. Ipiranga, 6681 2o andar. Porto Alegre RS Brasil 90619-900, Tel: 55 51 3320 3000 x. 2725, Fax: 55 51 3320 3312, e-mail: cbonorino@pucrs.br

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Hsp70 in the Stress Response

All cells respond to stress in a much conserved fashion, inducing or sometimes enhancing the synthesis of heat shock proteins (hsps), named following the original observation that they are induced at elevated temperatures in Drosophila (Ritossa 1963). Most of these proteins have constitutively expressed counterparts that function in non-stressed cells as molecular chaperones, organizing the folding of nascent peptides and translocating molecules across organelle membranes. In response to stress, however, these proteins are expressed more abundantly, and new isoforms appear (Parsell and Lindquist, 1994). The production of inducible stress proteins is regulated by heat shock transcription factors (HSFs), particularly HSF1, which is essential for their transcription in mammalian cells. Upon heat shock, HSF1 is phosphorylated by protein kinase CK2 and translocates to the nucleus, binding to heat shock elements (HSEs), and initiating Hsp gene transcription (Soncin et al., 2003).

Following stress, hsps rescue cells from death by refolding aggregated proteins and binding the proteins mediators that relay intracellular damage signals. Later verified in cells under stresses other than heat, such as hypoxia and reperfusion or ionizing radiation, the heat shock response was renamed the stress response, and hsps are sometimes referred to as stress proteins. Stress proteins are chaperones that interact with components of the survival and apoptotic pathways, preventing the activation of both caspase-mediated and caspase-independent cell death pathways (Mosser and Morimoto, 2004).

Hsps are grouped in families according to their molecular size. Each gene family has constitutive and inducible members, the most heavily studied of which is the hsp70 family. At least eight different members of the hsp70 family have been identified that play a central role in the folding and intracellular translocation of peptides. They differ in pattern of expression and cellular localization (Table 1). This ubiquitous distribution within a given cell almost certainly endows the Hsp70 family with the ability to operate at different points in multiple apoptotic pathways (Daugaard, Jaattela and Rohde, 2005).

Table 1. The Hsp70 family (adapted from Daugaard and Jaatela, 2005)

Protein	Synonyms	Locus	Cellular localization
Hsp70	Hsp72, Hsp70i,Hsp70-1(A),Hsp70-1(B)*	HSPA1AHSPA1B	Cytosol, nucleus, membranes
Hsc70	Hsp73	HSPA8	Cytosol, nucleus, lysosomes
Bip	Grp78	HSPA5	Endoplasmic reticulum
MtHsp70	Grp75	HSPA9	Mitochondria
Hsp70-6	Hsp70B	HSPA6	Cytosol, nucleus
Hsp70t	Hsp70-Hom	HSPA1L	Cytosol
Hsp70-2	HSPA2	HSPA2	Cytosol, nucleus

*99% amino-acid identity with HSP70-1(A)

Studies on cultured cell lines provided the earliest clues that tumors might upregulate Hsp70 in response to the stress of growing *in vivo*. The heat shock response appeared to be diminished in different cultured tumor lines (Mathur, 1994). The inducibility of Hsp70 by heat or irradiation also varied among cell lines (Muramatsu 1995). Gorzowski et al. (1995) found that many cultured tumor lines failed to induce Hsp70 upon heat shock because the Hsp70 genes were silenced by methylation. CH1 lymphomas, while in culture, did not express Hsp70 upon heat shock, even though they possessed functionally intact HSF1 and hsp70 genes. However, upon transplantation, they synthesized abundant Hsp70 (Davidson, 1995). These observations suggested that while tumor cells growing *in vitro* do not necessarily require Hsp70, this stress protein can endow cells with a decisive survival advantage *in vivo*.

We have made similar observations. While studying the growth dynamics of the B16F10 melanoma, we investigated the expression of Hsp70 by B16F10 cells at different stages of *in vitro* and *in vivo* growth (Figure 1). These cells did not express detectable Hsp70 while growing *in vitro* (Figure 1A, 2). Upon heat shock,

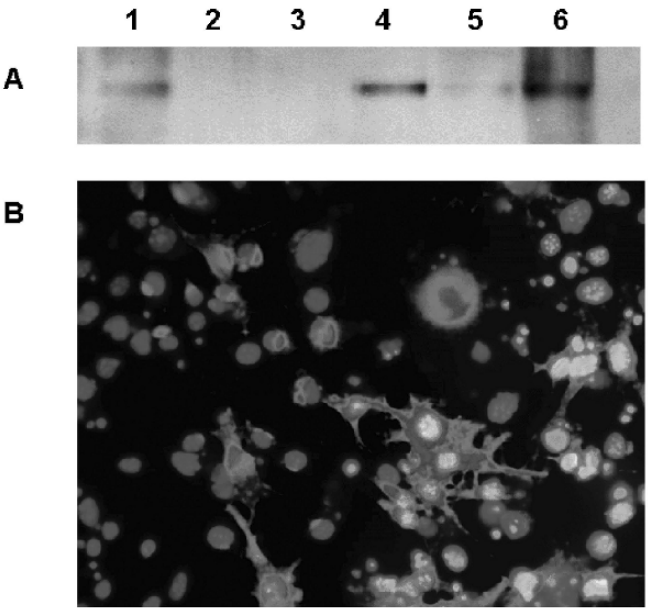

Figure 1. **In vitro and in vivo expression of Hsp70 by B16F10 cells.** A, Five micrograms of cell lysate of B16 F10 cells straight out of the culture dish, submitted for 2h to 39.9°C and 6h of recovery at 37°C. Alternatively, they were injected subcutaneously, in a C57BL/6 mouse, and after three or twenty days the mice were sacrificed and the tumor mass dissected. Samples were ran on an SDS-PAGE gel, transferred to a nitrocellulose membrane and incubated with a biotinylated anti-Hsp70 antibody, as presented: 1, B16F10 heat shocked; 2, B16F10 growing at 37°C; 3, normal skin, three days; 4, three day tumor mass; 5, normal skin, twenty days; 6, twenty day tumor mass. B, B16F10 cells growing on a coverslip placed inside the culture dish were treated as in (B), fixed in acetone, stained with Hoechst (blue) and biotin-anti-Hsp70/SA-Cy3 and visualized using a Zeiss immunofluorecence microscope (400X)

however, induction of Hsp70 was observed (Figure 1A, 1). Immunofluorescence assays showed that only a fraction of the cells in culture expressed Hsp70 in response to high temperature (Figure 1B). Within 3 days following transplantation to mice, however, production of Hsp70 was observed in the absence of any deliberate stress (Figure 1A, 3), and this persisted at least until day 20, both at the transplantation site and in lymph node metastases (Figure 1A, 6). Our observations suggest that upregulation of Hsp70 is necessary for *in vivo*, but not *in vitro*, tumor growth. Because it is likely that tumor cells in culture still produce considerable quantities of misfolded proteins, it is possible that Hsp70 is upregulated by tumor cells *in vivo* specifically to cope with major challenges associated with competition for nutrients, oxygen, and the immune system.

Protection from Apoptosis

Resistance to apoptosis is obviously a key advantage in tumorigenesis. Apoptosis can be initiated by extrinsic (receptor mediated) or intrinsic (mitochondria mediated) pathways, leading to the activation of a set of proteins known as caspases. Their activation is dependent on the oligomerization and association of these proteins in complexes, called apoptosomes. Hsps, and especially Hsp70, have been shown to bind many of the protein constituents of these complexes, and likely catalyze the conformational changes necessary for these proteins to assemble into apoptosomes (See: *Brunet et al*, Chapter 11, this volume). Different studies have shown that expression of Hsp70 by tumors is associated with protection from apoptosis by both major caspase-dependent pathways. Transfection of TNF-alpha-sensitive WEHI-S tumor cells with Hsp70 protects against apoptosis upstream of the mitochondria, after ligation of the TNF receptor (Jaattela et al., 1992). Transfection with Hsp70 also protected CCRF-CEM cells from apoptosis after Fas ligation, also acting upstream of the mitochondria, inhibiting cytochrome c release (Clemons et al., 2005). Both Saleh and collaborators (Saleh et al., 2000) and Beere and collaborators (Beere et al., 2000) showed that Hsp70 can bind to Apaf-1 and negatively regulate the recruitment of caspase 9 to the apoptosome.

Apoptosis can also occur through caspase-independent pathways. The apoptosis inducing factor (AIF) is a caspase independent death inducing factor that is released from mitochondria, like cytochrome c. In a cell-free system, AIF can bind nuclei and induce chromatin condensation and DNA loss. Hsp70 can antagonize AIF both in a cell free system, in concentrations similar to what is observed in the mitochondria intermembrane space (Ravagnan et al., 2001). Cells transiently transfected with AIF undergo apoptosis upon AIF overexpression, but this effect was inhibited by co-transfection with Hsp70, rescuing the AIF transfected cells from death. Hsp70 has also been shown to protect tumor cells from death induced from lysosomal degranulation, a caspase independent mechanism (Gyrd-Hansen, Nylandsted and Jaattela, 2004). More recently, it was shown that overexpression of Hsp70 can induce the expression of other stress proteins by enhancing HSF1 activation (Seo et al., 2006). In normal cells, excess production of hsps inhibits HSF1, in a negative feedback

mechanism. In this study, cells transfected with Hsp70 enhanced synthesis of other Hsps, in a mechanism dependent of HSF1. Hsp70 bound to MKP1and enhanced its phosphorylation, inhibiting ERK1/2 phosphorylation, which resulted in a more stable and dephosphorylated form of HSF1. That resulted in a positive feedback loop, enhancing Hsp expression. This suggests that in cancer cells that express high levels of HSF1, Hsp70 can increase resistance to apoptosis by positively regulating its own expression in cells.

If Hsp70 protects cells from apoptosis, one might expect that tumor cells will express higher quantities of inducible Hsp70 *in vivo*, relative to normal tissue. Indeed, this has been observed by numerous independent investigators (Ciocca, 1993; Protti, 1994; Lee C.S. 1994; Volm, 1995; Sugerman, 1995; Kaur and Ralhan, 1995; Seo et al., 1996; Torronteguy et al., 2006). In addition, Hsp70 production was negatively correlated with apoptosis in prostate cancer cells (Jones et al., 2004). High levels of Hsp70 in tumors growing in vivo protected tumor cells from treatment with anti-cancer drugs (Ciocca, 1992; Lee, 1992), especially cisplatin (Brozovic, Simaga and Osmak, 2001). This suggests that Hsp70 may be a useful predictive marker for resistance to chemotherapy, radiotherapy and hyperthermia (Ciocca and Calderwood, 2005).

Enhancement of Tumorigenesis

There are a number of reports indicating that overexpression of Hsp70 enhances tumorigenic potential (See: *Ciocca et al*, Chapter 2). In WEHI tumor cells transfected with Hsp70, tumorigenicity was positively correlated with Hsp70 expression levels. And while transfection with Hsp70 did not enhance their ability to form colonies in agar (a property of tumor cells) it rendered WEHI cells more resistant to killing by cytotoxic T cells and macrophages in vitro (Jaattela, 1995). It also enabled Rat-1 fibroblasts to lose contact inhibition and form the characteristic foci of oncogenically transformed cells in culture (Volloch and Sherman, 1999), suggesting that Hsp70 not only protects tumor cells from death, but also enhances their proliferation. Indeed, Hsp70 expression in MCF-7 breast cancer cells enhanced cell proliferation (Barnes et al., 2001), significantly shortening the mean doubling time by approximately two fold. This observation, together with a higher frequency of cells in second and third division metaphases at 42 and 69 hours, led the authors to conclude that Hsp70 appeared to be exerting its effect on MCF-7 cells primarily by shortening of the G_0/G_1 and S phases of the cell cycle. Transgenic mice expressing Hsp70 develop malignant T cell lymphomas (Seo et al., 1996), supporting the idea that Hsp70 confers proliferation advantages upon cells in vivo. Finally, Hsp70 can bind to proteins that are known regulators of the cell-cycle, such as p53 (Hainaut and Milner, 1992) and c-myc (Henriksson et al., 1992), so it is conceivable that Hsp70 influences cell proliferation by assisting in the proper folding of these key regulatory molecules. Given its major and ubiquitous roles in protecting tumor cells from apoptosis and enhancing proliferation, HSP70 is an attractive target for tumor therapy.

Neutralizing Hsp70 as a Therapeutic Tool for Cancer

The ability of some tumors to develop thermotolerance is attributed to Hsp70 (Subjeck, Sciandra and Johnson, 1982). Therefore, strategies to inhibit Hsp70 might enhance the efficacy of hyperthermia as a tumor therapy. Thus far, several different approaches have been applied to inhibit Hsp70, all with promising results. Nishimura et al. (Nishimura et al., 2000) found that stable transfection of a P19 carcinoma cell line with an antisense Hsp70 enhanced heat induced cell death. Inhibition of Hsp70 by antisense DNA also lead to massive death of breast cancer cultured cell lines, while it did not affect survival of non-tumorigenic breast epithelial cells or fibroblasts (Nylandsted et al., 2000). Adenovirus-encoded antisense Hsp70 erradicated human tumor xenografts in BALB/c nude mice (Nylandsted et al., 2002). Bcl-2 and Bcl-X_L, which protect tumor cells from different forms of cell death by inhibiting caspase activation, failed to rescue cells from death induced by Hsp70 depletion. However, many tumors can survive activation of caspases. Targeting the interaction between Hsp70 and the flavoprotein apoptosis inducing factor (AIF), involved in caspase-independent cell death, also sensitizes tumor cells to apoptosis induced by different stress stimuli (Schmitt et al., 2006). Consequently, a promising strategy might be to associate Hsp70 inhibition with classical anti-tumor treatments that activate caspase-dependent or –independent pathways of cell death. More recently, the inhibition of HSF-1 with RNAi (Rossi et al., 2006) lead to massive death in response to chemotherapy combined with hyperthermia. Altogether, these results indicate that Hsp70 plays a major role in tumor survival, and that neutralizing Hsp70 expression in tumors may constitute an important tool in cancer treatment.

HSP70 AS AN IMMUNE ACTIVATOR: FRIEND?

Hsp70 is composed of at least two different structural domains: the 44 kDa N-terminal contains a nucleotide binding site and possesses ATPase activity (Chappell et al., 1987). The 30 kDa C-terminal domain can bind unfolded polypeptides (Stevens et al., 2003). It is divided into an 18 kDa N-terminal subdomain (the peptide binding site) and a 10kDa C-terminal subdomain, which constitutes a lid over the peptide binding site. The full-length structure of Hsp70 is still unknown, however different studies provided evidence that these two domains work cooperatively. While ADP binding by the N-terminal domain correlates with peptide binding by the C-terminal domain, ATP binding favors release of the peptide by the C-terminal domain (McCarty et al., 1995). Thus, in vivo, ADP/ATP concentrations are likely to regulate chaperoning function of Hsp70.

The discovery that Hsp70 extracted from tumors could elicit specific anti-tumor immune responses by chaperoning tumor peptides into antigen presentation routes inaugurated a new and unexpected role for this protein (See *Gong & Calderwood*, Chapter 18, *Tamura et al*, Chapter 19). The pioneering studies of Srivastava and co-workers demonstrated that Hsps *per se* were not immunogenic, but when

purified from tumors by ADP chromatography, consequently in a form that was still associated with tumor peptides, they could be used to immunize against the original tumor (Srivastava, 1993). These studies suggested that although Hsp70 correlated with increased tumorigenesis, enhancing tumor survival, it also correlated with tumor immunogenicity, activating anti-tumor immune responses. While the role of Hsp70 in adaptive immunity, specifically in antigen presentation, is well documented, the idea that Hsp70 can also play a role in innate immunity is disputed. Some investigators have dismissed such a role due to technical flaws in experimental procedures. However, not all studies are easily dismissed and warrant a closer look. We discuss the evidence presented by these studies below.

Hsp70 and the Adaptive Response

Antigen presentation

Menoret et al. (1995) examined the immunogenicity of different clones derived from the same parental rat colon carcinoma. Variants that expressed high levels of Hsp70, but not other proteins such as Hsc70, MHC class I or II, were more readily rejected in vivo in a manner that was dependent upon alpha-beta+ T cells. Their results suggested that production of Hsp70 by tumor cells somehow facilitated the activation of an anti-tumor immune response. Support for their findings was provided by Binder et al. (2001), who demonstrated that cytosolic Hsp70 played a role in enhancing peptide presentation by MHC I molecules. By loading cell lines with either Hsp70-peptide complexes or free peptides, using liposomes to target them directly to the cytosol, they showed that the former were significantly more likely to be presented by class I molecules. Inhibition of Hsp70 by deoxyspergualin inhibited peptide presentation, and such inhibition could be reversed by supplying additional Hsp70. This study could provide an explanation for in vivo observations: tumor cells that expressed high levels of endogenous Hsp70 would be prone to generating more MHC class I-peptide complexes on their membranes, rendering the tumor more susceptible to recognition and lysis by CD8+ T cells. It also suggested that if exogenous Hsp70 could reach the cytosol of antigen presenting cells (APC), it could enhance antigen presentation.

The clearance of tumors by the immune system is performed mainly by CD8+T cells, through the activation of cytotoxic mechanisms upon recognition of specific peptides presented by MHC class I molecules. Studies using Hsp70-peptide complexes purified from tumors (Udono and Srivastava, 1993) or reconstituted in vitro (Blachere et al., 1993) to immunize mice demonstrated it was possible to induce CD8+ T cells that specifically recognized the tumor and mediated tumor regression. Such results were somewhat surprising because exogenous antigens are generally presented by MHC class II molecules. However, they can sometimes appear in MHC class I molecules. This phenomenon was previously described and named cross-presentation by Bevan and co-workers (Bevan, 1976a, 1976b; Bevan, 2006), while the generation of CD8+ T cell responses against antigens that reach APC via an exogenous route is referred to as cross-priming.

Additional studies provided evidence that chaperoning of peptides by Hsp70 can cross-prime T cells. Noessner and colleagues demonstrated that Hsp70 peptide complexes purified from a tyrosinase positive, but not a tyrosinase negative, human melanoma could deliver tyrosinase peptide to MHC class I molecules in dendritic cells (Noessner et al., 2002). Activation of a tyrosinase-specific CD8+ T cell clone was competitively inhibited by incubation with HSP70-peptide complexes that were purified from the tyrosinase negative melanoma. Hsp70-peptide complexes purified from tumors were shown to be internalized by bone marrow-derived DC and presented in MHC class I molecules (Ueda et al., 2004), and immunization with these complexes induced tumor specific CD8+ T cell clones. Massa and collaborators transfected tumor cell lines with secretable Hsp70, which resulted in increased tumor immunogenicity as well as generation of anti-tumor cytotoxic T cell lines (CTLs) (Massa et al., 2004). In addition to Hsp70, other hsps in tumor lysates, were necessary for cross priming against tumor antigens (Binder and Srivastava, 2005); when all hsps were depleted from a tumor lysate, cross-priming did not occur *in vivo*. Given the strong immunogenicity of Hsp-peptide complexes (very few picograms of Hsp70-peptide are sufficient to immunize mice), several groups explored the possibility that APC might possess specific receptors for stress proteins.

Receptors

While a saturable receptor-mediated mechanism seemed to apply to Hsc70 (Arnold-Schild et al., 1999) and gp96 (Wassenberg, Reed and Nicchitta, 2000), other groups found evidence for a receptor-independent pathway for Hsp70-peptide endocytosis (Fujihara and Nadler, 1999), confirming earlier observations of Hightower and Guidon (Hightower and Guidon, 1989) that Hsp70 could translocate across plasma membranes. Castellino and coworkers observed that Hsp70-peptide complexes could be transported across an endosomal membrane (Castellino et al., 2000). Their study demonstrated, however, that Hsp70-Ova complexes were endocytosed by dendritic cells in a receptor-mediated fashion, upon which it was subjected to least two distinct intracellular routes of degradation, one cytosolic and the other endocytic. The route was dependent on the sequence of the peptide, and culminated in class I presentation of the chaperoned peptide. Based on their data on saturability of Hsp70 surface binding, they predicted more that one receptor for Hsp70-peptide complexes.

Interestingly, different studies have implicated different receptors for Hsp70. The $\alpha 2$ macroglobulin receptor (CD91) was the first hsp receptor identified, initially for gp96 (Binder, Han and Srivastava, 2000), and later as a common receptor for Hsp70, gp96 and calreticulin in peritoneal exudate cells as well as murine bone marrow derived DC (Basu et al., 2001). In that study, following Hsp binding to CD91, Hsp-chaperoned peptides were re-presented via a proteasome-dependent mechanism, in a manner that was completely inhibited by $\alpha 2$ macroglobulin and anti-CD91, leading the authors to conclude that CD91 was the only receptor for these Hsps. However, CD14 (Asea et al., 2000) and TLR4 (Asea et al., 2002)

were also reported to be receptors for Hsp70. Delneste et al. observed that Hsp70 bound to the scavenger receptor LOX-1, a receptor for oxidized LDL, on human immature dendritic cells that neither expressed CD91 nor CD14 (Delneste et al., 2002), and such binding lead to internalization of Hsp70-peptide complexes and cross-presentation. Theriault and collaborators (Theriault et al., 2005) investigated binding of Hsp70 to different receptors such as CD91, TLR2 and TLR4, as well as to LOX-1. They demonstrated that only LOX-1 expression on transfected cell lines lead to significant binding of Hsp70. Surprisingly, Hsp70 was observed to bind also to non-APC cell lines, of epithelial/endothelial origin. Finally, CD40 was also reported to be an endocytic receptor for Hsp70 (Becker, Hartl and Wieland, 2002).

Other reports for multiple receptors for other mammalian hsps, as well as for their prokaryotic homologues (for a review, see (Binder, Vatner and Srivastava, 2004)) continue to puzzle investigators. The inconsistencies among the various reports on surface receptors for Hsp70 are still not completely understood. A common misconception is that, because Hsp70 is highly evolutionarily conserved, it will play the same roles in prokaryotes and eukaryotes, and consequently Hsp70 homologues should bind to the same molecules. One must consider that, if bacterial and mammalian Hsp70 present approximately 50% similarity, they also present 50% divergence. Another key issue is the uncertainty generated by the observations that one given receptor can bind different Hsps, or that different hsps can bind to the same receptor. If on the one hand these differences might be related to purification procedures (see below), on the other hand they might have structural causes. For example, if a given preparation of Hsp70 contained oligomers, instead of mainly monomers, Hsp70 could bind efficiently to low affinity receptors, due to an avidity effect. Indeed, Hsp70 has been reported to dimerize and oligomerize (Nemoto et al., 2006; Yamada et al., 2003), but it is still not clear how binding to the peptide influences dimer/oligomer formation, and which physiological conditions would influence the oligomers or monomers to be the most common extracellular forms in vivo.

In any case, the strong immunogenicity of Hsp70 suggests that this cytosolic protein can reach the extracellular milieu, and that it is advantageous for the cell (and consequently for the host) to specifically gather and internalize this protein. The ability to endocytose Hsp70 might have evolved due to issues that at least at first glance are unrelated to immunity. One example is that neurons can take up Hsp70 released by glial cells in response to stress, and that this correlates with stress protection for the neuron (Guzhova et al., 2001).

So how can we translate the results of the studies discussed above into the generation immune responses against a living tumor in vivo? Two main hypotheses guide most of the investigations on this subject: 1) that Hsp70 can somehow be released by viable tumor cells, binding to APCs; or, 2) that some tumor cells must die, releasing their intracellular contents including Hsp-peptide complexes that are taken up by APCs. There is some evidence for both scenarios. Although Hsp70 does not contain a leader sequence, a recent study shows that it can be released from viable prostate carcinoma cells through an active mechanism that involves lysosomal

translocation, as revealed by its sensitivity to lysosomal inhibitors (Mambula and Calderwood, 2006). Other studies point to a combination of Hsp70 release and cell death resulting on an important event for the generation of an anti-tumor response: the activation of innate immunity mechanisms.

Hsp70 and the Innate Response

Little is known about the generation of immune responses to tumors in vivo. Because tumor antigens are basically self proteins, central tolerance mechanisms are likely to ensure an extremely low precursor frequency of T cells able to recognize such antigens. Moreover, if tumor antigens are picked up by APC, the costimulatory signals delivered by the APC will determine the type of specific immune response generated. Antigen presentation in the presence of costimulatory molecules results in the activation of specific immune responses, while presentation in the absence of such molecules leads to tolerance (Steinman et al., 2005). Consequently, peptides chaperoned by Hsp should not be sufficient to elicit an anti-tumor immune response in vivo. On the contrary, signals that activate APCs to upregulate costimulatory molecules presumably must be provided, otherwise the presented tumor antigens will induce a state of self-tolerance in the acquired immune system and facilitate tumor growth.

Results of several studies have lead to the conclusion that Hsp70 alone could lead to upregulation of inflammatory mediators and/or costimulatory molecules after binding to receptors on APCs (Asea et al., 2000; Asea et al., 2002; Basu et al., 2000; Vabulas et al., 2002). As such, Hsp70 would could belong to a new class of natural endogenous adjuvants (Rock et al., 2005). Because many studies involving Hsps (as well as other candidate endogenous adjuvants) have demonstrated binding to TLRs 2 and 4, with activation of MyD88, the adaptor signaling molecule for several TLRs; and because most of these studies used recombinant molecules (Asea et al., 2000) (Asea et al., 2002; Vabulas et al., 2002), some critics have argued that this adjuvant effect might actually have been the result of endotoxin contamination, rather than an intrinsic biological property of Hsp70. For example, a study by Gao and Tsan (2003) demonstrated that when contaminant endotoxin was carefully removed, recombinant Hsp70 preparations lost their ability to induce TNF-alpha production by monocytes.

Consequently, conclusions from studies that used recombinant Hsp70 and verified binding to LPS receptors need to be re-evaluated. LPS is hard to remove, particularly from Hsp70, and if the preparations used were indeed contaminated by endotoxin, such studies may have actually provided some of the first evidence for a specific interaction between Hsp70 and LPS, when LPS binds its receptors. Recently, it was shown that Hsp70 can associate with LPS in membrane lipid rafts and participate in a TLR-LPS complex together with other LPS receptor molecules (Triantafilou and Triantafilou, 2003; Triantafilou and Triantafilou, 2004). This association might have implications during the immune response to bacteria, since Hsp70-LPS complexes appear to be targeted to the Golgi apparatus after endocytosis. However, during

the generation of an anti-tumor immune response, LPS or other TLR ligands are unlikely to be present. If TLR ligation by endogenous ligands does occur, current evidence suggests it may play a role in tissue regeneration (Jiang et al., 2005) rather than CD8+ T cell priming.

So which signals activate an anti-tumor response, and how can Hsp70 participate? Because cell death is likely to be a highly frequent event in the millieu surrounding tumors growing in vivo, it has been hypothesized that the release of intracellular constituents by dead cells could provide activating signals for APC. It has been known for some time that dead cells are immunostimulatory (review in (Rock et al., 2005)). Gallucci et al. found that a delayed type hypersensitivity reaction could be elicited upon immunization with ovalbumin mixed with killed cells (Gallucci, Lolkema and Matzinger, 1999). Shi and co-workers found that T cell responses were augmented even when dead cells were administered separately from the antigen, and that this effect was enhanced by about 10-fold when the cells were stressed or injured before dying (Shi and Rock, 2002). While Shi and Rock found uric acid to be one of the major stimulatory constituents released by dead cells (Shi, Evans and Rock, 2003), Basu et al. suggested that necrotic cell death leads to Hsp release in the supernatant (Basu, et al., 2000) and that Hsp70 purified from mouse cells induces bone marrow-derived DC maturation.

Using an engineered system that induced cell death in vivo, Vile and collaborators combined these ideas, and demonstrated that enhanced tumor immunogenicity was related to the release of Hsp70-peptide complexes from the dying tumor cells (Melcher et al., 1998). In a following study, Daniels and collaborators subcutaneously injected mice subcutaneously with a plasmid encoding Hsp70 together with DNA for the thymidine kinase of herpes simplex virus (HSVtk) (Daniels et al., 2004). Cells that express HSVtk DNA, when provided with gancyclovir (GCV) injection, transform it into a toxic form that is incorporated into the chromosomal DNA, leading to cell death. The treatment induced non-malignant melanocyte death, and induced immunity against transplanted B16 melanoma cells, while no effect was observed with plasmids encoding only HSVtk or Hsp70. Their results suggest a role for cell death signals together with Hsp70 in the activation of anti-tumor immune responses. At face value, these studies could simply indicate that cell death facilitates release of Hsp70. However, while individually proposed as endogenous adjuvants, death signals such as uric acid, DNA, nucleotides and Hsp70 could work together to generate anti-tumor responses in vivo.

Another interesting line of evidence suggests that Hsp70 can be expressed on the cell surface, serving as a target for NK cells (Botzler, Issels and Multhoff, 1996; Multhoff et al., 2001). Hsp70 has been observed on membranes of tumor cells but not normal cells (Hantschel et al., 2000). Specifically, Hsp70 on membranes of tumor cells correlated with a poor prognosis (Steiner et al., 2006), suggesting membrane expression of Hsp70 was characteristic of aggressive tumors. In principle, however, NK cells should more easily detect and lyse tumor cells with membrane Hsp70, providing signals that might activate APC. Lysis of tumor cells by NK cells might also enhance release of Hsp70-peptide complexes that could be taken up by

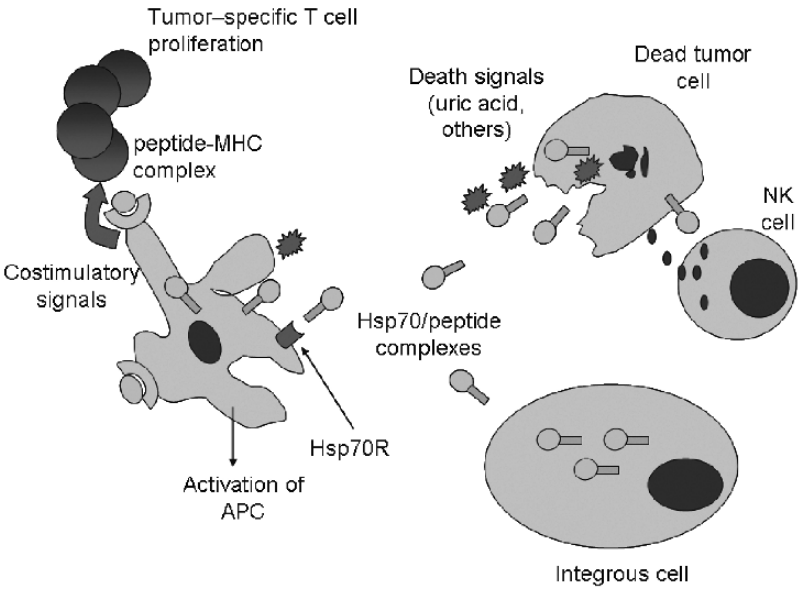

Figure 2. **Potentializing co-adjuvant effects of cell death and Hsp70.** Tumor cells that express high Hsp70 are likely to be protected against apoptosis, but cells with membrane expression of Hsp70 can be recognized and killed by NK cells. Upon tumor cell death, induced either by the intrinsic stresses of in vivo tumor growth including immune responses, Hsp70-peptide complexes are released to the extracellular medium. Also, integrous tumor cells can secrete Hsp70-peptide complexes. These complexes reach and are endocytosed by APC, through a receptor dependent or independent pathway. As a result, tumor peptides are directed to MHC molecules and presented on the cell surface. Other cell death signals like uric acid together with Hsp70 activate APC leading to upregulation of costimulatory signals, stimulating T cells in order to generate anti-tumor cell responses

APC, once again resulting in a favorable scenario for the generation of in vivo anti-tumor responses. A summary of the possibilities is schematically represented in Figure 2.

CONCLUSION AND PERSPECTIVES

Collectively, these observations suggest that the same Hsp70 that sometimes protects tumor growth may prove to be a useful tool for tumor elimination. Tumor cells growing in vivo face a dilemma: while the expression of high levels of Hsp70 may confer protection against apoptosis, it could also trigger tumor-specific immunity. A prediction is that primary tumors need to express sufficient Hsp70 to promote survival but low enough levels to escape immune surveillance. Advanced metastatic tumors, that successfully evade immunity, may be able to express higher Hsp70 levels, to cope with the stress associated with accumulated genetic mutations and increased division rate.

In our laboratory, we have observed that, in biopsies from breast cancer patients, expression of low levels of Hsp70 in primary tumors was significantly associated with development of metastasis, tumor relapse and/or death during a three-year follow-up (Torronteguy et al., 2006). Hsp70 has been found to be highly expressed in poorly differentiated (more aggressive) endometrial carcinomas (Nanbu et al., 1996), in poorly differentiated chondromas (Trieb et al., 2000), as well as in more highly malignant carcinoma cells (Tang et al., 2005). However, other studies did not find correlations between Hsp70 expression and the state of differentiation of the tumor (review in (Ciocca and Calderwood, 2005), so the influence of Hsp70 on immunogenicity versus tumorigenicity of the tumor remains elusive.

Further work on the structural properties of Hsp70 is needed to clarify the interaction of extracellular Hsp70 with other cells. The interaction of Hsp70 with receptors is central for tumor therapy design. For instance, it is still not known which subpopulations of APC are critical for the generation of anti-tumor responses. If the Hsp70 receptor is not optimally expressed by these APC, Hsp70-peptide complexes will need to be targeted so that anti-tumor responses can be efficiently generated.

Ongoing clinical trials focus either on the inhibition of Hsp70 to sensitize tumor cells to apoptosis, or on the immunization of cancer patients with hsp70-peptide complexes purified from their own tumors (an autologous and costumized vaccine). It is tempting to speculate that we could combine these two approaches. Hsp70 inhibitors could induce tumor death, and those death signals combined with the clearance of dead cells by APC could lead to T cell priming against the tumor. Later, immunization with Hsp70-peptide complexes could enhance tumor antigen presentation, culminating in the expansion those T cell clones in vivo. Finally, besides designing a vaccine that will lead to the generation of an anti-tumor response, it is necessary to determine the extent of this protective effect. We must further understand the dynamics and biochemistry of those reactions in vivo, so that we can optimally harness the potential anti-cancer properties of this protein.

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CHAPTER 11

HSP70 AND HSP27 AS PHARMACOLOGICAL TARGETS IN APOPTOSIS MODULATION FOR CANCER THERAPY

Heat shock proteins and Cancer

M. BRUNET, C. DIDELOT, S. SUBRAMANIAM, A.L. RÉROLE, A. DE THONEL AND C. GARRIDO*

INSERM UMR-866, Faculty of Medicine and Pharmacy, 7 Boulevard Jeanne d'Arc, 21039 Dijon, France

Abstract: The expression of heat shock proteins (HSP) HSP70 and HSP27 is induced in response to a wide variety of physiological and environmental insults including anticancer chemotherapy, thus allowing the cell to survive to lethal conditions. The cytoprotective effect of HSP70 and HSP27 is related to their ability to disable apoptosis. HSP70 and HSP27 both inhibit key apoptotic proteins at the pre- and post-mitochondrial level. HSP70 and/or HSP27 basal levels are unusually high in malignant cells, and both have been accused of participating in oncogenesis and/or in chemotherapy resistance. In rodent models, HSP70 or HSP27 over-expression increases tumor growth and metastatic potential. HSP70 and HSP27 depletion or inhibition frequently reduces the size of the tumors and even can cause their complete involution (for HSP70). In this chapter we will describe the effectors of the apoptotic machinery that interact with HSP70 or HSP27, and we will discuss the inhibition of HSP70 and HSP27 as a novel strategy of cancer therapy

Keywords: Heat shock proteins, apoptosis, cancer cell growth, cancer cell resistance

Abbreviations: HSP, heat shock proteins; AIF, apoptosis inducing factor; Apaf-1, Apoptosis protease activating factor-1; RO, Reactive oxygen species; PKC, Protein kinase C

*Corresponding author: INSERM UMR-866, 7, boulevard Jeanne d'Arc, Faculty of Medicine, 21039 Dijon, France, Tel: 33 3 80 39 32 84, Fax: 33 3 80 39 34 34, e-mail: cgarrido@u-bourgogne.fr

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INTRODUCTION: HSP70 AND HSP27 ARE MOLECULAR CHAPERONES

Mammalian HSPs are evolutionary conserved proteins that can behave as molecular chaperones for other cellular proteins. They have been classified into five families according to their molecular size: HSP100, HSP90, HSP70, HSP60 and small HSPs (15 to 30 kDa) including HSP27. Chaperones are instrumental for signaling and protein traffic, even in the absence of stress. However, the need of HSPs increases after proteotoxic damage. HSP70 and HSP27 are the most strongly and universally induced chaperones. They are strongly induced by different stresses such as heat, irradiation, oxidative stress, or anticancer chemotherapy (Garrido et al., 2001).

Under normal conditions, HSP70 functions as ATP-dependent molecular chaperone that assist the folding of newly synthesized polypeptides, the assembly of multi-protein complexes and the transport of proteins across cellular membranes (Beckmann et al., 1990; De Los Rios et al., 2006; Shi and Thomas, 1992). HSP70 contains two distinct functional regions (Carrello et al., 2004) : a peptide binding domain (PBD) and the amino-terminal ATPase domain (ABD) (Figure 1A). The PBD, that includes a carboxyl-terminal EEVD motif or chaperone motif, is responsible for substrate binding and refolding. The ABD, in turn, facilitates the release of the client protein after ATP hydrolysis (Mayer and Bukau, 2005) (Figure 1A). HSP70 chaperone activity is regulated by co-chaperones like Hip, CHIP or Bag-1. These co-chaperones bind to HSP70 and modulate its chaperone function by increasing or decreasing HSP70 affinity for substrates through the stabilization of the ADP or ATP bound state of HSP70. Under stressful conditions, elevated HSP70 levels allow cells to cope with increased concentrations of unfolded or denatured proteins (Nollen et al., 1999).

In contrast to HSP70, HSP27 is a ATP-independent chaperone that protect the cells from protein aggregation (Ehrnsperger et al., 1997). An interesting property of HSP27 is its capacity to oligomerize. HSP27 can form oligomers of up to 1000 kDa (Figure 2A). The affinity of HSP27 for the proteins to be chaperoned is modulated by their oligomerization status, the multimer being the binding competent state (Shashidharamurthy et al., 2005). The dimer of HSP27 is the building block for such multimeric complexes. The range of oligomerization size and the magnitude of chaperone activity increases as the temperature is increased (Bruey et al., 2000b; Lelj-Garolla and Mauk, 2006). HSP27 oligomerization is a highly dynamic process regulated by the phosphorylation of the protein (Garrido, 2002). Human HSP27 can be phosphorylated at three serine residues, and its dephosphorylation favors the formation of large oligomers (Parcellier et al., 2006; Theriault et al., 2004) (Figure 2A). HSP27 phosphorylation is a reversible process catalyzed by the MAPKAP kinases-2 and -3 in response to differentiating agents, mitogens, inflammatory cytokines such as TNF-α and IL-1-ß, some anticancer agents, hydrogen peroxide and other oxidants (Casado et al., 2007; Dorion and Landry, 2002; Vertii et al., 2006). However, phosphorylation is not the only process that modulates HSP27 oligomerization. Cell-cell contact, as that observed in confluent cultures *in vitro* or solid tumors *in vivo*, induces the formation of large HSP27 oligomers independently of the phosphorylation status of the protein (Bruey et al., 2000b).

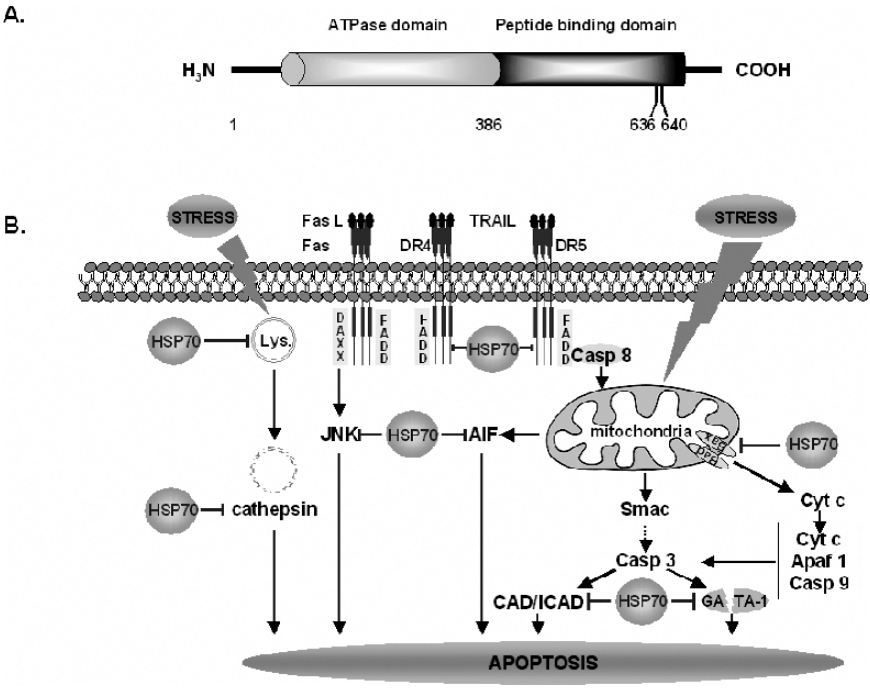

Figure 1. Simplified scheme of HSP70 structure and anti-apoptotic functions. **A**) HSP70 contains two distinct functional regions: the peptide binding domain (PBD) that includes a carboxyl-terminal EEVD motif or chaperone motif and is responsible for substrate binding and refolding, and the amino-terminal ATP binding domain or ATPase domain (ABD) that facilitates the release of the client protein. **B**) HSP70 is a decisive negative regulator of the mitochondrial pathway of apoptosis that can block apoptosis at different levels, at a pre-mitochondrial stage by inhibiting stress inducing signaling; at the mitochondrial stage by preventing mitochondrial membrane permeabilization through the blockage of Bax translocation; and, finally, at the post-mitochondrial level by interacting with AIF and Apaf-1 or by protecting essential nuclear proteins from caspase-3 cleavage

It has also been reported that HSP27 can be modified by methylglyoxal (MG), a dicarbonyl compound generated as a side product of glycolysis (Sakamoto et al., 2002). MG provokes the formation of large HSP27 oligomers by inducing the formation of Arg-pyrimidine, an MG-arginine adduct (Oya-Ito et al., 2006).

In this review, we will describe the different pathways through which HSP70 and HSP27 can modulate cancer cells apoptosis and tumorigenicity. The emerging strategies to inhibit these two chaperones in cancer therapy will also be discussed.

HSP70, HSP27 AND APOPTOSIS

HSP27 and HSP70 both have strong cytoprotective properties. Overexpressed HSP70 or HSP27 prevents apoptotic cell death triggered by various stimuli, including hyperthermia, oxidative stress, inhibition of tyrosine kinases by

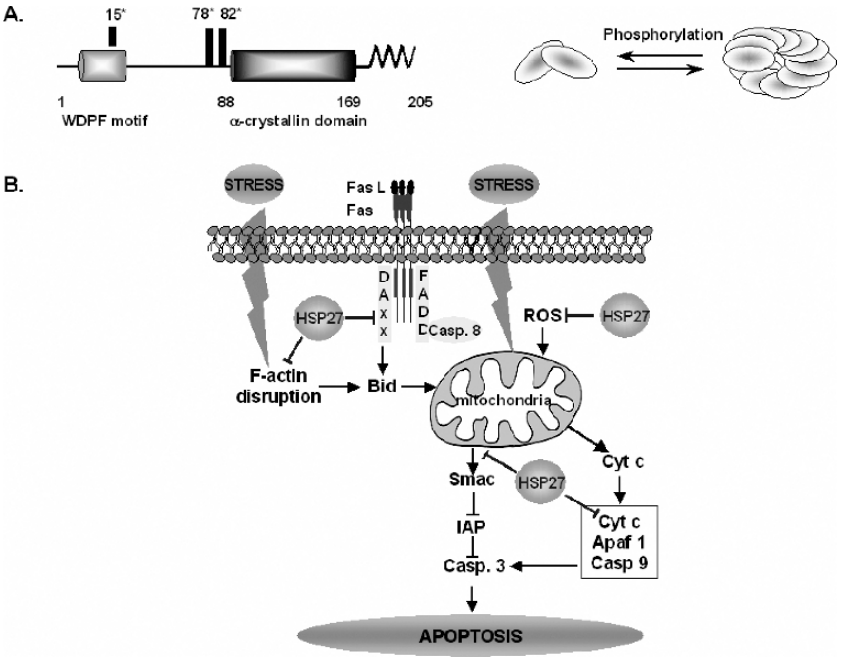

Figure 2. General scheme of HSP27 structure and anti-apoptotic role. A) HSP27 possesses a central domain called alpha-crystallin. This domain is well conserved among all the members of the small HSP family. The three serines that can be phosphorylated in human HSP27 and the aminoterminal WDPF motif are also indicated. This latter motif and the protein phosphorylation influence the oligomerization state of the protein. HSP27 can form oligomers of up to 1000 kDa. B) HSP27 can block cytochrome c-induced caspase activation at different stages, namely at the pre-mitochondrial level by inhibiting cytochrome c release indirectly through its action on F-actin, Bid or ROS and at the post-mitochondrial level through the sequestration of cytosolic cytochrome c. HSP27 may also influence apoptosis by favoring the ubiquitination/degradation of proteins like I-κBα or $p27^{kip1}$ under stressful conditions

staurosporine, ligation of the Fas/Apo-1/CD95 death receptor or addition of cytotoxic drugs (Garrido et al., 1996; Garrido et al., 1997; Mehlen et al., 1996; Parcellier et al., 2003a). (Also See: *Beere et al*, Chapter 8, this volume).

Apoptosis is a very frequent form of cell death observed in cancer cells for instance after treatment with chemotherapeutic agents. Two fundamentally different pathways of apoptosis can be distinguished, although crosstalk between the two signal transducing cascades exists (Figures 1B, 2B). The extrinsic pathway is triggered through plasma membrane proteins of the TNF receptor family known as death receptors and leads to the direct activation of caspases, in particular the receptor-proximal caspase-8. The intrinsic pathway involves intracellular stress signals that elicit the production or activation of pro-apoptotic molecules, which converge on the mitochondria to trigger their permeabilization. Outer mitochondrial membrane permeabilization leads to the release of caspase activators, in particular cytochrome *c*, which interacts with cytosolic apoptosis protease-activating factor-1

(Apaf-1) and pro-caspase-9 to form the apoptosome, the caspase-3 activation complex (Li et al., 1997). The flavoprotein apoptosis inducing factor (AIF) is another mitochondrial intermembrane protein released upon an apoptotic stimulus. AIF translocates to the nucleus and triggers caspase-independent nuclear changes upon activation of the intrinsic pathway (Daugas et al., 2000; Joza et al., 2001; Susin et al., 1999). Two additional mitochondrial proteins, Smac/Diablo and Htra2/Omi, activate apoptosis by neutralizing the inhibitory activity of the IAPs (inhibitory apoptotic proteins) that associate with and inhibit caspases (Du et al., 2000). Finally endonuclease G (EndoG) is an apoptotic DNase that is also released from the mitochondria intermembrane space to the cytosol upon an apoptotic stimulus (Garrido and Kroemer, 2004). HSP70 and HSP27 directly interact with several among these apoptotic effectors (Figures 1B, 2B), as will be discussed below.

HSP70 INHIBITS CASPASE-DEPENDENT AND CASPASE-INDEPENDENT APOPTOTIC PATHWAY

Induced expression of HSP70 enhances the ability of the cell to survive to a panoply of lethal conditions. Gene ablation studies demonstrate that HSP70 plays an important role in apoptosis. Cells lacking *hsp70.1* and *hsp70.3*, the two genes that code for inducible HSP70, are highly sensitive to apoptosis induced by a wide range of lethal stimuli (Schmitt et al., 2003). Ablation of the testis specific isoform of HSP70 (*hsp70.2*) results in germ cell apoptosis (Dix et al., 1996).

Elevated HSP70 levels block the apoptotic pathway at different levels, both upstream and downstream of the death-associated mitochondrial events (Figure 1B). At the pre-mitochondrial level, HSP70 inhibits stress-activated kinases. HSP70 binds to and functions as a natural inhibitory protein of c-Jun N-terminal Kinase (JNK1) (Park et al., 2001). The ATPase domain of HSP70 is dispensable for this binding (Mosser et al., 2000). HSP70 also appears to affect the Bid-dependent apoptotic pathway. HSP70-mediated protection against TNF-induced cell death is lost in Bid homozygous-deleted MEF cells (Mosser et al., 2000). It has been suggested that HSP70 could affect Bid-dependent cell death by inhibiting JNK activation, through a mechanism that is not fully elucidated (Gabai et al., 2000). In apoptosis induced by hyperosmolarity, HSP70 has been found to modulate JNK and ERK phosphorylation (Lee et al., 2005a). HSP70 has also been shown to bind to nonphosphorylated protein kinase C (PKC) *via* the kinase's unphosphorylated carboxyl-terminus, priming the kinase for rephosphorylation and stabilizing the protein(Gao and Newton, 2002). In macrophages, anti-sense HSP70 attenuated the anti-apoptotic effect of unmethylated CpG oligodeoxynucleotides; HSP70 depletion resulted in Bcl-xL down regulation and increased AIF translocation (Kuo et al., 2006).

At the mitochondrial level, HSP70 blocks heat-induced apoptosis by hindering Bax to translocate and to insert into the outer membrane, thereby preventing mitochondrial membrane permeabilization and the release of cytochrome *c* and AIF

(Stankiewicz et al., 2005). This HSP70 function depends on both the chaperone and the ATP hydrolytic domains (Ruchalski et al., 2006).

At the post-mitochondrial level, HSP70 has been reported to inhibit apoptosis downstream of the release of cytochrome *c* and upstream of the activation of caspase-3 (Li et al., 2000). This anti-apoptotic effect was explained by the HSP70-mediated modulation of the apoptosome. Indeed, HSP70 has been demonstrated to directly bind to Apaf-1, thereby preventing the recruitment of procaspase-9 to the apoptosome. The ATPase domain of HSP70 was described to be necessary for this interaction (Beere et al., 2000). Other rapports have shown that HSP70 interacts with procaspase-3 and procaspase-7 and prevents their proteolytic maturation, thereby inhibiting the caspase-dependent apoptotic signaling (Komarova et al., 2004). However, these results have been contradicted by a study in which the authors claimed that the inhibition of caspase-dependent apoptosis by HSP70 results from an inhibition of cytochrome c release from the mitochondria and not from direct effects of HSP70 on caspase activation (Steel et al., 2004).

HSP70 can also prevent caspase-independent pathways, because HSP70 prevents cell death in conditions in which caspase activation does not occur (Creagh et al., 2000; Ravagnan et al., 2001). We have observed that over-expression of HSP70 protects Apaf-$1^{-/-}$ cells from apoptotic cell death induced by serum withdrawal (Ravagnan et al., 2001), indicating that the cytochrome c/Apaf-1/caspase was not the sole pathway of the anti-apoptotic action of HSP70. Indeed, HSP70 directly binds to apoptosis inducing factor (AIF), inhibits AIF nuclear translocation and thereby AIF-induced chromatin condensation (Gurbuxani et al., 2003; Matsumori et al., 2005; Ravagnan et al., 2001; Ruchalski et al., 2006). A region between aminoacids 150 and 228 of AIF is necessary for the interaction that may require the ATPase function of HSP70 (Ruchalski et al., 2006). Of note, endogenous levels of HSP70 seem to be sufficiently high to control AIF-mediated apoptosis, because down-regulation of HSP70 by an anti-sense construct sensitized the cells to serum withdrawal and AIF (Ravagnan et al., 2001). AIF sequestration by HSP70 has been shown to reduce neonatal hypoxic/ischemic brain injury (Matsumori et al., 2005). HSP70 has also been shown to associate with EndoG and to prevent DNA fragmentation (Kalinowska et al., 2005). However, since EndoG can form complexes with AIF, the association EndoG/HSP70 described could be indirect and involve AIF as a molecular bridge.

HSP70 can also rescue cells from a later phase of apoptosis than any known survival-enhancing drug or protein. In TNFα-induced apoptosis, HSP70 does not prevent the activation of caspase-3 but prevents downstream morphological changes that are characteristic of dying cells like activation of phospholipase A2 and changes in nuclear morphology (Jaattela et al., 1998). During the final phases of apoptosis, chromosomal DNA is digested by the DNase CAD (caspase activated DNase) following activation by caspase-3. The enzymatic activity and proper folding of CAD has been reported to be regulated by HSP70, its co-chaperone HSP40 and ICAD, the inhibitor of CAD. ICAD recognizes an intermediate folding state conferred by HSP70-HSP40 (Sakahira and Nagata, 2002). It has also been reported

in TCR-stimulated T cells that HSP70 binds CAD and enhances its activity (Liu et al., 2003). Another final target of caspase-3 is the transcription factor GATA-1. We have demonstrated in erythroblasts that HSP70 in the nucleus can protect GATA-1 from caspase-3 cleavage. As a consequence, erythroblasts do not die by apoptosis but instead differentiate in red blood cells (Ribeil et al., 2007).

HSP70 can also act at the level of death receptors to mediate Bcr-Abl dependent resistance to apoptosis in human acute leukemia cells. HSP70 binds to the death receptors DR4 and DR5, thereby inhibiting the TRAIL-induced assembly and activity of death inducing signaling complex (DISC) (Guo et al., 2005b). Exposure of hematopoietic cells to TNFα induces the activity of the pro-apoptotic double-stranded RNA-dependent protein kinase (PKR). An inhibitor of PKR is the Fanconi anemia complementation group C gene product (FANCC). HSP70 interacts with the FANCC protein *via* its ATPase domain and, together with HSP40, inhibits TNFα-induced apoptosis through the ternary complex HSP70, FANCC and PKR (Pang et al., 2002; Pang et al., 2001).

An apoptosis regulatory protein interacting with HSP70 is Bag-1. Bag-1 may function as co-chaperone of HSP70, and simultaneously regulates the activity of proteins such as Bcl-2 and Raf-1. It has been shown that HSP70/Bag-1 regulates Raf-1/ERK kinase and cell growth in response to stress (Gotz et al., 2005; Song et al., 2001). Whether the HSP70-Bag-1 interaction is important for HSP70-mediated apoptosis regulation is elusive. Finally, HSP70 has been shown to inhibit lysosomal membrane permeabilization, thereby preventing the release of cathepsins, a group of proteases that are involved in apoptosis (Bivik et al., 2007; Gyrd-Hansen et al., 2004; Nylandsted et al., 2004). In colon cancer cells and murine fibroblasts (MEFs) HSP70 is found in the membrane of lysosomes. Depletion of HSP70 in those cells results in cysteine cathepsin-dependent death that is preceded by lysosomal destabilization and release of lysosomal constituents to the cytosol (Bivik et al., 2007; Gyrd-Hansen et al., 2004).

In summary**,** HSP70 is a decisive negative regulator of the mitochondrial pathway of apoptosis that can block cell death at several levels: at a pre-mitochondrial stage by inhibiting stress inducing signaling; at the mitochondrial stage, by preventing mitochondrial membrane permeabilization through the blockage of Bax translocation; and finally, at the post-mitochondrial level by interacting with AIF and Apaf-1 or by protecting essential nuclear proteins from caspase-3 cleavage (Figure 2A). All the reports that studied the domain of HSP70 involved in different interactions indicate that apoptotic partners of HSP70 bind to the PBD of the protein (see Figure 1). However, for some interactions/protective properties the ATPase function of HSP70 is needed but not for others. For instance, while the ATPase function seems to be needed for Apaf-1 (Beere et al., 2000) and AIF (Ruchalski et al., 2006), it is dispensable for JNK or GATA-1 binding/protection. In this way, in erythroblasts, in which HSP70 blocks apoptosis by protecting GATA-1 from caspase-3 cleavage, a HSP70 mutant that only contains the PBD is as efficient as wild type HSP70 to assure the protection of erythroblasts (Ribeil et al., 2006). Based on these data, we can hypothesize that HSP70 might chaperone proteins in an ATP-dependent as well as in an ATP-independent manner.

HSP27: An Inhibitor of Caspase-Dependent Apoptosis

Experimental depletion of HSP27 suggests that HSP27 mainly functions as an inhibitor of caspase activation. Knock-down of HSP27 by small interfering RNAs induces apoptosis through caspase-3 activation (Rocchi et al., 2005; Rocchi et al., 2006) (Also see: *Arrigo*, Chapter 4). This phenomenon can be explained by the reported ability of HSP27 to prevent the formation of the apoptosome and the subsequent activation of caspases (Garrido et al., 1999). Moreover, this may result from the capacity of HSP27 to sequester cytochrome *c* when released from the mitochondria into the cytosol, as demonstrated in leukemic U937, Jurkat T lymhoma cells, and Pro colon cancer cells treated with different apoptotic stimuli (Bruey et al., 2000a; Concannon et al., 2001; Paul et al., 2002). The heme group of cytochrome c is necessary but not sufficient for this interaction that involves amino-acids 51 and 141 of HSP27 and does not need the phosphorylation of HSP27 (Bruey et al., 2000a). When expressed at high intracellular levels, HSP can also interfere with caspase activation upstream of the mitochondria (Paul et al., 2002). This effect seems related to the ability of HSP27 to stabilize actin microfilaments. In L929 murine fibrosarcoma cells exposed to cytochalasin D or staurosporine, overexpressed HSP27 binds to F-actin (Guay et al., 1997) preventing the cytoskeletal disruption and Bid intracellular redistribution that precede cytochrome c release (Paul et al., 2002). In multiple myeloma cells treated with dexamethasone, HSP27 has also been shown to inhibit the mitochondrial release of Smac (Chauhan et al., 2003a) (Figure 2B). HSP27 has been described by us and other groups to have important anti-oxidant properties. This is related to its ability to maintain glutathione in its reduced (non-oxidized) form (Arrigo et al., 2005) and to decrease the abundance of reactive oxygen species (Garrido et al., 1997; Lee et al., 2005c). HSP27 inhibits radiation-induced apoptosis by directly interacting with PKC delta. This interaction, that results in HSP27 phosphorylation (Lee et al., 2005b), provokes a reduction in PKC delta-mediated reactive oxygen species production (Lee et al., 2005c). The anti-oxidant properties of HSP27 seem particularly relevant for HSP27-mediated cytoprotection in neuronal cells (Wyttenbach et al., 2002).

HSP27 also inhibits apoptosis by regulating upstream signaling pathways. Survival factors, such as nerve growth factor or platelet-derived growth factor, inhibit apoptosis by activating the phosphatidylinositol 3-kinase pathway (PI3-K). Activated PI3-K phosphorylates inositol lipids in the plasma membrane that attract the serine/threonine kinase Akt/PKB. Akt targets multiple proteins of the apoptotic machinery, including Bad and caspase-9 (Biggs et al., 1999; Ozes et al., 1999). HSP27 has been shown to bind the protein kinase Akt, an interaction that is necessary for Akt activation in stressed cells. In turn, Akt could phosphorylate HSP27, thus leading to the disruption of HSP27-Akt complexes (Rane et al., 2003). HSP27 also affects one of the downstream event elicited by the stimulation of CD95/Fas. The phosphorylated form of HSP27 directly interacts with Daxx, which connects Fas signaling to the protein kinase Ask1 that mediates caspase-independent cell death (Charette et al., 2000). HSP27 has recently been reported to induce cytoprotection in LNCaP tumor cells through the activators of the transcription

factor Stat3. HSP27 interacts with Stat3 and the protective effect induced by HSP27 overexpression in those cells was attenuated by Stat3 knockdown (Rocchi et al., 2005). Cytoprotection by HSP27 may also relie on the capacity of HSP27 to favour the proteasomal degradation of certain proteins under stress conditions. Two among the protein targets of HSP27 are the transcription factor nuclear factor-κB (NF-κB) inhibitor IκBα and the cyclin-dependent kinase inhibitor $p27^{kip1}$. In different cancer cells treated with etoposide or TNF-α, HSP27 favors the ubiquitination and subsequent degradation of IκBα. As a consequence, there is an increase in the activity of the survival factor NF-κB that contributes to the overall protective effect of HSP27 (Parcellier et al., 2003b). Under stressful conditions (serum depletion, staurosporine treatment) HSP27 also stimulates $p27^{kip1}$ ubiquitination/degradation. As a consequence, cells do not accumulate in the Go/G_1 phase of the cellular cycle but in the S-phase. Therefore, cells over-expressing HSP27 may be more ready to re-start proliferating once the stress conditions are over (Parcellier et al., 2006). Finally, HSP27 participates in the (de)phosphorylation reaction that controls the activity of the splicing regulator SRp38, thereby restoring the splicing activity inhibited after heat shock (Marin-Vinader et al., 2006).

In conclusion, HSP27 is able to block apoptosis at different stages because of its interaction with a number of partners implicated in the apoptotic pathways. The capacity of HSP27 to interact with one or another partner seems to be determined by the oligomerization/phosphorylation status of the protein, which, in turn, might depend on the type and the status of the cell. Depending on the physiological requirement of the cell, HSP27 may be phosphorylated and the equilibrium between large and small oligomers may shift. As a consequence, the affinity of the protein for one or another substrate increases, with profound consequences for apoptosis regulation. We have demonstrated *in vitro* and *in vivo* that for the HSP27 caspase-dependent antiapoptotic effect, large non-phosphorylated oligomers of HSP27 were the active form of the protein (Bruey et al., 2000b). Confirming this result, Oya-Ito et al (Oya-Ito et al., 2006) have recently published that the methylglyoxal modification of HSP27, which is reported to favor large oligomers, exacerbates the antiapoptotic caspase-inhibitory properties of HSP27. In contrast, phosphorylated and small oligomers of HSP27 are particulary efficient in binding to F-actin and Daxx (Charette et al., 2000; Guay et al., 1997), and it is the phosphorylated form of HSP27 that protects from neurotoxicity (Wyttenbach et al., 2002).

HSP70, HSP27 AND CANCER

The protective presence of HSP70 can be beneficial for the whole organism, if HSP70 is expressed in normal cells, for instance, as recently reported, in chondrocytes where gene transfer with HSP70 decreases the severity of osteoarthritis-induced lesions (Grossin et al., 2006) or in normal gastric cells, where HSP70 confers mucosal protection (Ebert et al., 2005). However, in cancer cells, HSP70 and/or HSP27 are negative prognostic markers (See: *Ciocca et al*, Chapter 2). Cells or tissues from a wide range of tumors have been shown to express unusually

high levels of HSP27 and/or HSP70. HSP27 and HSP70 have been shown to increase the tumorigenic potential of rodent cells transplanted into syngeneic hosts (Garrido et al., 1998; Jaattela, 1995). Both for HSP27 and HSP70 their tumorigenic potential seems to correlate with their antiapoptotic abilities. Rat colon cancer cells engineered to express human HSP27 were observed to form more aggressive and less apoptosis-prone tumors in syngenic animals than control cells (Bruey et al., 2000b; Garrido et al., 1998). HSP27 overexpression was also reported to increase the metastatic potential of human breast cancer cells inoculated into athymic (nude) mice (Lemieux et al., 1997). Conversely, antisense oligonucleotides designed to deplete HSP27 in prostate tumors enhance apoptosis and delay tumor progression (Rocchi et al., 2004). Concerning HSP70, antisense constructs of HSP70 have been shown to sensitize cancer cells to apoptosis and to eradicate tumors (glioma, breast and colon carcinomas) in several models (Gurbuxani et al., 2003; Li et al., 2000; Nylandsted et al., 2000). Thus, HSP27 and HSP70 contribute to tumorigenesis, at least in part, through their cytoprotective activity.

Clinically, in a number of cancers such as breast cancer, ovarian cancer, osteosarcoma, endometrial cancer and leukemias, an increased level of HSP27, relative to its level in non-transformed cells, has been detected (Parcellier et al., 2005). In ovarian tumors, HSP27 expression increases with the stage of the tumor (Geisler et al., 2004). In addition, the pattern of HSP27 phosphorylation in tumor cells is different from that observed in primary non-transformed cells (Ciocca et al., 1993). Consequently, the diversity of HSP27 isoforms may also represent an useful tumor marker, as recently demonstrated for human renal cell carcinomas (Sarto et al., 2004). Increased expression of HSP70 has been monitored in high-grade malignant tumors such as endometrial cancer, osteosarcoma and renal cell tumors (Nanbu et al., 1998; Santarosa et al., 1997; Wadhwa et al., 2006). HSP27 and HSP70 are associated to clinicopathologic parameters in hepatocarcinomas but not in non-neoplastic hepatocytes (Joo et al., 2005). The molecular basis for overexpression of HSPs in tumors is not completely understood. In some tumors, it may be explained by a suboptimal cellular environment, for instance due to hypoxia or nutrient stress (Garrido et al., 1998; Garrido et al., 1997). In other tumors, oncogenic mutations could create an increased requirement for chaperone activity to eliminate abnormally folded protein variants. Another possibility is the occurrence of gain-of-function mutations in transcription factors that increase heat shock promoter activity and induce a pseudo-hypoxic state. In adenocarcinoma cell lines, an increased level of heat shock transcription factor 1 (HSF1) was associated with an increased HSP70 and HSP27 protein level (Hoang et al., 2000). In breast carcinomas the signal transducer and activator of transcription 3 (Stat3) is constitutively activated. It has been demonstrated that Stat3 upregulates HSP27 in those cancer cells (Song et al., 2004). HSP70 is also rather abundant in Bcr-Abl human leukemia cells, and the GATA-RE element found in *hsp70* promotor is necessary for this accumulation (Ray et al., 2004).

Could HSPs be used for diagnostic or as prognostic markers? The ability of HSP27 and HSP70 to prevent apoptosis induced by several anticancer drugs as

well as other stimuli explains how these proteins could limit the efficacy of cancer therapy. High expression of HSP27 and HSP70 in breast, endometrial or gastric cancer has been associated with metastasis, poor prognosis and resistance to chemotherapy or radiation therapy (Brondani Da Rocha et al., 2004; Ciocca et al., 1993; Vargas-Roig et al., 1998). HSP70 contributes to hepatocarcinogenesis and tumor progression(Joo et al., 2005)**.** Overexpression of HSP27 confers chemoresistance in laryngeal cancer (Lee et al., 2007). In prostate cancer, HSP27 is an independent predictor of clinical outcome. A low expression of HSP27 is associated with a delay in prostate tumor progression (Rocchi et al., 2004). Expression of HSP27 in primary breast cancers is associated with short survival for node-negative patients (Thanner et al., 2005). Similar conclusions were reached in patients with ovarian carcinoma where decreased HSP27 staining was related to decreased survival (Geisler et al., 2004). HSP27 has been suggested as a predictor of the response to vinorelbine chemotherapy in non-small cell lung cancer patients (Berrieman et al., 2006). Recently, it has been demonstrated that evaluation of the soluble level of HSP27 in human serum may be a useful biomarker for breast cancers or hepatocellular carcinoma (De and Roach, 2004; Feng et al., 2005). HSP27 is a potential therapeutic target in advanced prostate cancer (Rocchi et al., 2005). Enhanced expression of HSP27 is associated with poor clinical outcome in patients with prostate cancer following hormonal therapy (Miyake et al., 2006). Furthermore, also in prostate cancer, HSP27 has been shown to be involved in cell invasion by increasing matrix metalloproteinase type 2 activity (Xu et al., 2006). HSP27 and HSP70 have been associated with major adverse prognostic factors in acute myeloid leukemia (Thomas et al., 2005). HSP70, along with PSA, are good tumor markers to identify patients with early stage prostate cancer (Abe et al., 2004).

Nonetheless, HSP27 or HSP70 are not universal markers of poor prognosis. Even though HSP70 levels correlate with malignancy in osteosarcoma and renal cell tumors, its expression is paradoxically associated with improved prognosis (Santarosa et al., 1997; Trieb et al., 1998). In oral squamous cell carcinoma, cases with reduced expression of HSP27 that were more aggressive and poorly differentiated were found (Lo Muzio et al., 2004). Similarly, reduced expression of HSP27 exhibits an independent association with the poorest overall survival rate in head and neck squamous cell carcinoma (Lo Muzio et al., 2006). In breast cancers, HSP27 expression has been associated with increased invasiveness but decreased cell motility (Lemieux et al., 1997). The *hsp27* gene contains an imperfect estrogen-responsive element and can be induced by estrogen treatment in breast cancer cells (Ciocca et al., 1993; Oesterreich et al., 1997). Overexpressed HSP27 in series of breast cancers has been correlated with the expression of estrogen receptors, small tumor size and a low proliferation index (Hurlimann et al., 1993). Despite this, clinico-pathological studies attempting to correlate HSP27 protein level in breast cancers after hormone therapy with tumor progression and clinical outcome have provided contradictory results (Ciocca et al., 1993; Nakopoulou et al., 1995). It is therefore possible that the chemo-protective effects of HSPs, under certain

circumstances, may be bypassed by a variety of other modulators of drug resistance in human tumors. It should be noted, however, that HSP27 phosphorylation status is not taken into account in most *in vivo* studies. Knowledge of the diversity of HSP27 phosphorylation species might represent useful markers, as it has been demonstrated in renal cell carcinomas, in which HSP27 phosphorylation is associated with differentiation (Tremolada et al., 2005) or in patients with hepatocellular carcinoma; in which attenuated phosphorylation of HSP27 correlates with tumor progression (Yasuda et al., 2005). Futhermore, in breast cancer cells the level of phosphorylated HSP27 correlated with the cells' resistance to vincristin (Casado et al., 2007). In non-small lung cancer cells it is the amount of argpyrimidine-modified HSP27 that should be evaluated as a prognostic factor (van Heijst et al., 2006).

HSP70 AND HSP27: PHARMACOLOGICAL TARGETS IN CANCER THERAPY

Constitutively high HSP expression is a property of and essential for the survival of at least some cancers. Neutralizing HSPs is therefore an attractive strategy for anticancer therapy. Unfortunately, the only specific HSP inhibitors that are available for clinical trials target HSP90 (See: *Neckers*, Chapter 13, *Whitesell*, Chapter 14, *Kamal et al*, Chapter15, *Workman*, Chapter16, *Calderwood et al*, Chapter 18). HSP90 can be inhibited by the benzoquinone ansamycin antibiotic geldanamycin and its analogue 17-AAG (17-allylamino-17-deemethoxygeldanamycin) (Neckers, 2002; Neckers and Ivy, 2003). The fact that geldanamycin and 17-AAG selectively kills cancer cells has been rationalized by assuming that tumor cells, as compared to their normal counterparts, would exhibit a stressed phenotype, with an enhanced dependency on the cytoprotective action of HSP90. Accordingly, in tumors, HSP90 is present entirely in multichaperones complexes with high ATPase activity, whereas HSP90 from normal tissues is in an uncomplexed state (Kamal et al., 2003).

HSP90 inhibitors like 17-AAG have been shown to have an important side effect: they induce the expression of HSP70, thereby reducing the desired therapeutic effect (Demidenko et al., 2006; Guo et al., 2005a). The use of products like KNK437, a benzylidine lactam inhibitor of HSP70 and thermotolerance, attenuated 17-AAG mediated induction of HSP70 and increases 17-AAG induced apoptosis. However, KNK437 is an unspecific inhibitor of HSP70 (Guo et al., 2005a).

We and others have reported that HSP70 antisense constructs have chemosensitizing properties and may even kill cancer cell lines (in the context of adenoviral infection) in the absence of additional stimuli (Gurbuxani et al., 2003; Nylandsted et al., 2000; Zhao and Shen, 2005). The cytotoxic effect of HSP70 down-modulation is particularly strong in transformed cells, yet undetectable in normal, nontransformed cell lines or primary cells (Schmitt et al., 2003). Studies in Bcr-Abl human leukemia cells reveal that HSP70 is a promising therapeutic target for reversing drug resistance, presumably due to its ability to inhibit apoptosis upstream and downstream of the mitochondria (Guo et al., 2005b; Ray et al., 2004). Unfortunately,

thus far no small molecules that would selectively inhibit HSP70 are available. Since HSP70 blocks apoptosis at the post-mitochondrial level by inactivating the apoptosome as well as AIF, strategies targeting HSP70 may be especially effective in overcoming tumor cell resistance. We have recently demonstrated that rationally engineered decoy targets of HSP70 derived from AIF can sensitize cancer cells to apoptosis induction by neutralizing HSP70 functions. These AIF-derived peptides all mimic a domain of the AIF protein (amino acids 150 to 228) that is required for HSP70 binding (Gurbuxani et al., 2003). Hence, they bind to HSP70 but lack an autonomous pro-apoptotic function. Experiments using different cancer cell lines (leukemia, colon cancer, breast cancer and cervix cancer) demonstrate that several among these peptides strongly increase the sensitivity to chemotherapy *in vitro*. This effect was merely related to their ability to neutralize endogenous HSP70, because this pro-apoptotic activity was lost in HSP70-negative cells (Schmitt et al., 2003). *In vivo*, in syngeneic rat colon cancer and mouse melanoma models, one of these inhibitors, called ADD70 (for AIF derived decoy for HSP70), decreased the size of the tumors, strongly reduced their metastatic potential and led to complete and permanent cure after treatment with the anticancer agent cisplatin (Schmitt et al., 2006). These anti-tumor effects were associated with an important infiltration of $CD8^+$ T lymphocytes into the tumors expressing ADD70 (Schmitt et al., 2006). We have also demonstrated for colon cancer and melanoma cells that a combination of our HSP70 inhibitor, ADD70, and the HSP90 inhibitor 17-AAG (currently undergoing phase II clinical trials), as it will be extensively described in others chapters of this book, is particularly efficient in mediating chemosensitization. Future will tell whether a similar strategy may allow for the chemosensitization of HSP70-expressing human tumors. This strategy could be used in combination with HSP90 inhibitors.

Concerning HSP27, phosphorothioate HSP27 antisense oligonucleotides can enhance apoptosis and delay tumor progression in prostate cancer (Rocchi et al., 2004; Teimourian et al., 2006)**.** Taxol has been suggested to overcome drug resistance to etoposide, colcemid and vincristine in ovarian and uterine cancer cells *in vitro* by inhibiting HSP27 expression (Tanaka et al., 2004). Antisense strategies have also demonstrated that lymphomas and multiple myelomas can be sensitized to chemotherapeutic drugs like dexamethasone and bortezomib. In dexamethasone-resistant cell lines, HSP27 is overexpressed. Its downregulation by siRNA restores the apoptotic response to dexamethasone by triggering caspase activation (Chauhan et al., 2003a). We have demonstrated that HSP27 participates in proteins ubiquitination/proteasomal degradation and that this effect contribute to its protective functions by enhancing the activity of proteins like NF-κB (Parcellier et al., 2003b). Bortezomib, which is currently in clinical use for the treatment of multiple myeloma, has been shown to induce apoptosis in several cancer cell lines. HSP27 confers bortezomib resistance and depletion of HS27 sensitizes cells to bortezomib-induced apoptosis (Chauhan et al., 2003a; Chauhan et al., 2003b). It is therefore tempting to conclude that a combinational therapy using bortezomib together with an inhibitor of HSP27 would have additive or synergistic anti-cancer effects.

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CHAPTER 12

HEAT SHOCK PROTEIN 90: THE CANCER CHAPERONE

LEN NECKERS*

Urologic Oncology Branch, National Cancer Institute, Bethesda, MD 20892, USA

Abstract: Heat shock protein 90 (Hsp90) is a molecular chaperone required for the stability and function of a number of conditionally activated and/or expressed signaling proteins, as well as multiple mutated, chimeric, and/or over-expressed signaling proteins, that promote cancer cell growth and/or survival. Hsp90 inhibitors are unique in that, although they are directed towards a specific molecular target, they simultaneously inhibit multiple cellular signaling pathways. By inhibiting nodal points in multiple overlapping survival pathways utilized by cancer cells, combination of an Hsp90 inhibitor with standard chemotherapeutic agents may dramatically increase the *in vivo* efficacy of the standard agent. Hsp90 inhibitors may circumvent the characteristic genetic plasticity that has allowed cancer cells to eventually evade the toxic effects of most molecularly targeted agents. The mechanism-based use of Hsp90 inhibitors, both alone and in combination with other drugs, should be effective toward multiple forms of cancer. Further, because Hsp90 inhibitors also induce Hsf-1-dependent expression of Hsp70, and because certain mutated Hsp90 client proteins are neurotoxic, these drugs display ameliorative properties in several neurodegenerative disease models, suggesting a novel role for Hsp90 inhibitors in treating multiple pathologies involving neurodegeneration

Keywords: Molecular chaperone, Hsp90, cancer, protein folding

INTRODUCTION

Cancer is a disease of genetic instability. Although only a few specific alterations seem to be required for generation of the malignant phenotype, at least in colon carcinoma there are approximately 10,000 estimated mutations at time of diagnosis (Hahn and Weinberg 2002; Stoler, et al., 1999). This genetic plasticity of cancer cells allows them to frequently escape the precise molecular targeting of a single signaling node or pathway, making them ultimately non-responsive to molecularly targeted therapeutics. Even Gleevec™ (Novartis Pharmaceuticals Corp.), a well-recognized clinically active Bcr-Abl tyrosine kinase inhibitor, can eventually lose

*Corresponding author: Urologic Oncology Branch, NCI, 9000 Rockville Pike, Bldg. 10/CRC, Room 1-5940, Bethesda, MD 20892-1107, Tel: 301-496-5899, Fax: 301-402-0922, e-mail: len@helix.nih.gov

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its effectiveness under intense, drug-dependent selective pressure, due to either mutation of the drug interaction site or expansion of a previously existing resistant clone (La Rosee, et al., 2002). Most solid tumors at the time of detection are already sufficiently genetically diverse to resist single agent molecularly targeted therapy (Kitano, 2003). Thus, a simultaneous attack on multiple nodes of a cancer cell's web of overlapping signaling pathways should be more likely to affect survival than would inhibition of one or even a few individual signaling nodes. Given the number of key nodal proteins that are Hsp90 clients (see the website maintained by D. Picard, http://www.picard.ch/DP/downloads/Hsp90interactors.pdf, as well as several excellent reviews (Nardai, et al., 2006; Sreedhar, et al., 2004; Zhao, et al., 2005)), inhibition of Hsp90 may serve the purpose of collapsing, or significantly weakening, a cancer cell's safety net (Also see: *Pratt et al*, Chapter 1, *Whitesell*, Chapter 13, *Kamal et al*, chapter 14, *Matts & Caplan*, Chapter 16). Indeed, following a hypothesis first proposed by Hanahan and Weinberg several years ago (Hanahan and Weinberg, 2000), genetic instability allows a cell to eventually acquire six capabilities that are characteristic of most if not all cancers. These are [1] self-sufficiency in growth signaling; [2] insensitivity to anti-growth signaling; [3] ability to evade apoptosis; [4] sustained angiogenesis; [5] tissue invasion and metastasis; and [6] limitless replicative potential. As is highlighted in Figure 1, Hsp90 plays a pivotal role in acquisition and maintenance of each of these capabilities. Several excellent reviews provide an in depth description of the many signaling nodes regulated by Hsp90 (Bagatell and Whitesell, 2004; Chiosis, et al., 2004; Goetz, et al., 2003; Isaacs, 2005; Isaacs, et al., 2003; Workman, 2004; Zhang and Burrows, 2004).

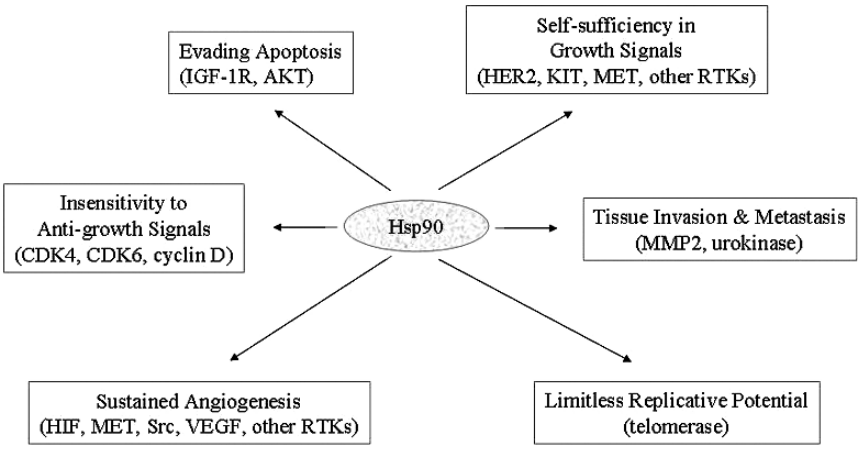
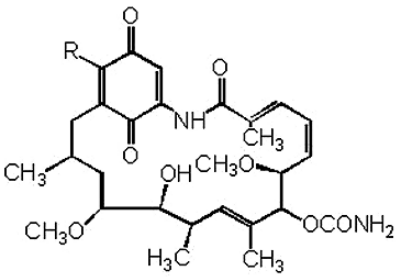


Figure 1. Hsp90 function is implicated in establishment of each of the hallmarks of cancer as first proposed by Hanahan and Weinberg (Hanahan and Weinberg, 2000). Importantly, Hsp90 function may also permit the genetic instability on which acquisition of the six hallmarks depends

Cancer cells survive in the face of frequently extreme environmental stress, such as hypoxia and acidosis, as well as in the face of the exogenously applied environmental stresses of chemotherapy or radiation. These stresses tend to generate free radicals that can cause significant physical damage to cellular proteins. Given the combined protective role of molecular chaperones toward damaged proteins and the dependence of multiple signal transduction pathways on Hsp90, it is therefore not surprising that molecular chaperones in general, and Hsp90 in particular, are highly expressed in most tumor cells. However, Hsp90 may be elevated in tumor cells and may provide a unique molecular target therein for an additional reason. Using *Drosophila* and *Arabidopsis* as model systems, Lindquist and colleagues have shown that an ancient function of Hsp90 may be to permit accumulation at the protein level of inherent genetic mutations, and thus the chaperone may play a pivotal role in the evolutionary process itself (Queitsch, et al., 2002; Rutherford and Lindquist, 1998). Extrapolating this hypothesis to genetically unstable cancer cells, it is not a great leap to think that Hsp90 may be critical to their ability to survive in the presence of an aberrantly high mutation rate.

The benzoquinoid ansamycin antibiotics, first isolated from the actinomycete, *Streptomyces hygroscopicus var. geldanus var. nova* (DeBoer, et al., 1970) include geldanamycin (GA) and its semi-synthetic derivatives, 17-allylamino-17-demethoxygeldanamycin (17-AAG) and the more water-soluble 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) (see Figure 2).



Compound	R Group
17-Allylaminogeldanamycin (17-AAG)	CH_2=$CHCH_2NH$-
17-Aminogeldanamycin (17-AG)	NH_2
17-Dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG)	$(CH_2)_2NCH_2CH_2NH$-
Geldanamycin	CH_3O-

Figure 2. The chemical structures of geldanamycin, 17-AAG, its biologically active metabolite 17-AG, and 17-DMAG, highlighting the unique substitutions to the quinone moiety of the pharmacophore that characterize each molecule

These small molecules inhibit the chaperone function of the heat shock protein Hsp90 (Schulte and Neckers, 1998) and are currently being evaluated in phase 1 and 2 clinical trials. The parent compound, GA, is broadly cytotoxic in the NCI 60-cell line screen (Supko, et al., 1995); its poor solubility and unacceptable liver toxicity in dogs precluded testing in humans. Because 17-AAG is less toxic than GA in rats (Page, et al., 1997) and caused growth inhibition in breast (Paine-Murrieta, et al., 1999), melanoma (Burger, et al., 1998), and ovarian mouse xenograft models, the National Cancer Institute (NCI) initiated phase 1 trials in 1999.

Hsp90: A CHAPERONE OF ONCOGENES

Several recent, excellently detailed reviews of the mechanics of Hsp90 function are in the scientific literature (Bagatell and Whitesell, 2004; Chiosis, et al., 2004; Prodromou and Pearl, 2003; Siligardi, et al., 2004; Wegele, et al., 2004; Zhang and Burrows, 2004). For the purposes of the current update on Hsp90-directed therapeutics, suffice it to say that Hsp90 is a conformationally flexible protein that associates with a distinct set of co-chaperones in dependence on nucleotide (ATP or ADP) occupancy of an amino-terminal binding pocket in Hsp90. Nucleotide exchange and ATP hydrolysis (by Hsp90 itself, with the assistance of co-chaperones) drive the so-called Hsp90 chaperone machine to bind, chaperone, and release client proteins. Indeed, identification of the GA binding site as a nucleotide pocket favoring purines led Chiosis and colleagues to design a series of highly potent purine scaffold Hsp90 inhibitors with markedly improved drug-like properties (Chiosis, et al., 2003b; Chiosis, et al., 2002; He, et al., 2006; Llauger, et al., 2005). Workman and colleagues used a high-throughput screen based on inhibition of Hsp90 ATPase activity to identify 3,4-diarylpyrazoles as a novel class of Hsp90 inhibitors (Cheung, et al., 2005; Dymock, et al., 2005). By employing biochemical evaluation and crystallography, these investigators found that pyrazole inhibitors of Hsp90 provide a platform for extensive derivatization and provide an attractive starting point for hit to lead exploration.

The Hsp90 inhibitors currently in clinical trial (17-AAG and 17-DMAG), as well as those under development, all share the property of displacing nucleotide from the amino terminal pocket in Hsp90, and therefore short-circuiting the Hsp90 chaperone machine, much as one would stop the rotation of a bicycle wheel by inserting a stick between the spokes. Cycling of the chaperone machine is critical to its function. The Hsp90 inhibitors, by preventing nucleotide-dependent cycling, interfere with the chaperone activity of Hsp90, resulting in targeting of client proteins to the proteasome, the cell's garbage disposal, where they are degraded (Neckers, 2002). Even if the proteasome is inhibited, client proteins are not rescued from Hsp90 inhibition, but instead accumulate in a misfolded, inactive form in detergent-insoluble subcellular complexes (An, et al., 2000).

Hsp90 Inhibitors Target Mutated and Chimeric Proteins Uniquely Expressed in Certain Cancers

Hsp90 characteristically chaperones a number of mutated or chimeric kinases that are key mediators of disease. Thus, anaplastic large cell lymphomas are characterized by expression of the chimeric protein NPM-ALK, which originates from a fusion of the nucleophosmin (*NPM*) and the membrane receptor anaplastic lymphoma kinase (*ALK*) genes. The chimeric kinase is constitutively active and capable of causing malignant transformation (Fujimoto, et al., 1996). Bonvini and colleagues have shown that NPM-ALK kinase is an Hsp90 client protein, and that GA and 17-AAG destabilize the kinase and promote its proteasome-mediated degradation in several anaplastic large cell lymphoma cell lines (Bonvini, et al., 2002).

FLT3 is a receptor tyrosine kinase that regulates proliferation, differentiation and survival of hematopoietic cells. FLT3 is frequently expressed in acute myeloid leukemia, and in 20 percent of patients with this cancer the tumor cells express a FLT3 protein harboring an internal tandem duplication in the juxtamembrane domain. This mutation is correlated with leukocytosis and a poor prognosis (Naoe, et al., 2001). Minami and colleagues have reported that Hsp90 inhibitors cause selective apoptosis of leukemia cells expressing tandemly duplicated FLT3. Further, these investigators reported that mutated FLT3 was an Hsp90 client protein and that brief treatment with multiple Hsp90 inhibitors resulted in the rapid dissociation of Hsp90 from the kinase, accompanied by the rapid loss of kinase activity together with loss of activity of several downstream FLT3 targets including MAP kinase, Akt, and Stat5a (Minami, et al., 2002). Minami et al. propose that Hsp90 inhibitors should be considered as promising compounds for the treatment of acute myeloid leukemia characterized by tandemly duplicated FLT3 expression.

BCR-ABL ($p210^{Bcr-Abl}$) is an Hsp90 client protein that is also effectively inhibited by the novel tyrosine kinase inhibitor imatinib (An, et al., 2000; Druker, et al., 1996; Shiotsu, et al., 2000). While imatinib has proven very effective in initial treatment of patients with chronic myelogenous leukemia, a majority of patients who are treated when their disease is in blast crisis stage (e.g., advanced) eventually relapse despite continued therapy (Sawyers, et al., 2002). Relapse is correlated with loss of BCR-ABL inhibition by imatinib, due either to gene amplification or to specific point mutations in the kinase domain that preclude association of imatinib with the kinase (Shah, et al., 2002). Gorre and colleagues have reported the very exciting finding that BCR-ABL protein that was resistant to imatinib remained dependent on Hsp90 chaperoning activity and thus retained sensitivity to Hsp90 inhibitors, including GA and 17-AAG. Both compounds induced the degradation of "wild-type" and mutant BCR-ABL, with a trend indicating more potent activity toward mutated imatinib-resistant forms of the kinase (Gorre, et al., 2002). These findings were recently confirmed by other investigators (Nimmanapalli, et al., 2002), thus providing a rationale for the use of 17-AAG in treatment of imatinib-resistant chronic myelogenous leukemia.

Mutations in the proto-oncogene c-*kit* cause constitutive kinase activity of its product, KIT protein, and are associated with human mastocytosis and gastrointestinal stromal tumors (GIST). Although currently available tyrosine kinase inhibitors are effective in the treatment of GIST, there has been limited success in the treatment of mastocytosis. Treatment with 17-AAG of the mast cell line HMC-1.2, harboring the Asp816Val and Val560Gly KIT mutations and the cell line HMC-1.1, harboring a single Val560Gly mutation, causes both the level and activity of KIT and downstream signaling molecules AKT and STAT3 to be down-regulated following drug exposure (Fumo, et al., 2004). These data were validated using Cos-7 cells transfected with wild type and mutated KIT. 17-AAG promotes cell death of both HMC mast cell lines. In addition, neoplastic mast cells isolated from patients with mastocytosis and incubated with 17-AAG *ex vivo* are selectively sensitive to Hsp90 inhibition as compared to the mononuclear fraction as a whole. These data provide compelling evidence that 17-AAG may be effective in the treatment of c-*kit* related diseases including mastocytosis, GIST, mast cell leukemia, sub-types of acute myelogenous leukemia and testicular cancer.

More recently, several groups have reported that mutated B-Raf and mutated epidermal growth factor receptor (EGFR) develop strong dependence on Hsp90 and thus acquire marked sensitivity to Hsp90 inhibitors (da Rocha Dias, et al., 2005; Grbovic, et al., 2006; Shimamura, et al., 2005). Since B-Raf is mutated in approximately 60% of melanomas and to a lesser degree in other cancers (Rajagopalan, et al., 2002), and since cells expressing mutated B-Raf appear to be dependent on its activity for their survival, Hsp90 inhibitors may have wide applicability in melanoma. Indeed, results of a recent clinical trial support this hypothesis (Banerji, et al., 2005). Similarly, the EGFR mutations described in a small percentage of non-small cell lung cancer patients also confer Hsp90 dependence and sensitivity to Hsp90 inhibitors (Shimamura, et al., 2005). While these patients initially respond to EGFR inhibitor therapy, they almost invariably become refractory with time. However, even tumors refractory to EGFR inhibitors remain very sensitive to Hsp90 inhibitors, suggesting that Hsp90 inhibitor therapy may be an efficacious second-line therapy in these patients (Shimamura, et al., 2005).

Hsp90 Inhibitors Target the Androgen Receptor in Prostate Cancer

Androgen receptor continues to be expressed in the majority of hormone-independent prostate cancers, suggesting that it remains important for tumor growth and survival. Receptor over-expression, mutation, and/or post-translational modification may all be mechanisms by which androgen receptor can remain responsive either to low levels of circulating androgen or to anti-androgens. Vanaja et al. have shown that Hsp90 association is essential for the function and stability of the androgen receptor in prostate cancer cells (Vanaja, et al., 2002). These investigators reported that androgen receptor levels in LNCaP cells were markedly reduced by GA, as was the ability of the receptor to become transcriptionally active in the presence of synthetic androgen. In addition, Georget et al. have shown

that GA preferentially destabilized androgen receptor bound to anti-androgen, thus suggesting that the clinical efficacy of anti-androgens may be enhanced by combination with an Hsp90 inhibitor (Georget, et al., 2002). These investigators also reported that GA prevented the nuclear translocation of ligand-bound androgen receptor, and inhibited the transcriptional activity of nuclear-targeted receptors, implicating Hsp90 in multiple facets of androgen receptor activity. Finally, Solit and colleagues have reported that 17-AAG caused degradation of both wild type and mutant androgen receptors and inhibited both androgen-dependent and androgen-independent prostate tumor growth in nude mice (Solit, et al., 2002). Importantly, these investigators also demonstrated the loss of Her2 and Akt proteins, two Hsp90 clients that are upstream post-translational activators of the androgen receptor, in the tumor xenografts taken from 17-AAG-treated animals.

Hsp90 Inhibitors Exert Anti-Angiogenic Activity by Promoting Oxygen- and VHL-Independent Inactivation and Degradation of Hypoxia Inducible Factor 1 α (HIF-1α) Leading to Inhibition of VEGF Expression

Hypoxia inducible factor-1α (HIF-1α) is a nuclear transcription factor involved in the transactivation of numerous target genes, many of which are implicated in the promotion of angiogenesis and adaptation to hypoxia (for a review, see (Harris, 2002)). Although these proteins are normally labile and expressed at low levels in normoxic cells, their stability and activation increase several-fold in hypoxia. The molecular basis for the instability of these proteins in normoxia depends upon VHL, the substrate recognition component of an E3 ubiquitin ligase complex that targets HIF-1α for proteasome-dependent degradation (Maxwell, et al., 1999). Hypoxia normally impairs VHL function, thus allowing HIF to accumulate. HIF-1α expression has been documented in diverse epithelial cancers and most certainly supports survival in the oxygen-depleted environment inhabited by most solid tumors.

VHL can also be directly inactivated by mutation or hyper-methylation, resulting in constitutive over-expression of HIF in normoxic cells. In hereditary von Hippel-Lindau disease there is a genetic loss of VHL, and affected individuals are predisposed to an increased risk of developing highly vascular tumors in a number of organs. This is due, in large part, to deregulated HIF expression and the corresponding up-regulation of the HIF target gene vascular endothelial growth factor (VEGF). A common manifestation of VHL disease is the development of clear cell renal cell carcinoma (CC-RCC) (Seizinger, et al., 1988). VHL inactivation also occurs in nonhereditary, sporadic CC-RCC.

HIF-1α interacts with Hsp90 (Gradin, et al., 1996), and both GA and another Hsp90 inhibitor, radicicol, reduce HIF-dependent transcriptional activity (Hur, et al., 2002, Isaacs, et al., 2002). Hur et al. demonstrated that HIF protein from radicicol-treated cells was unable to bind DNA, suggesting that Hsp90 is necessary for mediating the proper conformation of HIF and/or recruiting additional cofactors. Likewise, Isaacs et al. reported GA-dependent, transcriptional inhibition of VEGF.

Additionally, GA down-regulated HIF-1α protein expression by stimulating VHL-independent HIF-1α proteasomal degradation (Isaacs, et al., 2002; Mabjeesh, et al., 2002).

HIF-1α induction and VEGF expression has been associated with migration of glioblastoma cells *in vitro* and metastasis of glioblastoma *in vivo*. Zagzag et al., in agreement with the findings described above, have reported that GA blocks HIF-1α induction and VEGF expression in glioblastoma cell lines (Zagzag, et al., 2003). Further, these investigators have shown that GA blocks glioblastoma cell migration, using an *in vitro* assay at non-toxic concentrations. This effect on tumor cell motility was independent of p53 and PTEN status, which makes Hsp90 inhibition an attractive modality in glioblastoma, where mutations in p53 and PTEN genes are common and where tumor invasiveness is a major therapeutic challenge.

Dias et al. have recently reported that VEGF promotes elevated Bcl2 protein levels and inhibits activity of the pro-apoptotic caspase-activating protein Apaf in normal endothelial cells and in leukemia cells bearing receptors for VEGF (Dias, et al., 2002). Intriguingly, these investigators show that both phenomena require VEGF-stimulated Hsp90 association (e.g., with Bcl2 and Apaf), and that GA reverses both processes. Thus, GA blocked the pro-survival effects of VEGF by both preventing accumulation of anti-apoptotic Bcl2 and blocking the inhibition of pro-apoptotic Apaf.

Hsp90 Inhibitors Target MET and RET Receptor Tyrosine Kinases

The Met receptor tyrosine kinase is frequently over-expressed in cancer, and is involved in angiogenesis, as well as in the survival and invasive ability of cancer cells. A recent report by Maulik et al. has demonstrated a role for Met in migration and survival of small cell lung cancer (Maulik, et al., 2002). Met is an Hsp90 client protein, and these investigators went on to show that GA antagonized Met activity, reduced the Met protein level, and promoted apoptosis in several small cell lung cancer cell lines, even in the presence of excess Met ligand.

Hypoxia potentiates the invasive and metastatic potential of tumor cells. In an important recent study, Pennacchietti and colleagues reported that hypoxia (via two HIF-1α response elements) transcriptionally activated the Met gene, and synergized with Met ligand in promoting tumor invasion. Further, they showed that the pro-invasive effects of hypoxia were mimicked by Met over-expression, and that inhibition of Met expression prevented hypoxia-induced tumor invasion (Pennacchietti, et al., 2003). Coupled with an earlier report describing induction of HIF-1 transcriptional activity by Met ligand (Tacchini, et al., 2001), these data identify the HIF-VEGF-Met axis as a critical target for intervention using Hsp90 inhibitors, either alone or in conjunction with other inhibitors of angiogenesis. As Bottaro and Liotta recently pointed out (Bottaro and Liotta, 2003), the sole use of angiogenesis inhibitors to deprive tumors of oxygen might produce an unexpectedly aggressive phenotype in those cells that survived the treatment. These authors speculated that combination of Met inhibitors with anti-angiogenesis agents should therefore be

beneficial. We would suggest that combination of an anti-angiogenesis drug with an Hsp90 inhibitor would not only potentiate the anti-tumor effects obtained by inhibiting angiogenesis, but would also break the HIF-Met axis by simultaneously targeting both Hsp90-dependent signaling proteins.

Mutation of a related receptor tyrosine kinase, RET, is associated with human cancer and several human neuroendocrine diseases. Point mutations of RET are responsible for multiple endocrine neoplasia type 2 (MEN2A, MEN2B, and familial medullary thyroid carcinoma [FMTC]). Somatic gene rearrangements juxtaposing the TK domain of RET to heterologous gene partners are found in papillary carcinomas of the thyroid (PTC) (Ichihara, et al., 2004; Jhiang, 2000; Santoro, et al., 2002).

Possible effects of 17-AAG on RET activity and cell growth of the TT MTC cell line have been examined (Cohen, et al., 2002). Following treatment with 17-AAG, RET tyrosine kinase activity was inhibited by nearly 80%, as was the rate of cell growth. Thus, 17-AAG should be considered as an attractive pharmacologic agent for use as systemic therapy in patients with recurrent metastatic MTC for which non-surgical therapy has been ineffective.

Combined Inhibition of Hsp90 and the Proteasome Disrupt the Endoplasmic Reticulum and Demonstrate Enhanced Toxicity Toward Cancer Cells

Proteasome-mediated degradation is the common fate of Hsp90 client proteins in cells treated with Hsp90 inhibitors (Mimnaugh, et al., 1996; Schneider, et al., 1996). Proteasome inhibition does not protect Hsp90 clients in the face of chaperone inhibition – instead client proteins become insoluble (An, et al., 2000; Basso, et al., 2002). Since the deposition of insoluble proteins can be toxic to cells (French, et al., 2001; Waelter, et al., 2001), interest has arisen in combining proteasome inhibition with inhibition of Hsp90, the idea being that dual treatment will lead to enhanced accumulation of insoluble proteins and trigger apoptosis. This hypothesis is particularly appealing since a small molecule proteasome inhibitor has demonstrated efficacy in early clinical trials (Aghajanian, et al., 2002; L'Allemain, 2002). Initial experimental support for such an hypothesis was provided by Mitsiades et al. (Mitsiades, et al., 2002), who reported that Hsp90 inhibitors enhanced multiple myeloma cell sensitivity to proteasome inhibition. Importantly, transformed cells are more sensitive to the cytotoxic effects of this drug combination than are non-transformed cells. Thus, 3T3 fibroblasts are fully resistant to combined administration of 17-AAG and Velcade™ at concentrations that prove cytotoxic to 3T3 cells transformed by *HPV16* virus encoding viral proteins E6 and E7 (Mimnaugh, et al., 2004). In the same study, Mimnaugh et al. demonstrated that the endoplasmic reticulum is one of the main targets of this drug combination. In the presence of combined doses of both agents that show synergistic cytotoxicity, these investigators noted a nearly complete disruption of the architecture of the endoplasmic reticulum. Since all secreted and transmembrane proteins must pass through this organelle on

their route to the extracellular space, it is not surprising that a highly secretory cancer such as multiple myeloma would be particularly sensitive to combined inhibition of Hsp90 and the proteasome. One might speculate that other highly secretory cancers, including hepatocellular carcinoma and pancreatic carcinoma, would also respond favorably to this drug combination.

Hsp90 Inhibitors Sensitize Cancer Cells to Radiation

Gius and colleagues have reported that 17-AAG potentiates both the *in vitro* and *in vivo* radiation response of cervical carcinoma cells (Bisht, et al., 2003). An enhanced radiation response was noted when cells were exposed to radiation within 6 to 48 hours after drug treatment. Importantly, at 17-AAG concentrations that were themselves non-toxic, Hsp90 inhibition enhanced cell kill in response to an otherwise ineffective radiation exposure (2 Gray) by more than one log. Even at moderately effective levels of radiation exposure (4-6 Gray), addition of non-toxic amounts of 17-AAG enhanced cell kill by more than one log. Importantly, the sensitizing effects of 17-AAG observed in the cervical carcinoma cells were not seen in 3T3 cells, but were observed in *HPV16-E6* and *-E7* transformed 3T3 cells. The authors demonstrated convincingly that the effect of 17-AAG was multi-factorial, since several pro-survival Hsp90 client proteins were rapidly down-regulated upon drug treatment. *In vitro* findings were confirmed by a murine xenograft study in which the anti-tumor activity of both single and fractionated radiation exposure was dramatically enhanced by treatment with 17-AAG, either 16 hours prior to single radiation exposure or on days 1 and 4 of a 6 day period during which the animals received fractionated radiation exposure. Machida and colleagues reported similar findings for lung carcinoma and colon adenocarcinoma cells *in vitro* (Machida, et al., 2003). Thus, 17-AAG has been validated as a potential therapeutic agent that can be used at clinically relevant doses to enhance cancer cell sensitivity to radiation. It is reasonable to expect that other Hsp90 inhibitors will have a similar utility.

Targeting Hsp90 on the Cancer Cell Surface

Recently, Becker and colleagues reported that Hsp90 expression is dramatically upregulated in malignant melanoma cells as compared to benign melanocytic lesions, and that Hsp90 is expressed on the surface of 7 out of 8 melanoma metastases (Becker, et al., 2004). Eustace et al. have identified cell surface Hsp90 to be crucial for the invasiveness of HT-1080 fibrosarcoma cells *in vitro* (Eustace and Jay, 2004; Eustace, et al., 2004). Taken together, these data implicate Hsp90 as an important determinant of tumor cell invasion and metastasis. Indeed, in the Eustace et al. study, the investigators demonstrated that GA covalently affixed to cell impermeable beads was able to significantly impair cell invasion across a Matrigel-coated membrane. These findings have been confirmed using a polar (and thus cell impermeable) derivative of 17-DMAG in place of GA-beads (Neckers et al., unpublished

observations). Coincident with its inhibitory effects on cell invasiveness, cell impermeable GA also antagonized the maturation, via proteolytic self-processing, of the metalloproteinase MMP2, a cell surface enzyme whose activity has been previously demonstrated as essential to cell invasion. Further, these investigators demonstrated that Hsp90 could be found in association with MMP2 in the culture medium bathing the HT-1080 cells. It is intriguing to speculate that association with Hsp90 on the cell surface is necessary for the self-proteolysis of MMP2. Thus, a possible chaperone function for cell surface Hsp90 may be directly implicated in tumor cell invasiveness and metastasis. As such, cell surface Hsp90 may represent a novel, perhaps cancer-specific target for cell-impermeant Hsp90 inhibitors.

METABOLISM OF 17-AAG AND 17-DMAG *IN VIVO*

In human or murine hepatic microsome assays, 17-aminogeldanamycin (17-AG), a diol, and an epoxide are the three major metabolites of 17-AAG (Egorin, et al., 1998). The 17-AAG diol was the major metabolite in human hepatic microsomes, followed by 17-AG; in contrast, 17-AG was the most abundant metabolite in murine microsomes. Acrolein, a nephrotoxin, is a potential by-product of the 17-AG metabolite. Finally, the epoxide is probably formed by addition of oxygen across the double bond of the allylamino side chain. CYP3A4 enzymatic metabolism is responsible for 17-AG and epoxide formation. Microsomal epoxide hydrolase catalyzes the conversion of the diol to 17-AG, which does not undergo further microsomal metabolism. 17-AAG metabolites are active and may have clinical significance. The biologically active epoxides and acrolein may induce toxic effects in humans (Egorin, et al., 1998). Pharmacodynamic studies show that the 17-AG metabolite (see Figure 2) is as active as 17-AAG in decreasing cellular $p185^{erbB2}$ in human breast cancer SKBr3 cells in culture (Schnur, et al., 1995). 17-AG causes growth-inhibition in six human colon cancer lines and three ovarian cancer cell lines (Kelland, et al., 1999).

In contrast to 17-AAG, 17-DMAG appears to be only minimally metabolized by CYP3A4 (Egorin, et al., 2002). Therefore, intestinal CYP3A4 should not impede 17-DMAG's oral activity. 17-AG does not appear to be a metabolite of 17-DMAG based on the lack of conversion at the 17 position of the compound. The marked metabolic differences between 17-AAG and 17-DMAG suggest that they may have distinct toxicity profiles and therapeutic indices.

WHY ARE TUMOR CELLS UNIQUELY SENSITIVE TO HSP90 INHIBITION?

It is apparent, from both preclinical and clinical observations, that Hsp90 inhibitors can be administered *in vivo* at doses and schedules that significantly impact tumor growth but with minimal target related toxicity to normal tissues. This is the case for several small molecule inhibitors, including 17-AAG and 17-DMAG, the synthetic purine mimetic PU24FCl, and it even applies to a novel peptidomimetic inhibitor

of the N-terminal Hsp90 nucleotide binding site, shepherdin (Banerji, et al., 2005; Eiseman, et al., 2005,; Plescia, et al., 2005; Vilenchik, et al., 2004; Xu, et al., 2003). Since Hsp90 is highly expressed in most, if not all normal tissues, these findings require an explanation. Indeed, when murine model systems are examined *in vivo*, Hsp90 inhibitors are found to concentrate in tumor tissue, while being rapidly cleared from normal tissue with a half-life similar to that of drug in plasma (Banerji, et al., 2005; Eiseman, et al., 2005; Vilenchik, et al., 2004; Xu, et al., 2003). The Hsp90 inhibitor 17-AAG also has been reported to actively concentrate in tumor cells *in vitro* (Chiosis, et al., 2003a).

Since preferential accumulation of these Hsp90 inhibitors in tumor vs. normal tissue may provide the observed therapeutic (or at least biologic) index, it is important to understand the reason for this phenomenon. A possible explanation put forth by Kamal and colleagues suggests that enhanced drug binding to tumor cell Hsp90 reflects the activity state of the Hsp90 chaperone machine in tumor vs. normal cells (Kamal, et al., 2003). They proposed that enhanced the ATPase activity of the chaperone in tumor cells, which is dependent on preferential recruitment of Hsp90 to a multi-component chaperone complex, is responsible for the increased affinity of Hsp90 inhibitors in tumor cells.

Others have reported that expression of NAD(P)H:Quinone Oxidoreductase I (NQO1), also known as DT-diaphorase, dramatically enhances cellular sensitivity to 17-AAG (Banerji, et al., 2005; Kelland, et al., 1999). NQO1 generates the hydroquinone version of 17-AAG, which has recently been reported to bind more tightly to Hsp90 when compared to 17-AAG itself (Guo, et al., 2005). Further, the presence of NQO1 in a cell seems also to lead to increased total accumulation of intracellular ansamycin molecules, presumably reflecting the increased water solubility of the 17-AAG dihydroquinone and its decreased propensity to cross membranes. Thus, by this model NQO1 serves to trap 17-AAG in cells while simultaneously enhancing its Hsp90 binding affinity. Intriguingly, these investigators and others have shown that the presence of NQO1 in tumor cells dramatically affects cellular sensitivity to 17-AAG (Banerji, et al., 2005; Guo, et al., 2005; Kelland, et al., 1999). Since high levels of NQO1 have been observed in diverse tumor types (e.g., liver, lung, colon, breast) as compared to normal tissues of the same origin (Belinsky and Jaiswal, 1993), these data suggest an explanation for the disparate sensitivity of tumor and normal tissue to 17-AAG. However, the similar preference of other Hsp90 inhibitors, such as the synthetic purine analog PU24FCl and the peptidomimetic shepherdin, for tumor cells remains to be explained. Several groups are currently examining altered states of post-translational modification of Hsp90 in tumor vs. normal cells as a possible contributing factor to this phenomenon.

CLINICAL TRIAL DATA

A phase 1 Institute of Cancer Research (UK) phase 1 trial of 17-AAG in malignant melanoma used a once weekly administration schedule. The starting dose was 10 mg/m²/week administered IV once weekly in a cohort of three patients. Doses were

doubled in each succeeding cohort (Banerji, et al., 2001). Adverse events included grade 1/2 nausea and grade 1/2 fatigue in 3 and 9 of the first 15 patients, respectively. One patient experienced grade 3 vomiting at the 80 mg/m²/week dose. Grade 3 nausea and vomiting occurred in two of six patients treated at the 320 mg/m²/week dose, following which the dose was escalated by 40% to 450 mg/m²/week (Banerji, et al., 2002). A total of 28 patients have been treated to date on this trial. Among the six patients treated at the 320–450 mg/m²/week dose range, two patients showed stable disease for 27 and 91 weeks, respectively.

Pharmacodynamic marker analysis of tumor biopsies done before and 24 hours after treatment in nine patients showed depletion of the Hsp90 client c-Raf in four of seven samples (where the marker was expressed), and cdk4 (Hsp90 client) depletion and Hsp70 induction in eight of the nine samples (Banerji, et al., 2003). At the highest dose level, pharmacokinetic analysis indicated a $t_{½}$ of 5.8±1.9 h, V_{dss} of 274±108 L, clearance of 35.5±16.6 L/h, and C_{max} of 16.2±6.3 μM (Banerji, et al., 2003), which is above the levels of 375 nM to10 μM reported to inhibit Hsp90 *in vitro* (Burger, et al., 2000). Although a maximal tolerated dose was not established in this trial, the dose/schedule that will be taken forward to phase II evaluation is likely to be 450 mg/m²/week, as there was evidence of tumor target inhibition at that dose level (Banerji, et al., 2003). Updated results of this phase I trial have recently been published (Banerji, et al., 2005).

Hsp90 inhibitors are a class of agents that affect a diverse group of client proteins involved in oncogenesis. Many of these clients are expressed in a disease-specific fashion. The development of these inhibitors as biomodulators is complex and not necessarily governed by standard approaches. The clinical approach taken with the Hsp90 inhibitors was to proceed simultaneously with single agent phase 2 studies as well as disease-specific combinations that would be used to evaluate the biomodulatory effects of 17-AAG and 17-DMAG. As these studies mature and reach completion, the role of Hsp90 inhibitors in the treatment of cancer should be better defined with regard to their activity and molecular targeted effects.

HSP90 INHIBITORS IN NEURODEGENERATIVE DISEASES

Unfolded or misfolded proteins have exposed hydrophobic segments that render them prone to aggregation. Protein aggregates are toxic to the cell (Taylor, et al., 2002), and molecular chaperones, especially Hsp70, bind to hydrophobic surfaces of misfolded proteins to insure their continued solubility or to promote their degradation by the proteasome (Hershko and Ciechanover, 1998). Under pathologic conditions, the level of misfolded proteins may exceed the ability of the cell to either maintain them in a soluble form or to degrade them, allowing aggregation to proceed (Cohen, 1999; Zoghbi and Orr, 2000). Protein aggregates have been found in most chronic neurodegenerative diseases (Kakizuka, 1998; Taylor, et al., 2002), as well as in global and focal ischemia and in hypoglycemic coma (Hu, et al., 2000; Ouyang and Hu, 2001) . Thus, pharmacologic induction of molecular chaperones in general, and Hsp70 in particular, may be ameliorative in these cases. The Hsp90

inhibitors currently in clinical trial, 17-AAG and 17-DMAG, have the property of inducing Hsp70 in normal cells and tissues, via disruption of Hsp90 sequestration of the heat shock transcription factor Hsf1 (Ali, et al., 1998; Kim, et al., 1999; Lu, et al., 2002), and so they have become of interest in this regard.

Giffard and colleagues have reported that GA, an Hsp90 inhibitor structurally related to 17-AAG and 17-DMAG, via its ability to induce Hsp70, reduces protein aggregation in a rodent model of global ischemia and blocks apoptotic astrocyte death induced by glucose deprivation (Giffard, et al., 2004). These investigators showed that GA-treated astrocyte cultures were twice as viable as untreated cultures after 24 hours of glucose deprivation, and they make the point that, because Hsp70 can block both apoptotic and necrotic cell death, it is an intriguing target for anti-ischemic therapy.

The progressive loss of dopaminergic neurons in the substantia nigra is the defining pathogenic feature of Parkinson disease (PD). α-Synuclein is mutated in rare familial forms of PD and it is a major component of the pathologic protein aggregates characteristic of the disease (Kruger, 2004; Polymeropoulos, et al., 1997; Tofaris and Spillantini, 2005). Expression of normal as well as mutant α-synuclein in *Drosophila melanogaster* causes selective loss of dopaminergic neurons (Feany and Bender, 2000), and this can be completely prevented by raising the level of Hsp70 by transgenic expression (Auluck, et al., 2002a). Thus, dopaminergic neurons may be sensitive to compromised chaperone levels. A recent study demonstrated that pharmacologic enhancement of Hsp70, via GA administration to adult *Drosophila* during a 3-week period, completely protected their dopaminergic neurons from α-synuclein-induced toxicity (Auluck and Bonini, 2002b). Moreover, in contrast to the findings of a previous study, which treated developing flies in a similar fashion, prolonged exposure of adult flies to effective doses of GA caused no noticeable deleterious effects (Rutherford and Lindquist, 1998). Given the complete protection of dopaminergic neurons afforded by GA, the authors of this study propose that GA and its derivatives warrant a careful examination as cytoprotective agents for treating PD and other neurodegenerative diseases.

17-AAG has recently been shown to ameliorate polyglutamine-mediated motor neuron degeneration (Waza, et al., 2005). Because mutated androgen receptor (AR) is a pathogenic gene product in spinal and bulbar muscular atrophy (SBMA), Waza and colleagues examined whether 17-AAG could potentiate degradation of the polyglutamine-expanded mutant AR. These investigators found that administration of 17-AAG markedly ameliorated motor impairments in the SBMA transgenic mouse model without detectable toxicity and reduced the amount of detectable monomeric and aggregated mutated AR protein. As expected, polyglutamine-expanded AR showed a higher affinity for Hsp90 as compared to wild-type AR, and it was preferentially degraded in the presence of 17-AAG in both cells and transgenic mice. 17-AAG also mildly induced Hsp70 in this model. These investigators suggest that 17-AAG thus provides a novel therapeutic approach to SBMA by promoting the degradation of a pathologic mutant Hsp90 client protein.

Finally, two groups have reported that, using both mouse and *Drosophila* models of Huntington's disease (HD), pharmacologic induction of Hsp70 with Hsp90 inhibitors provides a useful therapeutic strategy. Hay et al. report that a progressive decrease in Hsp70 and other chaperones in brain tissue contributes to disease pathogenesis in the R6/2 mouse model of HD (Hay, et al., 2004). Both radicicol and GA were able to maintain chaperone induction for at least three weeks and were able to improve the detergent-soluble properties of polyglutamine-containing aggregates over this time course. Meanwhile, Agrawal and colleagues have shown, using a *Drosophila* model of HD (flies transgenically express glutamine-expanded human huntingtin protein), that feeding affected flies GA alone or in combination with a histone deacetylase inhibitor (suberoylanilide hydroxamic acid, SAHA) strongly suppresses the degeneration of photoreceptor neurons while causing no overt toxicity in control flies (Agrawal, et al., 2005). Intriguingly, we and others have recently shown that several classes of histone deacetylase inhibitors, including SAHA, have the unexpected property of inhibiting Hsp90 by promoting its hyperacetylation (Bali, et al., 2005; Yu, et al., 2002). Thus, in the *Drosophila* HD model the observed beneficial activity of each agent alone as well as the synergistic activity of their combination suggests that Hsp90 inhibition (and/or the resultant increase in Hsp70) is the primary mechanism of action of both drugs.

The apparent *in vivo* safety and efficacy of these benzoquinone ansamycin Hsp90 inhibitors in several models of neurodegeneration considerably extend the therapeutic application of these drugs (and perhaps other Hsp90 inhibitors) beyond oncology. Whether the primary mechanism is degradation of an Hsp90-dependent polyglutamine-expanded mutant protein, the pharmacologic induction of Hsp70, or a combination of the two processes, Hsp90 inhibitors have a promising future in the treatment of neurodegenerative pathologies.

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CHAPTER 13

TARGETING HSP90 FUNCTION TO TREAT CANCER: MUCH MORE TO BE LEARNED

LUKE WHITESELL* AND CATHERINE A. McLELLAN
Whitehead Institute for Biomedical Research Cambridge, MA 02142, USA

Abstract: Molecular chaperones or so-called heat shock proteins act as central integrators of protein homeostasis within cells. Among the major chaperones, however, Hsp90 is unique because it is not required for the biogenesis of most polypeptides. Instead, it oversees a surprisingly diverse network of conformationally labile client substrates that regulate signaling pathways and gene expression. Many of the processes modulated by Hsp90 are dysregulated in cancer cells including cell cycle control, apoptosis and chromatin re-modeling. Over the past decade, much of the progress achieved in understanding the complex role of Hsp90 in cancer biology has been made possible by the discovery of several natural product antitumor antibiotics that selectively inhibit Hsp90 function. These compounds have the ability to accomplish what most molecularly targeted anticancer therapies do not –the simultaneous disruption of multiple processes critical to tumor cell growth and survival. Now, great enthusiasm exists over the prospect of targeting Hsp90 function to treat cancer. New chemotypes with improved pharmacology are being developed and clinical trials have demonstrated that Hsp90 function can be inhibited in cancer patients without undue acute toxicity. Remarkable progress has been made, but much more remains to be learned if we are to succeed in the challenge of defining safe and effective ways to exploit Hsp90 inhibition in the treatment of patients

Keywords: Chaperone, geldanamycin, heat shock protein, chemotherapy

It is much more difficult to be convincing about ignorance concerning disease mechanisms than it is to make claims for full comprehension, especially when the comprehension leads, logically or not, to some sort of action.
Lewis Thomas in "On Magic in Medicine", The Medusa and the Snail, 1979

*Corresponding author: Whitehead Institute Room 663 9 Cambridge Center, Tel: 617-452-3542, Fax: 617-258-6691, e-mail: whitesell@wi.mit.edu

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INTRODUCTION

Tremendous progress in understanding the specific molecular genetic lesions that underlie the initiation and progression of human cancers has made possible the development of targeted therapeutics that do not rely on the generalized disruption of DNA metabolism and cell division for their activity. Of particular interest have been inhibitors of cellular signal transduction pathways involving kinases and hormone receptors. Unique within this rapidly expanding array of small molecules and proteins, have been compounds that target the functions of heat shock protein 90 (Hsp90). This multifunctional molecular chaperone regulates the post-translational stability and function of a distinct but diverse set of "client" proteins known to be critically involved in oncogenesis (also See: *Pratt et al*, Chapter 1, *Neckers*, Chapter 12, *Kamal et al*, Chapter 14). Inhibition of Hsp90 leads to depletion of many of these oncogenic clients by stimulating their enhanced degradation by the ubiquitin-proteasome system. Most of the current enthusiasm driving the discovery and development of Hsp90 inhibitors has been generated by their potential to accomplish what many molecularly targeted anticancer therapies do not –the simultaneous disruption of multiple signaling pathways critical to tumor cell growth and survival. Such a combinatorial attack on the oncogenic clients of Hsp90 has been proposed to represent a "rational approach" to addressing the heterogeneity and complexity of the numerous genetic defects characteristic of most clinical cancers. Decades of careful investigation encompassing many different fields has provided a wealth of knowledge about Hsp90 and its interaction with its client proteins. Nevertheless, our understanding of Hsp90 function and how it is altered in various cancers remains far from complete. In particular the impact of altering Hsp90 function on cellular processes other than signaling, such as protein homeostasis, energy metabolism, chromatin re-modeling and DNA repair has been given much less consideration. A better understanding of Hsp90 function at a more global level will be required for understanding its role in oncogenesis and for the efficient development of Hsp90 inhibitors as useful cancer chemotherapeutics Soti, 2005 #739}. In this regard, targeting Hsp90 in the treatment of cancer presents its own unique challenges. But it can also serve as an excellent paradigm for thinking more generally about molecular interventions as "perturbagens" that alter not just their target, but rather the function of entire systems to generate their desired therapeutic effects(Lamb et al., 2006; Ramanathan et al., 2005a).

Interest in Hsp90 inhibition as a therapeutic strategy is high with an average of 5 to 10 new cancer-related research papers appearing each week in PubMed. Academic labs, biotechnology firms and large pharmaceutical corporations are generating a wealth of new compounds with potent and selective Hsp90 inhibitory activity. Many recent reviews are available summarizing the pre-clinical and clinical data generated as these compounds are evaluated (Cullinan and Whitesell, 2006; Goetz et al., 2003; Isaacs et al., 2003; Sharp and Workman, 2006). Here we provide a brief summary of Hsp90 structure and function to provide context and describe some of the more recent progress in identifying and developing new inhibitors of this chaperone. To avoid repetition and provide a somewhat different perspective,

however, this chapter will highlight the most important gaps in our understanding of how Hsp90 function is subverted to enable oncogenesis and how the altered utilization of Hsp90 by cancer cells might impact attempts to target it for treatment. The goal is to stimulate further investigation into these areas and make the ongoing clinical development of Hsp90 inhibitors as anticancer drugs less empiric, more efficient and hopefully more successful.

HSP90 STRUCTURE AND BIOCHEMICAL FUNCTION

The Hsp90 family in mammalian cells consists of four members: the cytoplasmic chaperone which includes both inducible (Hsp90α in humans, Hsp86 in mice) and constitutive (HSP90β/Hsp84) isoforms; the endoplasmic reticulum chaperone, Grp94 (glucose regulated protein 94); and the mitochondrial homologue, TRAP1 (Tumour necrosis factor Receptor-Associated Protein 1)(Csermely et al., 1998). An unusual member of the HSP90 family, 75kDHSP90N has been identified which possesses a putative myristoylation signal and associates with RAF-1 in a RAS-independent manner (Grammatikakis et al., 2002). Relatively little attention has been given to the effects of Hsp90 inhibitors on the function of non-cytoplasmic family members, which bind the classical Hsp90 inhibitors geldanamycin and radicicol (Lawson et al., 1998; Rosser and Nicchitta, 2000; Schulte et al., 1999).

In all eukaryotes examined to date, cytosolic Hsp90 is an essential protein and under basal conditions constitutive Hsp90 expression can account for up to 1–2% of total cellular protein, a level that seems to exceed the level required for homeostasis under basal conditions (Borkovich et al., 1989). The prokaryotic homolog of Hsp90, *HtpG* is not an essential gene in bacteria. Although it shares sequence similarity and domain organization with Hsp90, none of the co-chaperones that act with Hsp90 in eukaryotic cells have been found in prokaryotes. Indeed, recent structural insights indicate that HtpG functions in a manner with important differences from that of eukaryotic Hsp90 (Richter and Buchner, 2006; Shiau et al., 2006)

The so-called 'inducible' isoform of Hsp90 in mice and humans is actually expressed under normal conditions; however under stress conditions a 2–3 fold increase in its level is observed, presumably in response to the accumulation of damaged clients (Jerome et al., 1991; Quraishi et al., 1996; Xiao and Lis, 1989). To date, no differences in biochemical function have been described for the two mammalian Hsp90 isoforms (Sreedhar et al., 2004). The proteins are 76% identical and are the likely result of gene duplication about 500 million years ago (Moore et al., 1989). However, the proteins might not be entirely redundant, since a knockout mouse strain for Hsp90β in which Hsp90α remains unperturbed displays embryonic lethality (Voss et al., 2000).

Hsp90 exists primarily as a dimer in cells with each monomer made up of three functional domains, an intensively studied N-terminal ATP-binding domain; a middle region that completes the ATPase site; and a C-terminal dimerization domain containing an EEVD tetratricopeptide repeat (TPR)-binding motif (Pearl and Prodromou, 2000). A representation of the functional domains of Hsp90

that also depicts the location of binding sites for regulatory small molecules is provided in Figure 1.

Of note, biochemical evidence from several labs has suggested the existence of a second cryptic ATP binding site in the chaperone's C-terminal domain that is revealed by nucleotide occupancy of its N-terminal site (Garnier et al., 2002; Soti et al., 2002; Soti et al., 2003). The role of this putative site in regulating overall function is not well understood.

Hsp90 forms several distinct super-protein complexes with an array of co-chaperones and accessory proteins, such as Hsp70, Hsp40, Hop/p60, p23, the large immunophilins, and Cdc37/p50. The interaction between Hsp90 and the immunophilins, Hsp70, Hop and Cdc37, occurs within the C-terminal domain (Pratt and Toft, 2003). In contrast, the recently identified co-chaperone Aha1 binds to the middle domain of Hsp90 where it stimulates Hsp90 ATPase activity (Lotz et al., 2003; Meyer et al., 2004). Complex formation appears to regulate and direct Hsp90 interactions with specific types of client proteins (Chadli et al., 2006; Prodromou et al., 1999). Interaction with the Hsp90-based machinery maintains these conformationally labile clients in a dynamic so-called "poised" state –properly localized and ready for ligand binding, activation, translocation, etc. Should the client protein

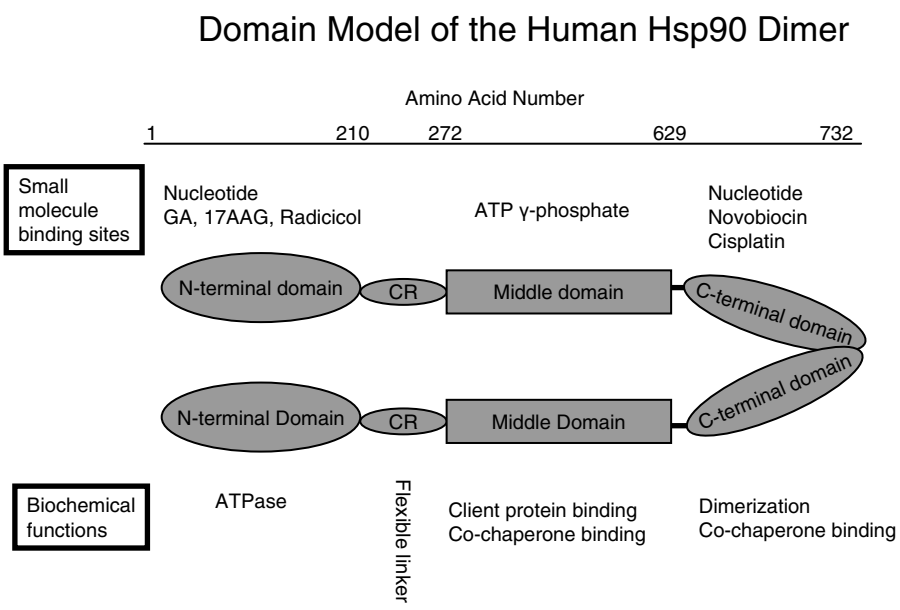

Figure 1. Structure of Hsp90. The approximate positions in the amino acid sequence of human Hsp90 that define its functional domains are indicated above the cartoon representation of the dimer. "CR" refers to a region with many charged residues that acts as a flexible linker between the N-terminal and Middle domains of Hsp90 in vertebrates. The biochemical functions ascribed to the various domains are indicated just below the cartoon while the locations where various small molecules bind Hsp90 and modulate its function are indicated below these. GA, geldanamycin; 17AAG, 17allylaminogeldanamycin

fail to attain its proper conformation, or should it fail to encounter a requisite ligand or binding partner, the Hsp90 machinery can alternatively present clients for degradation, most commonly via the ubiquitin-26S proteasome system (Marques et al., 2006; Xu et al., 2002). Largely based on the work of D. Smith and colleagues (Smith et al., 1995), a simplified representation of how the Hsp90-based chaperone machinery regulates the function and turnover of classical clients such as steroid hormone receptors is presented in Figure 2.

Iterative cycles of loading and discharging substrates and accessory proteins rely on the intrinsic ATPase activity of Hsp90 (Pratt and Toft, 2003). The N-terminal ATP-binding pocket of Hsp90 consists of an unusual adenine-nucleotide-binding motif known as the Bergerat fold (Dutta and Inouye, 2000). This structural motif belongs to the GHKL super-family (bacterial Gyrase, Hsp90, histidine Kinase, MutL). It has no similarity to the ATPase domains found in other kinases or the

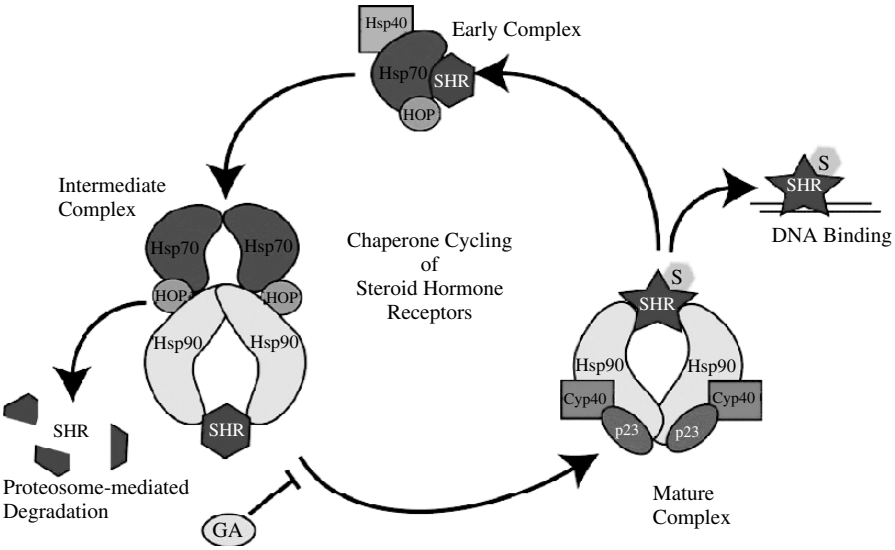

Figure 2. An Hsp90-based chaperone machine regulates the maturation, function and turnover of steroid hormone receptors. Newly synthesized steroid hormone receptor (SHR) associates with Hsp70, Hsp40 and the co-chaperone HOP to form an early complex. Hsp90 then binds to this complex and displaces Hsp40 to form an intermediate complex. In an ATP-dependent step, Hsp90 fully exposes the hormone binding domain of the SHR, the co-chaperone p23 stabilizes this ATP-bound Hsp90 conformation and a large immunophilin (e.g. Cyp40, as depicted here for the case of the estrogen receptor) fills the open tetratricopeptide (TPR) acceptor site on Hsp90 to complete a mature complex. In the absence of steroid hormone ligand ("S"), the receptor is eventually released from the complex to undergo another cycle of ATP-dependent chaperone interactions. Upon binding "S", however, the receptor undergoes additional conformational changes that lead to release of chaperone components and tight binding to DNA hormone response elements and recruitment of the necessary co-activators to drive transcription. The binding of geldanamycin (GA) and other Hsp90 inhibitors to the N-terminus of the chaperone prevents the formation of mature complexes and normal cycling. As a result, the SHR accumulates in an intermediate complex that targets it for ubiquitination and proteasome-mediated degradation

chaperone Hsp70 which makes possible its highly selective targeting by small molecules. The binding and hydrolysis of ATP trigger conformational changes in Hsp90, switching it between a "closed" and an "open" state. A model for Hsp90 cycling in which the chaperone undergoes transient dimerization of its N-terminal domains in concert with association of the N-terminal and middle domains following ATP binding is supported by a recent report of the first crystal structure for full-length yeast Hsp90 in complex with a co-chaperone (p23/Sba1) and a non-hydrolyzable ATP analog (Ali et al., 2006). An absolute requirement for cooperation between the ATPase activities of each monomer during the chaperoning of clients by human Hsp90, however, has been questioned (McLaughlin et al., 2006).

Although Hsp90 is thought to interact with its clients via hydrophobic binding surfaces that change conformation as a result of ATP binding and hydrolysis, it remains unclear exactly where and how clients bind to the various domains of Hsp90. So far, the only structural data addressing this issue consist of electron micrographs of Hsp90 bound to a kinase in association with the kinase specific co-chaperone Cdc37 (Vaughan et al., 2006). Within the limits of the resolution provided by electron microscopy, it appears that this complex is surprisingly asymmetric. Reconstructions indicate that contacts between the kinase and the Hsp90 dimer are restricted to just one of the monomers while Cdc37 interacts with the other monomer.

PHARMACOLOGICAL INHIBITION OF HSP90

The discovery by Whitesell et al in 1994 of the natural product geldanamycin as the first potent and selective inhibitor of Hsp90 greatly enhanced efforts by many groups to understand Hsp90 function at a molecular level (Whitesell et al., 1994). The discovery of another structurally distinct natural product inhibitor, radicicol followed a few years later (Schulte et al., 1999). Unfortunately, the poor solubility, limited bioavailability and extensive hepatic metabolism of these compounds limits their use to *in vitro* systems (Schnur et al., 1995; Soga et al., 1999). Extensive efforts from both academic and industrial groups, however, have made possible the development of an array of new synthetic chemotypes and semi-synthetic derivatives of known natural products with improved pharmacological properties that retain *in vivo* activity (Janin, 2005). These compounds share the ability to bind the N-terminal ATPase site of Hsp90 with higher affinity than the natural nucleotides and prevent the chaperone from cycling between its ADP- and ATP-bound conformations. Table 1 summarizes the major chemical classes that have been reported and their status as candidates for further development as anticancer drugs.

Although Hsp90 is ubiquitously expressed in normal tissues and tumors, recent laboratory studies have begun to shed light on why a useful therapeutic index might exist for Hsp90 inhibitors. Kamal et al. have shown that Hsp90 in cancer cells exists predominantly in multi-protein complexes whereas in normal cells it remains largely free (Kamal et al., 2004; Kamal, 2003). The complexed, presumably activated form of Hsp90 in tumors appears to possess higher affinity for 17AAG, although

Table 1. Hsp90 Inhibitors

Class	Example	Source	Binding site	Development Status
Macrolide	Radicicol	Natural product	N-terminal ATP binding pocket	N.A.
	Radicicol 6 oxime	Modified radicicol	N-terminal ATP binding pocket	Preclinical development dropped
Benzoquinone ansamycin	Geldanamycin	Natural product	N-terminal ATP binding pocket	N.A.
	Herbimycin A	Natural product	N-terminal ATP binding pocket	N.A.
	Macbecin I/II	Natural product	N-terminal ATP binding pocket	N.A
	17AAG	Semi-synthetic geldanamycin	N-terminal ATP binding pocket	In clinical trial
	17DMAG	Semi-synthetic geldanamycin	N-terminal ATP binding pocket	In clinical trial
	CNF-1010*	Semi-synthetic GA analog	N-terminal ATP binding pocket	In clinical trial
	KOS-953**	Semi-synthetic GA analog	N-terminal ATP binding pocket	In clinical trial
	IPI-504	Chemical reduction product of 17AAG	N-terminal ATP binding pocket	In clinical trial
Purine-like	CNF2024	Synthetic	N-terminal ATP binding pocket	In clinical trial
Bi-substituted Pyrazole	CCT-018159	Synthetic	N-terminal ATP binding pocket	Late pre-clinical
Peptidomimetic	Shepherdin	Synthetic	N-terminal ATP binding pocket	Preclinical development
Coumarin	Novobiocin	Natural product	C-terminal cryptic ATP binding pocket	Lead development
DNA cross linker	Cisplatin	Synthetic	C-terminal cysteines	N.A.
Histone deacetylase inhibitor	Depsipeptide	Natural product	No direct contact	In clinical trial
	Vorinostat	Synthetic		

* CNF-1010 is17AAG in a proprietary intravenous formulation developed by Conforma Therapeutics.
** KOS-953 is 17AAG in an intravenous formulation developed by Kosan Biosciences.
N.A.: Not applicable.

this finding has been questioned by others (Ge et al., 2006; Gooljarsingh et al., 2006). The mechanism responsible for the increase in Hsp90 complex formation in tumor cells is not clear, but could result from their burden of mutated client proteins which require greater assistance from the Hsp90 machinery to achieve and maintain function than their wild type counterparts (Gorre et al., 2002; Xu et al., 1999). Additional explanations for the enhanced activity of 17AAG against tumor cells as compared to non-transformed cells have been proposed. These include increased reduction of the drug in tumors to its dihydroquinone form which has higher affinity for Hsp90 and a protracted K-off rate (Guo et al., 2006; Maroney et al., 2006). Hsp90 inhibitors have also been shown to be selectively retained in the tumor burdens of xenografted mice as compared to their normal systemic tissues for reasons that are not entirely clear, but could involve higher Hsp90 expression and/or drug affinity in the tumors (Sydor et al., 2006; Zhang et al., 2006).

Work by many groups has shown that inhibition of the N-terminal ATPase of Hsp90 alters many, if not all, of its normal functions (Roe et al., 1999). In particular, drug-bound Hsp90 recruits E3 ubiquitin ligases such as CHIP (Carboxy-terminus of Hsp70 Interacting Protein) to the many client proteins that are normally chaperoned by Hsp90-containing multi-protein complexes (Xu et al., 2002). This recruitment leads to enhanced proteasome-mediated degradation of many Hsp90 clients and a decline in their cellular levels (Figure 2). Drug-mediated inhibition of the N-terminal ATPase activity of Hsp90, however, does not deplete all Hsp90 clients. In fact, it has been shown to actually activate some such as heat shock factor 1 (Hsf1), topoisomerase II and the wild type tyrosine kinase c-Src (unlike mutant v-Src which is destabilized). Furthermore, drug-mediated inhibition is not functionally equivalent to constitutive genetic knockout, which is lethal in all eukaryotes examined. Conditional genetic approaches to altering Hsp90 function that might be more relevant to drug effects in multi-cellular organisms have only been reported in *S. cerevisiae*, *C. elegans* and *D. melanogaster*. In these model organisms, impairment of chaperone function results in cell cycle arrest, dauer developmental arrest and male sterility respectively, in a manner that is dependent on the extent of Hsp90 compromise (Birnby et al., 2000; Nathan and Lindquist, 1995; Yue et al., 1999).

In addition to small molecules that bind the N-terminal ATPase site of Hsp90, several alternative approaches to Hsp90 inhibition have been reported. For example, the peptidomimetic compound shepherdin was designed to disrupt the interaction of the anti-apoptotic protein survivin with Hsp90. Shepherdin makes extensive contacts within the N-terminus of Hsp90 and can destabilize several Hsp90 client proteins as well as survivin. It is cell-permeable and selectively induces apoptosis in tumor cells (Plescia et al., 2005). The ATPase cycle of Hsp90 can also be inhibited by acetylation of lysine residues within the protein. Histone deacetylase 6 (HDAC6) co-purifies with Hsp90 and HDAC6 knockdown promotes the depletion of several known Hsp90 clients (Kovacs et al., 2005). Thus, it appears that HDAC inhibitors such as vorinostat (suberoylanilide hydroxamic acid) which are currently undergoing clinical evaluation based on their ability to alter chromatin structure

and gene expression might also exert anticancer activity in part through Hsp90 inhibition (Bali et al., 2005; George et al., 2005; Richon, 2006).

Novobiocin, a coumarin-based inhibitor of bacterial DNA gyrase binds to Hsp90's putative C-terminal ATP binding site and alters Hsp90 conformation thereby impairing Hsp90-client interactions and possibly dimerization, albeit only at relatively high concentrations (Allan et al., 2006; Marcu et al., 2000; Yun et al., 2004). Novobiocin derivatives that possess better potency and Hsp90 selectivity have been reported, but further improvements will be required to make them clinically relevant drug candidates (Burlison et al., 2006). Cisplatin a commonly used DNA cross-linking chemotherapeutic has been reported to form adducts with reactive cysteines within the C-terminus of Hsp90 in a region overlapping its putative ATP/novobiocin binding site (Itoh et al., 1999; Soti et al., 2002). Unlike geldanamycin and other N-terminal binding compounds, cisplatin treatment preferentially enhances the depletion of client steroid hormone receptors in preference to tyrosine kinases in drug-treated cells (Rosenhagen et al., 2003). However, effects are only observed at concentrations of cisplatin higher than those typically required for its DNA cross-linking activity and cytotoxicity. The microtubule poison docetaxel has been reported to impair Hsp90 function by causing its dissociation from tubulin, thereby stimulating proteasome-mediated degradation of the chaperone itself, but the extent to which this novel activity contributes to the established anticancer activity of docetaxel is not clear (Murtagh et al., 2006). The chemopreventative compound epigallocatechin gallate (EGCG) found in green tea has also been reported to bind Hsp90 and disrupt the function of the aryl hydrocarbon receptor, a known Hsp90 client protein Palermo, 2005 #742}.

HSP90 AND CANCER BIOLOGY: NOT JUST KINASES

The well recognized clients of Hsp90 such as tyrosine kinases and steroid hormone receptors provide strong evidence that this chaperone plays a key role in oncogenic, aberrantly activated, signal transduction pathways. Although it has provided a very useful entry point for efforts to understand Hsp90, signal transduction is far from the whole story. As an important corollary, it is also misleading to regard Hsp90 inhibitors as simply "multi-specificity" super kinase/signaling inhibitors for several reasons. First, the array of proteins thought to interact with Hsp90 is expanding rapidly to include many other entities than kinases and steroid hormone receptors. A recent study using global proteomic and genomic methods in yeast to map Hsp90 interactions has identified an extended network consisting of 198 putative physical interactions and 451 putative genetic and chemical genetic interactions detected by screening a library of 4700 viable yeast gene mutants for hypersensitivity to the classical inhibitor geldanamycin (Zhao et al., 2005). A well-annotated and frequently updated list of bona fide Hsp90 client proteins is maintained as a web page by the laboratory of D. Picard (http://www.picard.ch/downloads.htm).

Only recently recognized, an important consequence of this expanded range of clients is that Hsp90 chaperone function impacts more than just the post-translational

regulation of protein stability. Hsp90 participates in the disassembly of transcriptional regulatory complexes (Freeman and Yamamoto, 2002). At least in yeast, it can modulate transcription and DNA repair through effects on clients such as Slt2 and Ssl2 (Flom et al., 2005; Piper et al., 2006). Intriguing findings also show that compromising Hsp90 in Drosophila can induce epigenetic alterations in gene expression and heritable alterations in chromatin state (Ruden et al., 2003; Sollars et al., 2003). Consistent with these findings, Hsp90 has been implicated in the chaperoning of DNA helicase complexes involved in chromatin re-modeling (Zhao et al., 2005) and the histone methyltransferase SMYD3 (Hamamoto et al., 2004).

A second issue is that Hsp90 inhibition does not always lead to client inhibition. Early on, it was appreciated that Hsp90 inhibitors induce robust up-regulation of heat shock protein gene expression via activation of Hsf1, the primary transcriptional regulator of the heat shock response (Hegde et al., 1995; Whitesell et al., 2003). This response in peripheral blood cells has actually served as a sensitive pharmacodynamic endpoint for drug action in many of the clinical trials of Hsp90 inhibitors that have been reported so far (Banerji et al., 2005; Goetz et al., 2005). Under basal conditions, Hsf1 exists in the cytosol of mammalian cells as a monomer in complex with an Hsp90-containing multichaperone complex. Rather than inducing the degradation of Hsf1, Hsp90 inhibitors release the transcription factor from this complex and stimulate its translocation to the nucleus where it can activate gene expression (Guo et al., 2001). Such a release or activation effect has also been reported for the Hsp90 client topoisomerase II. Here, activation associated with Hsp90 inhibition actually sensitizes tumor cells to conventional topoisomerase II poisons such as etoposide (Barker et al., 2006). While Hsp90 inhibition typically does result in the depletion of mutated or over-expressed oncogenic tyrosine kinases, it has recently been shown to activate wild type Akt and Erk and to potentiate Akt activation induced by insulin and insulin-like growth factor I (Meares et al., 2004). It does this, at least in part by transiently activating rather than disrupting the function of wild type c-Src tyrosine kinase which then phosphorylates Cbl to recruit PI3-kinase to the membrane (Koga et al., 2006). Geldanamycin can also increase the activity of ErbB2 kinase prior to stimulating its degradation, again by activating c-Src which phosphorylates Y877 in the activation loop of ErbB2 (Xu et al., 2007).

A third issue is that the effects of Hsp90 inhibitors can vary dramatically in relation to the extent of inhibition achieved. An example of an Hsp90 client that displays variant behavior in response to Hsp90 inhibition is HIF-1α, a key component of the heterodimeric complex that plays a central role in regulating tumor neovascularization (Isaacs et al., 2002; Kaur et al., 2004; Sanderson et al., 2006). Surprisingly, exposure to nanomolar concentrations of the clinically relevant Hsp90 inhibitors 17AAG and 17DMAG increased the expression of HIF1-dependent genes such as VEGF while exposure to higher (micromolar) concentrations destabilized HIF-1α and inhibited gene expression as expected (Ibrahim et al., 2005). Bimodal effects of 17AAG on vessel formation were also seen in an *in vivo* angiogenesis model. These findings raise concerns over chronic Hsp90 inhibitor therapy as a potential anti-angiogenic strategy and suggest that appropriate dosing could

be a critical factor in the treatment of patients. Another consideration particularly relevant to chronic dosing is the potential impact of Hsp90 inhibition on immune functioning. Geldanamycin has been shown to profoundly inhibit T-cell activation *in vitro* (Yorgin et al., 2000), and even modest reduction of Hsp90 levels in B lymphocytes by genetic techniques impairs their activation and IgM production (Shinozaki et al., 2006). Clinical trials of 17AAG and 17DMAG have demonstrated relatively little acute bone marrow toxicity/myelosuppression, but no data have been reported regarding the potential immunosuppressive effects of either intermittent high dose exposure or chronic low dose exposure to Hsp90 inhibitors.

A fourth issue that has received relatively little attention is the effect of Hsp90 inhibitors on the function of tumor suppressor proteins. For example, the tumor suppressor LKB1 is an Hsp90 client protein that can be markedly depleted by Hsp90 inhibition (Boudeau et al., 2003; Nony et al., 2003). Mutations within LKB1 are responsible for Peutz-Jeghers syndrome (PJS), a condition characterized by the presence of hamartomatous polyps in the gastrointestinal tract and susceptibility to the development of a variety of cancers, including those of the breast, pancreas, intestine, testis and cervix. Recent work has shown that LKB1 regulates cellular metabolism; in the absence of proper LKB1 activity, cells become growth factor independent for proliferation (Shaw et al., 2004a). Treatment of cells with Hsp90 inhibitors destabilizes the Hsp90-LKB1 interaction thereby promoting LKB1 degradation via the proteasome. To date, however, the effects that Hsp90 inhibition might have on tumor cell metabolism and LKB1-dependent tumorigenesis remain unclear (Shaw et al., 2004b). Recent data also demonstrate that Hsp90 chaperones both wild type and mutant forms of p53, the most frequently mutated tumor suppressor in human cancers. Hsp90 inhibition exerts variant effects on p53 function depending on its mutational status (Muller et al., 2004; Sepehrnia et al., 1996); inhibiting Hsp90 impairs the activation of wild type p53, thereby disrupting its ability to act as a tumor suppressor. On the other hand, Hsp90 inhibition destabilizes mutant p53 isoforms that can exert dominant negative effects on the normal function of wild type p53 (Blagosklonny et al., 1996; Whitesell et al., 1997).

Clearly, the ability to disrupt multiple cancer-promoting signaling pathways is an attractive and unique feature of Hsp90 inhibitors, but it might come at the price of impairing the function of important tumor suppressors or inappropriately activating tumor promoting pathways. For example, 17AAG treatment of mice bearing human breast cancer xenografts reduced primary tumor growth, but enhanced the occurrence of bony metastases (Price et al., 2005). Such considerations suggest that the net outcome in a particular patient might well depend on the particular constellation of molecular genetic defects driving aberrant proliferation and survival in their tumor burden at the time therapy is initiated. Indeed, the Hsp90-dependency of the kinase ZAP-70 is reported to be highly conditional, based on the status of the cell in which it is expressed. It behaves as an inhibitor-sensitive Hsp90 client in chronic lymphocytic leukemia cells, but not in normal peripheral blood lymphocytes (Castro et al., 2005). Much additional work needs to be done to address these important issues as Hsp90 inhibitors undergo clinical development.

CANCER EVOLUTION

As a consequence of its effects on both signal transduction and gene expression, Hsp90 seems to have an important, but previously unrecognized, role in evolutionary processes. In the model organisms *D. melanogaster* and *A. thaliana,* Hsp90 can act to conceal inherent genetic variation within populations of organisms (Rutherford and Lindquist, 1998; Queitsch et al., 2002). As a consequence of its protein chaperoning activity, Hsp90 permits polymorphic variants of critical signaling molecules and transcription factors to retain uniform "wild type" biochemical activity. This "buffering" at the protein level by Hsp90 funnels complex developmental processes into discrete, well-defined outcomes despite underlying genotypic variation, and it appears to be essential for the robust expression of uniform phenotypes under basal conditions (Ruden et al., 2005). Under stressful conditions, however, some unstable client proteins of Hsp90 are likely to become even more unstable. This problem creates an increased demand for Hsp90 to facilitate the refolding of its usual client proteins as well as new, stress-destabilized clients. The accumulated genetic variation in certain individuals can thereby exceed the buffering capacity of Hsp90 and produce diverse, but genotype-specific phenotypes (Sangster et al., 2004). In this way, previously hidden genetic variation becomes available for natural selection to enhance the survival of distinct genotypes within a population (Rutherford, 2003).

A tumor can be viewed from an evolutionary perspective as a large, genetically and epigenetically heterogeneous population of cells (Merlo et al., 2006). We have proposed that Hsp90 acts as a biochemical buffer at the protein level for this extensive heterogeneity to maintain cell viability and limit phenotypic variation (Bagatell and Whitesell, 2004; Whitesell and Lindquist, 2005). During the natural history of cancer progression, however, canalization of the malignant phenotype could break down when Hsp90 capacity is exceeded as a result of normal ageing, an increasing load of mutant and/or misfolded oncoproteins, or the hostile tumour microenvironment—or perhaps, all these factors in concert. Epigenetic instability and phenotypic diversity within the tumour cell population would increase and accelerate the emergence of invasive, metastatic and drug-resistant biologies (Feinberg et al., 2006; Gatenby and Vincent, 2003). Such an evolutionary view of tumor progression fits well with the clinical behavior of many malignancies and suggests that reliable control of cancers in patients is likely to be achieved only by limiting their ability to adapt and evolve. How Hsp90 inhibition might impact tumor evolution is not known. On a cautionary note, however, compromising Hsp90 function in established cancers, especially to a sub-lethal extent could work to reveal their underlying genotypic diversity and increase epigenetic instability, thereby accelerating the process of malignant progression in highly unpredictable ways. Little or no data are available to address these issues currently, but work is underway in our group and by others to better define the role(s) of Hsp90 in cancer evolution.

WHAT HAS BEEN LEARNED IN THE CLINIC

Although Hsp90 provides a mechanistically appealing target for the treatment of cancer, the feasibility of inhibiting Hsp90 function in patients is just now being

established. A particular concern has been that drugs affecting such an essential chaperone could have prohibitive side effects due to impairment of essential cellular functions, but phase I clinical trials of 17AAG have demonstrated that Hsp90 function can be modulated in cancer patients with tolerable acute toxicity (Banerji et al., 2005; Goetz et al., 2005; Grem et al., 2005; Munster et al., 2001; Ramanathan et al., 2005b). In these trials, plasma concentrations in excess of the mean IC50 value for the NCI panel of 60 human tumor cell lines *in vitro* (120nM) could be achieved for over 24 hours, and pharmacodynamic alterations in Hsp90 client protein levels, such as Raf and Cdk4, were induced in both peripheral blood lymphocytes and tumor biopsies following drug administration. Importantly, myelosuppression was not a dose-limiting toxicity in these trials which supports the feasibility of combining Hsp90 inhibitors with conventional cytotoxic agents in Phase Ib trials.

In addition to Hsp90 itself, the cellular stress response appears to be an important determinant of drug sensitivity in pre-clinical studies. As mentioned above, Hsp90 inhibitors alter the multi-chaperone complexes associated with Hsf1, the dominant transcription factor controlling induction of the stress response, thereby stimulating Hsf1-activated heat shock gene expression. Induction of this heat shock response provides a measure of protection to cells from the toxicity of Hsp90 inhibitors (Bagatell et al., 2000), while tumors that fail to up-regulate heat shock protein levels appear particularly sensitive to Hsp90 inhibition (Clarke et al., 2000). Induction of the stress response as measured by increased levels of a highly inducible Hsp70 isoform (Hsp72) in peripheral blood lymphocytes has been demonstrated in patients receiving 17AAG. Given the cytoprotective effect of the stress response, however, it might prove important to administer Hsp90 inhibitors in a pulsed fashion with sufficient time between doses to allow drug-stimulated stress responses to resolve, thereby avoiding the induction of a tolerant, relatively drug resistant state in tumors. Alternatively, concurrent pharmacological inhibition of the heat shock response could be used to enhance the activity of Hsp90 inhibitors (Whitesell et al., 2003). Whether Hsf1 inhibitors would increase the anticancer efficacy of Hsp90 inhibitors or primarily increase toxicity for normal tissues remains unknown. It should become possible to test these concepts shortly because interest in manipulating the heat shock response to treat non-oncological disorders such as hypoxic-ischemic injury and neurodegenerative protein aggregation diseases has led to identification of both inducers and inhibitors of Hsf1 activation (Hansen et al., 1997; Mulholland et al., 2001; Westerheide et al., 2006).

An unanticipated but particularly worrisome problem in the clinical development of Hsp90 inhibitors has been the observation of cardiac electrical conduction abnormalities and life-threatening arrhythmias in a few patients receiving 17AAG and 17DMAG. This toxicity might be directly related to Hsp90 inhibition because the cardiac potassium channel HERG has been shown to be an Hsp90 client and its function can be impaired by geldanamycin treatment of ventricular myocytes *in vitro*. Given the complex medical problems typical of patients with recurrent/refractory cancers, other factors may be involved in predisposing patients to this toxicity, but intensive cardiac rhythm monitoring of all patients has now being incorporated into trials of these agents. In pediatric phase I trials of 17AAG, acute respiratory distress syndrome (ARDS) was observed as a rare, but severe adverse

event, perhaps resulting from drug-induced alterations in pulmonary surfactant metabolism. Based on this experience, caution has been urged in the use of 17AAG in patients with extensive pulmonary metastatic disease (Bagatell et al., 2007).

As the first Hsp90 inhibitors are just emerging from phase I safety and dose finding studies, limited information regarding clinical activity is available. No objective responses have been reported using an Hsp90 inhibitor as a single agent, but prolonged stable disease has been documented in several patients with advanced melanoma (Banerji et al., 2005; Ronnen et al., 2006). In multiple myeloma, a phase Ib study of 17AAG combined with the proteasome inhibitor bortezomib was shown to be well-tolerated and an overall response rate of 57% in heavily pre-treated patients, some of them highly bortezomib resistant was reported (Richardson, P et al. American Society of Hematology, 48th Annual Meeting, 2006). Enhanced antitumor activity and an unusual pattern of cytopathic changes had already been demonstrated for this combination *in vitro* (Mimnaugh et al., 2004). Based on these findings, it is tempting to speculate that the unusual sensitivity of multiple myeloma to the simultaneous inhibition of Hsp90 and proteasome function results from the unique demands on protein homeostasis imposed by the synthesis and processing of immunoglobulins by these tumor cells.

THE WAY AHEAD

A clear understanding of how the Hsp90-based chaperone machinery is altered in cancers and contributes to the malignant phenotype has yet to be achieved despite efforts now going back over 20 years. Many critical questions remain with great relevance to the exploitation of Hsp90 as an anticancer target. For example: 1) Hsp90 itself is frequently found over-expressed, but not mutated in many tumors (Jameel et al., 1992; Nanbu et al., 1998; Trieb et al., 2000). A simple view is that tumor cells proliferate more rapidly and therefore possess a greater requirement for client proteins to be processed. But are there tissue-specific differences in requirements for Hsp90 and what it does (e.g. cardiac HERG channels)? Are these differences conserved in cancers of different histologies to confer unique susceptibility to Hsp90 inhibition (e.g. multiple myeloma)? Does the hypoxic, acidotic, nutrient-deprived microenvironment of many tumors constitute a proteotoxic stress that drives compensatory up-regulation of chaperone expression, Hsp90 included? Could such up-regulation predict or even alter the anticancer activity of an Hsp90 inhibitor, independent of the expression of specific oncogenic clients?

2) Based on work in a variety of model organisms, Hsp90 clearly plays a critical role in supporting and canalizing normal developmental processes (Ruden et al., 2003; Sangster et al., 2004). Does this have implications for the aberrant expansion and impaired differentiation of tissue stem cells that are increasingly recognized as the key target of malignant transformation in many cancers (Feinberg et al., 2006; Ruden et al., 2005)? Could Hsp90 inhibition alter such stem cell processes, either for better or worse? 3) Most cancers progress from benign, relatively well-differentiated tumors to increasingly invasive and metastatic cancers characterized by profound

genomic instability and the accumulation of numerous genetic alterations (Merlo et al., 2006; Sjoblom et al., 2006; Stoler et al., 1999). Does the role of Hsp90 shift as cells move through the initiation phase to the progression phases of tumorigenesis? If so, inhibiting Hsp90 could have profoundly different effects on early stage lesions than on advanced cancers (Whitesell and Lindquist, 2005). Might it be important to assess the functional status of Hsp90 in a tumor prior to initiating therapy with an inhibitor? Would absolute expression level, extent of association with co-chaperones or relative drug binding affinity be useful endpoints for evaluation? Would any of these Hsp90-centric endpoints be more predictive of response than the expression of a particular oncogenic client protein?

CONCLUSIONS

Since their initial discovery over a decade ago, remarkable progress has been made in developing Hsp90 inhibitors as anticancer agents. Along the way, these compounds have proven very useful in exploring the complex role of Hsp90 in many biological processes. To become truly useful chemotherapeutics, however, two major challenges remain. The first is to improve the poor pharmacological properties of first generation, natural product-based agents while retaining target specificity. While this problem is far from trivial, the diversity of approaches being pursued and the progress achieved so far make success in this area very likely. The more daunting challenge is to define the appropriate way in which to exploit Hsp90 inhibition for the treatment of specific cancers. Except in relatively rare circumstances, it seems unlikely that Hsp90 inhibitors will prove useful as primary cytoreductive agents to shrink bulk disease. Rather, their known biological activities and early clinical results suggest they are likely to be safer and more effective when given in combination with other molecularly targeted or conventional agents. An unresolved issue remains the effect of Hsp90 inhibition on the intrinsic evolvability of cancers, which, at present, is a major barrier to curative therapy. Understanding how Hsp90 couples cellular physiology to internal and external environmental contingencies has much to teach us about how cancers work. Based on what has been learned so far, however, it is clear that Hsp90 as a therapeutic target in cancer and other diseases is here to stay.

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CHAPTER 14

SMALL-MOLECULE HSP90 INHIBITORS: APPLICATIONS IN CANCER AND NEURODEGENERATIVE DISEASES

ADEELA KAMAL, SRINIVAS KASIBHATLA, MARCO BIAMONTE, HONG ZHANG, LIN ZHANG, KAREN LUNDGREN, MARCUS F. BOEHM AND FRANCIS J. BURROWS*

Biogen Idec, 5200 Research Place, San Diego, CA 92122, USA

Abstract: Hsp90 is a pleiotropic cytoplasmic chaperone that regulates an ever-growing list of client proteins. Hsp90 clients include many, but not all, kinases, steroid receptors and a number of transcription factors. Many clients display heightened Hsp90 dependence when mutated and mutation can also create *de novo* Hsp90 dependence. A common factor that underlies the selective therapeutic activity of Hsp90 modulators in malignant and neurodegenerative disease is the presence of inherited or acquired mutant proteins at the core of disease pathophysiology that drives the progressive activation of the cellular Hsp90 pool, leading to increased drug sensitivity. The benzoquinoid ansamycin geldanamycin was identified as the first Hsp90 inhibitor in 1995, and its semi-synthetic analogue 17-AAG is currently in Phase II clinical trials. Several groups have synthesized sizeable libraries of ansamycin drugs, but all share the limitations of hepatotoxicity and challenging pharmaceutics, suggesting that the full potential of this drug target would only be realized through synthetic medicinal chemistry. In the last five years, a plethora of academic and industrial programs have been initiated and the most advanced small-molecule Hsp90 inhibitor drug is already in Phase I clinical trials. In this chapter we will review these efforts, focusing on the potential of small-molecule Hsp90 modulators in cancer and neurodegenerative diseases

Keywords: Heat shock protein 90, tumor selectivity, cancer therapeutics, molecular chaperones, Hsp90 small-molecule inhibitors, neurodegenerative diseases

INTRODUCTION

Hsp90 belongs to a family of proteins called molecular chaperones that regulate protein folding and influence protein degradation (See *Pratt et al*, Chapter 1, this volume). Hsp90 is not a general chaperone like Hsp70 that folds all proteins,

*Corresponding author: Biogen Idec, 5200 Research Place, San Diego, CA 92122, Tel: 858-401-8136, e-mail: francis.burrows@biogenidec.com

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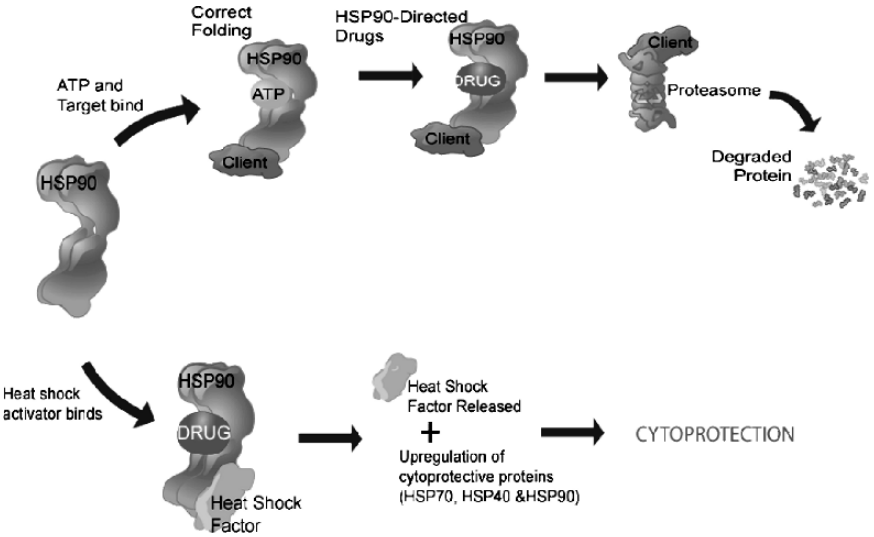


Figure 1. **Mechanism of action of Hsp90 inhibitor drugs.** Hsp90, when ATP bound, is involved in the correct folding and stabilization of Hsp90 client proteins which get degraded in presence of Hsp90 inhibitor drugs. Hsp90 inhibitors also cause release of heat shock factor (HSF1) which translocates to the nucleus and induces expression of cytoprotective proteins

but instead Hsp90 is involved in the conformational maturation of key signal transduction proteins, referred to as "client" proteins. Hsp90 client proteins include receptor tyrosine kinases (HER-2/neu, EGFR, IGF-R1), intracellular signaling kinases (Raf, Akt, IKK), cell cycle regulators (CDK4, CDK6), nuclear steroid receptors (androgen, estrogen and progesterone receptors), and mutant/fusion proteins (Flt3, v-src, BCR-Abl, NPM-ALK). Hsp90 inhibition results in the proteasome-mediated degradation of oncogenic client proteins, thus making Hsp90 an attractive target for cancer therapy (Figure 1). In addition, the triggering of the heat-shock response pathway leading to marked upregulation of a set of chaperones including Hsp70 and the degradation of pathogenic client proteins have stimulated interest in the use of Hsp90 inhibitors in neurodegenerative diseases. In this chapter, we will review Hsp90 biology and discuss the development of novel, small-molecule inhibitors of Hsp90 for cancer and neurodegenerative diseases.

ONCOGENIC HSP90 CLIENT PROTEINS AND SIGNALING PATHWAYS

Hsp90 influences the activity and stability of many client proteins that function as key regulators in cellular growth, differentiation and apoptotic pathways (Also see: *Neckers*, Chapter12, *Whitesell*, Chapter13, *Holmes et al*, Chapter 15; *Matts & Caplan*, Chapter16). The more than 100 known Hsp90 clients regulate multiple

single transduction pathways that are aberrantly activated in human cancers. In the following section, we focus on certain critical oncogenic proteins and pathways (Figure 2) that are affected by Hsp90, including growth factor receptors (EGFR and HER-2), Bcr-abl and FLT3, the PI3K/AKT pathway, Ras/Raf/ERK pathway and other pathways, and discuss the potential of blocking their oncogenic propensity through inhibition of Hsp90.

Growth Factor Receptors

EGFR and HER-2 are receptor tyrosine kinases of HER family that play critical roles in cell proliferation. Overexpression or mutation of either protein has been recorded in a variety of malignancies, including HNSCC, glioblastoma, NSCLC, breast cancer and ovarian cancer (Menard et al., 2001; Zalutsky, 1997). In the past decade, great efforts have been made in developing HER-2 and EGFR inhibitors, including trastuzumab (Herceptin), cetuximab (Erbitux), erlotinib (Tarceva), gefitinib (Iressa) but beneficial responses were limited to patients with HER-2 gene amplification or mutant EGFR expression in certain tumor types, such as breast cancer and NCSLC(Albanell et al., 2003; Arteaga, 2006; Eberhard et al., 2005). Further studies in resistant cell lines revealed that major downstream signaling pathways, including AKT and Raf-1 pathways were still active due to the expression of compensatory receptor tyrosine kinase or some other mutations the tumor acquired to adapt to the

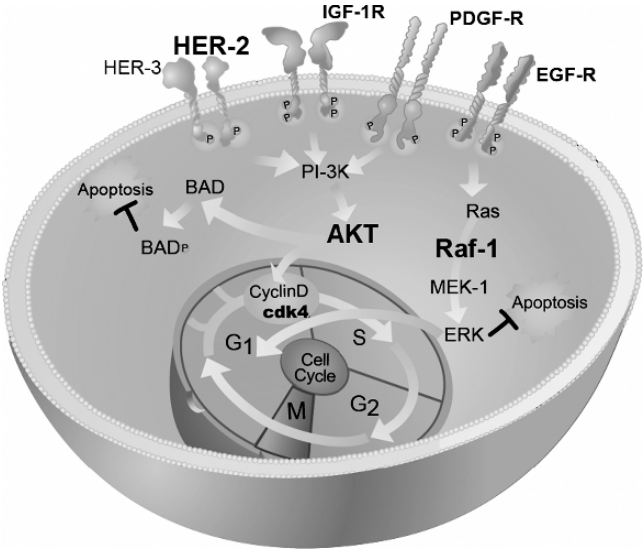


Figure 2. **Hsp90 clients are in multiple signal transduction pathways.** Many key signaling molecules in a variety of oncogenic pathways are Hsp90 clients (shown in bold) that drive cellular proliferation and inhibit apoptosis

new environment (Arteaga, 2003; Bianco et al., 2003). These adaptations bypassed the suppression of the target and maintained tumor growth, indicating that tumor cells can circumvent inhibition of one target and activate an alternative pathway to support proliferation and survival, underscoring the potential value of pleiotropic agents targeting multiple proteins or pathways. Hsp90 inhibitors inhibit tumor cell growth in both high and low HER-2 cells, although they are more potent in HER-2 overexpressing lines. In some cells, both EGF-R and HER-2 are sensitive Hsp90 clients, whose degradation can be detected at early time points upon Hsp90 inhibition (Basso et al., 2002b; Xu et al., 2001), whereas others have found that EGF-R degradation follows a more indolent course (Citri et al., 2004). Therefore, Hsp90 inhibitors may provide more robust and long-lasting antitumor effects by inhibiting multiple signaling pathways and several studies in breast cancer patients with high HER-2 levels are proceeding in clinical trials.

Bcr-Abl

The fusion protein Bcr-Abl, known as Philadelphia Chromosome, is expressed in approximately 95% of cases of Chronic Myeloid Leukemia (CML) and is the target of the first small molecule tyrosine kinase inhibitor, imatinib (Gleevec). Although CML patients respond remarkably well to imatinib, patients become resistant to the drug mostly due to mutations in the kinase domain that decrease imatinib binding affinity and enhance oncogenic potency (Nimmanapalli et al., 2002). Both wild type and mutant Bcr-Abl, including the most common T315I mutant Bcr-Abl are Hsp90 clients (Gorre et al., 2002; Nimmanapalli et al., 2001). Inhibition of Hsp90 causes degradation of Bcr-Abl and suppresses tumor growth in a variety of hematopoietic tumor lines, including those that are resistant to imatinib (Nimmanapalli et al., 2001; Nimmanapalli et al., 2002; Shiotsu et al., 2000) suggesting that Hsp90 inhibitors could become a preferred treatment for Gleevec-resistant CML, or be used in first line in combination with imatinib or other Abl kinase inhibitors to prevent the development of resistance.

Flt3

FMS -like tyrosine kinase 3 (Flt3), a receptor tyrosine kinase, plays a crucial role in several hematopoietic malignancies, including acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL) and myeloid lymphoblastic leukemia (MLL). Flt3 in AML is mutated as internal tandem duplication (Flt3 ITD) or a point mutation in the tyrosine kinase domain (Flt3 TKD); both types of mutations cause constitutive Flt3 activation and are associated with poor prognosis (Stirewalt and Radich, 2003). Both wild type and mutant Flt3 are sensitive to Hsp90 inhibitors which suppress Flt3 phosphorylation, induce cell cycle arrest and apoptosis, and prolong the survival of mice with mutant Flt3 ITD malignancy (Naoe et al., 2001; Yao et al., 2003).

PI3K/AKT Pathway

Because of its central role in cell proliferation and survival, the PI3K/AKT pathway (Figure 2) has attracted much attention in drug development for anti-cancer therapy. AKT is abnormally activated in a variety of human malignancies, including breast, prostate, lung, pancreatic, ovarian and colorectal carcinomas, through persistent upstream signaling, AKT amplification or loss of its negative regulator, PTEN (Hennessy et al., 2005; Vivanco and Sawyers, 2002). Numerous efforts have been made in developing drugs targeting this pathway, with inhibitors for mTOR, an AKT substrate, being the most advanced in clinical studies. The mTOR inhibitor, temsirolimus (CCI-779), showed anti-tumor activity in AKT over-expressing patients (Dancey, 2002) but responses only occur in a small number of patients due to insufficient inhibition of the pathway, emphasizing the need for novel clinical strategies. Several studies have shown that Akt relies on Hsp90 for its stability and activity (Basso et al., 2002a; Basso et al., 2002b; Sato et al., 2000), existing in an active conformation in an Hsp90-cdc37 complex that facilitates the phosphorylation of AKT by PDK1 (Basso et al., 2002a). In HER-2 overexpressing cell lines, 17-AAG inhibits AKT phosphorylation prior to the loss of AKT expression, probably due to more rapid-acting HSP90-dependent effects on the upstream regulator HER-2 (Fujita et al., 2002). In animal models, 17-AAG inhibits the growth of tumors with active AKT when it is administered at doses capable of suppressing AKT phosphorylation (Basso et al., 2002a; Solit et al., 2003). All these findings imply that Hsp90 inhibitors function as pan-inhibitors of the pathway and could have a better outcome against cancers exhibiting aberrant PI3K/AKT activation.

Ras/Raf/MEK

Another pathway that occupies center stage in cell proliferation and survival is the Ras/Raf/MEK pathway (Figure 2). Mutations of Ras and Raf are some of the most common mutations in human tumors. One Raf isoform, B-Raf, is mutated in 70% and 36% of melanomas and papillary thyroid tumors, respectively (Cohen et al., 2003; Davies et al., 2002). Developing inhibitors of the Ras/Raf/MEK pathway has been extensively explored in both preclinical and clinical studies. Among these, the multi-kinase Raf-1 inhibitor sorafenib (Bay43-9006) showed promise in renal cell carcinoma (RCC) and many other tumor types although it is likely that its anti-tumor activity is mediated by inhibition of several receptor tyrosine kinases (RTKs) involved in tumor progression, such as VEGFR-2, VEGFR-3, PDGFR-β, Flt3, c-KIT and p38α (Gollob et al., 2006). In contrast, a highly specific MEK inhibitor, CI-1040, was suspended after phase II trials due to lack of response (Sebolt-Leopold et al., 2003). Raf isoforms and its downstream effector, MEK, are both Hsp90 clients, and Hsp90 inhibitors deplete both proteins from tumor cells (Jaiswal et al., 1996; Schulte et al., 1996). Based on this, clinical trials of Hsp90 inhibitors have been carried out in melanoma patients harboring Raf-driven tumors, and Raf depletion and clinical responses have been reported (Pacey et al., 2006).

Other Pathways

In addition to those mentioned above, several other signaling pathways linked to tumor progression are also associated with chaperoning activity of Hsp90, such as the NF-κB, Wnt/β-Catenin and JAK-STAT pathways. The NF-κB activating kinase IKK is an Hsp90 client (Chen et al., 2002) and β-Catenin is regulated by IKK via phosphorylation and ubiquitin-dependent degradation (Lamberti et al., 2001). In addition, STAT3 binds to Hsp90 and requires Hsp90 complex for its stabilization (Sato et al., 2003). Disruption of the interaction with geldanamycin abolished the activation of STAT3 in IL-6 treated 293T cells. Thus, Hsp90 inhibitors have the potential to be utilized in NF-κB driven tumors and tumors with aberrant Wnt/β-Catenin and JAK-STAT pathways.

HSP90 ACTIVATION IN MALIGNANT CELLS

Even though Hsp90 is an abundant protein that constitutes 1–2% of all the protein in the cell, Hsp90 inhibitor drugs selectively destroy tumor cells over normal cells. This was also evidenced in the phase I clinical trials for 17-AAG, where the drug was found to induce client protein degradation at well-tolerated doses. Recent data have revealed that the therapeutic selectivity of Hsp90 inhibitors results from the presence of a predominantly high-affinity, activated form of Hsp90 in tumors whereas the Hsp90 in normal tissues is a low-affinity, inactive form (Kamal et al., 2003). Hsp90 is activated in tumor cells apparently as a result of the accumulation of unstable mutant client proteins driving cancer growth. Tumor Hsp90 is maintained in an activated conformation by multichaperone protein complexes that enhance Hsp90's enzymatic activity. These complexes enable a 100-fold greater binding affinity for 17-AAG compared to the uncomplexed inactive form of Hsp90 that resides in normal cells (Figure 3). High-affinity binding was observed in a wide range of tumor cell lines and clinical cancer tissues, and was closely linked to

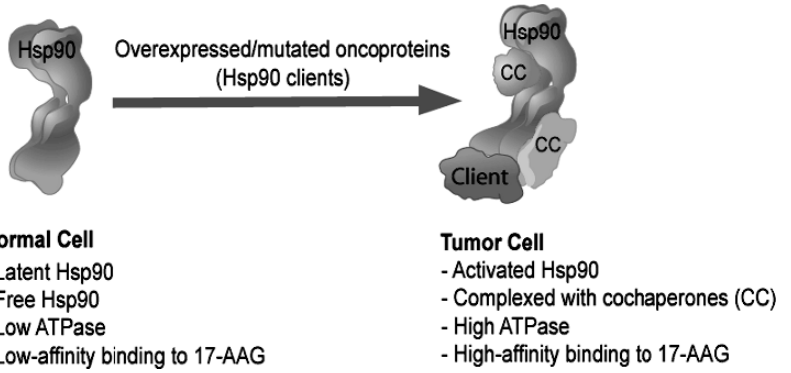


Figure 3. **Model for therapeutic selectivity of Hsp90 inhibitors in tumor cells.** The Hsp90 in normal cells is in a latent form that has low ATPase enzymatic activity, and low-affinity binding to the Hsp90 inhibitor 17-AAG whereas the Hsp90 in tumor cells is in an activated state and exists in a complexed form with co-chaperone proteins, and exhibits increased ATPase activity and high-affinity binding to 17-AAG

the killing activity of 17-AAG in cells. The increased binding affinity of tumor Hsp90 for 17-AAG also explains the ability of Hsp90-directed drugs to selectively accumulate in tumors, compared to normal tissues. More recently, a synthetic Hsp90 inhibitor, PU24FCl, was shown to bind to tumor Hsp90 with much higher affinity than Hsp90 derived from normal cells (Vilenchik et al., 2004).

In our hands, all active ansamycin-based Hsp90 inhibitors display a similar (20–40 fold) selectivity for the activated form of Hsp90. By contrast, synthetic Hsp90-binding drugs may be completely unselective (as is radicicol) but can show upto 400-fold preference for activated Hsp90. A recent report suggested an alternative explanation for varying binding affinities of ansamycins based on a two-stage binding model where low-affinity binding (~1 uM) of geldanamycin to uncomplexed Hsp90 converted to a high-affinity state after more than 6 hours (Gooljarsingh et al., 2006). The pharmacologic relevance of this observation is questionable since geldanamycin is cleared from normal tissues in 1–3 hours, but these data could imply that Hsp90 in inherently flexible and that protein-drug interactions may exploit dynamic states.

Taken together, these results suggest that Hsp90 "usage" (measured by binding-affinity) could be a true predictor of the Hsp90 dependence of cancer cells. Furthermore, accumulation of mutant proteins in advanced cancers would further increase the demand for Hsp90 chaperoning activity, driving the observed amplification of the Hsp90 gene and making tumor cells even more Hsp90-dependent. Indeed, one particularly exciting possibility is that, since most mutant proteins in cancer cells use Hsp90 to compensate for their structural instability, Hsp90 activation could itself become an independent prognostic tool to help oncologists decide how aggressively to treat patients on an individual basis.

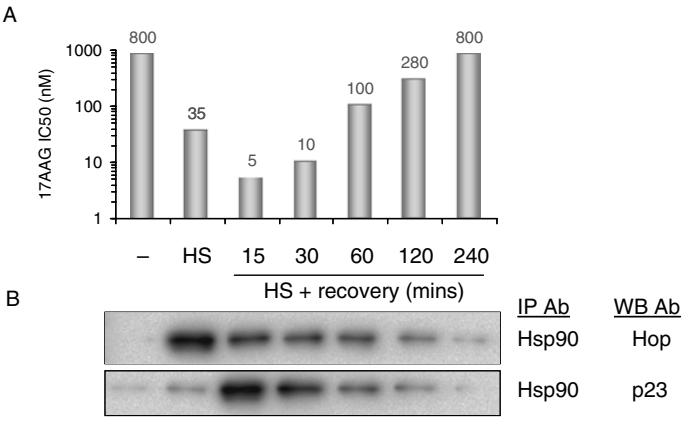
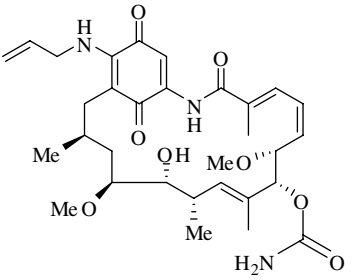


Figure 4. **Heat-shock induces Hsp90 activation in human normal dermal fibroblast cells**. Heat-shock (HS) of normal fibroblasts for 30 minutes at 42°C increases 17-AAG binding affinity to Hsp90 (B) and increases formation of multichaperone complexes with the co-chaperones Hop and p23 sequentially. If the cells are allowed to recover after the HS for 15–240 minutes, the Hsp90 is not longer activated. (IP Ab = antibody used for immunoprecipitating; WB Ab = antibody used for Western blots)

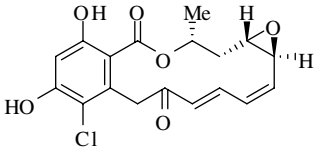
The model for the molecular basis of tumor selectivity of Hsp90 binding drugs as described above suggests that in normal cells, Hsp90 is in a free uncomplexed form that has low-affinity to Hsp90-binding drugs, which therefore accumulate poorly in normal tissues, and normal tissues have low drug sensitivity. In cancer cells, overexpression of oncoprotein clients causes Hsp90 to become increasingly engaged in active chaperoning. The Hsp90 is in a complexed form with cochaperones that has high-affinity binding to Hsp90 inhibitor drugs, which therefore show good accumulation in tumors, and thus tumor cells have high drug sensitivity. This model also suggests that the affinity change of Hsp90 can be driven not only by client protein overexpression but also by stressful conditions in normal cells. Indeed, we have preliminary data that not only overexpression of two different Hsp90 clients, v-src and EGFR, increases 17AAG binding affinity but also heat-shock of normal fibroblasts increases 17-AAG binding affinity and increases complexed Hsp90 (Figure 4).

NATURALLY-OCCURRING HSP90 INHIBITORS

Hsp90 function is regulated by the help of co-chaperone proteins that participate in ordered series of dynamic multi-protein complexes, linked to the ATPase cycle of Hsp90. Client proteins bind to Hsp90 in an "intermediate complex" containing Hsp70, Hsp40, Hip and Hop which upon ATP binding and hydrolysis, forms a "mature complex" containing p23 and immunophilins that mediate the conformational maturation of the client (Kamal et al., 2004). Hsp90 inhibitor drugs, such as the ansamycin antibiotic geldanamycin or the fungal macrolide radicicol, bind to a conserved pocket in the N-terminal ATP-binding domain of Hsp90, inhibiting ATP binding and hydrolysis, and locking Hsp90 in the "intermediate complex" from which the client protein gets ubiquitinated and targeted to the proteasome for degradation.



17-AAG



Radicicol

A semi-synthetic product of geldanamycin, 17-allylamino-17-desmethoxygeldanamycin (17-AAG) is currently in Phase II clinical trials for cancer (Grem et al., 2005). Although this compound represents a major advancement in Hsp90-directed therapy, natural product derivatives based on geldanamycin engender a number of difficulties (Supko et al., 1995). In particular, geldanamycin derivatives present

significant formulation and delivery challenges associated with their relative insolubility in aqueous media. Moreover, the dose limiting toxicity appear to be hepatic, an off-target effect due to the presence of the reactive quinone moiety. These limitations and liabilities have encouraged researchers to develop small-molecule Hsp90 inhibitors that are structurally unrelated to the first generation natural products. We and others have recently identified a number of synthetic analogs of Hsp90 inhibitors that are described below (Chiosis et al., 2002; Chiosis and Rosen, 2002; Chiosis et al., 2001; Drysdale et al., 2003; Dymock et al., 2004; Dymock et al., 2005; He et al., 2006; Kasibhatla et al., 2005a; Kasibhatla et al., 2005b; Kasibhatla et al., 2005c; Kasibhatla et al., 2005d; Kasibhatla et al., 2003; Llauger et al., 2005; Vilenchik et al., 2004; Wright et al., 2004; Zhang et al., 2006).

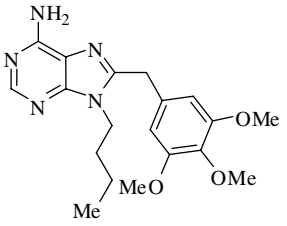
SMALL-MOLECULE HSP90 INHIBITORS

Purine Based Inhibitors

The first purine-based inhibitor analogs were represented by 8-benzylpurine **1** (PU3) analogs (Chiosis and Rosen, 2002; Chiosis et al., 2001). Purine **1** was designed based on the known binding modes of ATP and geldanamycin. The adenine ring of PU3 was envisioned to mimic the adenine ring of ATP, while the trimethoxybenzene moiety was introduced to capture the same network of hydrogen bonds in which the quinone ring of geldanamycin is involved. A recent crystal structure of the Hsp90-PU3 complex (Dymock et al., 2004) indicates that while the adenine ring fulfills its intended role, the trimethoxybenzene ring actually exploits a pocket not occupied by geldanamycin. The cellular activity of these Hsp90 inhibitors is measured by looking at the cellular degradation of the HER-2 client protein in tumor cells. PU3 proved to be a weak Hsp90 inhibitor (HER-2 IC50 = 38 μM), and subsequent structure-activity relationship (SAR) optimization led to the identification of **2** (PU24FCl), which showed low micromolar potency in the HER-2 degradation assay *in vitro* (HER-2 IC_{50} = 1.7 μM). The compound **2** showed relatively weak *in vivo* efficacy mainly due to low *in vitro* potency and poor pharmacokinetic properties (Vilenchik et al., 2004). The discovery of the 8-benzyladenines led us (Kasibhatla et al., 2003) and subsequently others (Llauger et al., 2005) to design 8-arylsulfanyladenines, exemplified by 8-(2-iodo-5-methoxy-phenylsulfanyl)-9-pent-4-ynyl-9H-purin-6-ylamine (**6**).

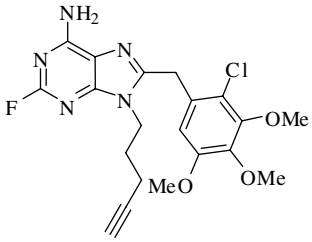
The SAR studies leading to **6** are summarized in Table 1. The 2,5-dimethoxybenzene **3** was superior to the prototypic 3,4,5-trimethoxy pattern of **1** (Table 1, entries 1,2). Replacing the 2-MeO group by iodine led to an increase in activity (Table 1, entry 3). Next, the linker connecting the benzene ring to the adenine ring was optimized. The compounds with a NH or O as linker are inactive, and it was assumed that only the CH_2 linker could be tolerated. However, upon introduction of a sulfur (-S-) linker we observed that the sulfur atom was not only tolerated (Table 1, entry 4), but was superior to the original CH_2 linker (Table 1, entry 2).

Compound **6** exhibited good *in vitro* potency in several cell-based assays, but did not show *in vivo* efficacy due to lack of oral absorption. We hypothesized that the



1 PU3

HER-2 IC50: 38 M



2 PU24FCl

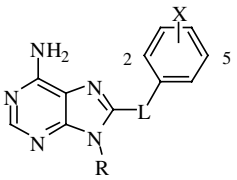
1.7 M

low aqueous solubility of **6** was the main reason for its poor oral bioavailability. We therefore sought to incorporate ionizable amino groups in the N(9) side-chain of the inhibitor. The best results were obtained when the amino N atom was separated by 2 or 3 methylene units from the adenine ring, and was further substituted with a bulky alkyl group, represented by compound **7**. The most important feature of these compounds, besides their potency, was their dramatically increased water-solubility. Once converted to their H_3PO_4 salt, these amines were remarkably soluble in water (>5 mg/mL), and could therefore be readily administered to rodents in standard aqueous solutions.

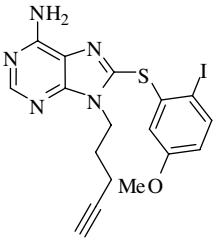
To further probe into the key aryl binding site Zhang et. al. explored many heteroaryl groups such as benzothiazole (**8**), pyridinothiazole (**9**) and benzoxazoles (**10**) (Zhang et al., 2006). Introduction of a Cl atom in position 7' led to a nearly 30-fold potency gain as compared to H at that position.

The chemistry to produce inhibitors **3–10** has been previously described in detail (Biamonte et al., 2006; Zhang et al., 2006). Briefly, adenine (Scheme 1, 11) was alkyated at N(9) by a variety of alkyl halides (Cs_2CO_3, DMF, rt-70 °C). Treatment

Table 1. Optimization of the benzene-ring substituents, and of the linker

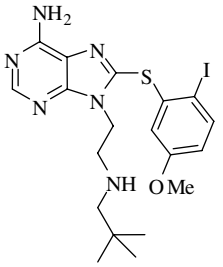


Entry	ID	R	L	X	HER-2 IC_{50} [textmuM]
1	1	Bu	CH_2	3,4,5-triMeO	40
2	3	Bu	CH_2	2,5-diMeO	12
3	4	Bu	CH_2	2-I, 5-MeO	5.0
4	5	Bu	S	2,5-diMeO	3.5
5	6	Pent-4-ynyl	S	2-I, 5-MeO	0.28



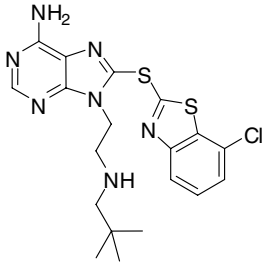
6

HER-2 IC50: 0.28 M



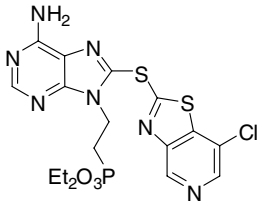
7

0.09 M



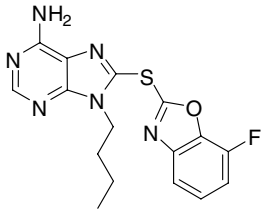
8

HER-2 IC50: 0.035 μM



9

0.03 μM

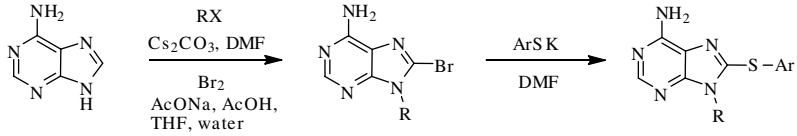


10

5.0 μM

with bromine in an acetic acid buffer afforded 8-bromoadenine **12,** and the bromine atom was displaced by thiophenols and 2-mercaptobenzothiazoles **13**.[16]

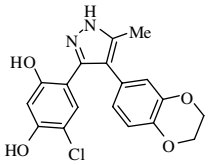
Scheme 1



11 **12** **13**

Pyrazole Based Inhibitors

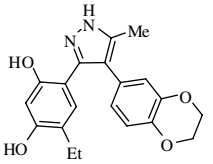
Drysdale *et al*. described pyrazole-based small molecule inhibitors series, which retains the important hinge binding resorcinol moiety found in radicicol (Drysdale et al., 2003). They have determined from the cocrystal structure that these compounds also bind to the N terminal domain of Hsp90. The SAR indicated that the compounds especially with 5-amide group (**16**) displayed good fluorescent polarization binding potency (FP IC_{50}, 0.025 μM) and cell-based activity (ATPase IC_{50}, 0.14 μM) (Dymock et al., 2005). However, no *in vivo* efficacy data on these compounds has been reported.



14

FP IC_{50}: 0.21 M

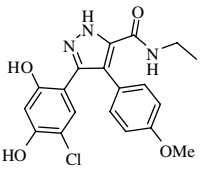
ATPase IC_{50}: 0.65 M



15

0.21 M

0.65 M



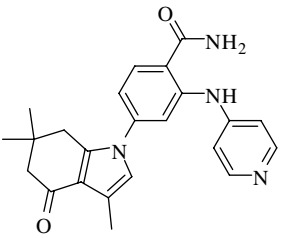
16

0.21 M

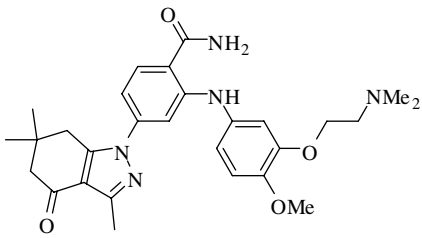
0.65 M

Tetrahydroindolone, Tetrahydroindazolone Based Inhibitors

Recently a series of tetrahydroindolone and related compounds was disclosed as Hsp90 inhibitors in a patent application (Huang et al., 2006). As demonstrated by the patent application, a 2-substituted benzamide moiety is necessary at the 1-position of the tetrahydroindolone/tetrahydroindazolone as represented by **17** and **18**. These compounds showed good potency in their cell proliferation assay (IC50 <0.5 μM) but the exact potencies were not disclosed. No in vivo efficacy data is available for these compounds.



17



18

APPLICATION OF HSP90 INHIBITORS IN NEURODEGENERATIVE DISEASES

Neurodegenerative diseases are characterized by the progressive loss of neurons and the majority of such conditions are associated with the formation of insoluble protein deposits. Polyglutamine-expanded proteins form aggregates in Huntington's disease, whereas tau-containing neurofibrilliary tangles and β-amyloid containing plaques are characteristic of Alzheimer's disease. In Parkinson's disease, α-synuclein containing aggregates are found in Lewy bodies. Molecular chaperones such as Hsp70 are very commonly found in these abnormal protein aggregates and are thought to protect against disease progression by promoting the formation of less toxic aggregates and/or targeting misfolded proteins to the proteasome for degradation. Expression of Hsp70 and other cytoprotective chaperones (the component

of the "heat –shock response") is controlled by the transcription factor heat shock factor 1 (HSF1) which normally resides in the cytoplasm in a latent form sequestered by Hsp90 (Zou et al., 1998). In response to cellular stress, HSF1 is released from Hsp90, translocates to the nucleus, and activates Hsp70 gene transcription (Figure 1). Furthermore, Hsp90 inhibitors, such as geldanamycin, also cause release of HSF1 and thereby induce Hsp70, thus making Hsp90 inhibitors very attractive for upregulating Hsp70 and being neuroprotective (Soti et al., 2005).

Indeed, a large body of data in the literature supports the concept that mobilization of the heat-shock response using Hsp90 inhibitors protects against neurotoxic insults in a variety of models of neurodegenerative diseases. For instance, geldanamycin is an effective post-treatment neuroprotective agent in a cell culture model of glutamate-induced oxidative toxicity (Xiao et al., 1999) and also protects the brain from focal ischemia in an *in vivo* rat model (Lu et al., 2002). Geldanamycin has also been shown to reduce injury due to oxygen-glucose deprivation in primary astrocyte cultures and mouse hippocampal organotypic cultures (Ouyang et al., 2005). There is evidence that Hsp90 inhibitors could be utilized in Huntington's disease since nanomolar concentrations of geldanamycin activated a heat-shock response and inhibited huntingtin protein aggregation in a cell-culture model (Sittler et al., 2001), and huntingtin mutant flies fed on the drug were completely protected from neuronal loss in a *Drosophila* model of Huntington's disease (Agrawal et al., 2005). Similarly, α-synuclein mutant flies fed on geldanamycin were fully protected from neuronal loss in a Drosophila model of Parkinson's disease (Auluck and Bonini, 2002), and geldanamycin treatment also protected against the neurotoxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in a mouse model of Parkinson's disease (Shen et al., 2005). More recently, the geldanamycin derivative 17-AAG has been shown to ameliorate polygutamine-mediated motor neuron degeneration in a transgenic mouse model for spinal and bulbar muscular atrophy (SBMA) (Waza et al., 2005). Collectively, these studies suggest that Hsp90 inhibitors could be used to modulate the heat shock response and provide protective function in damaged cells in the brain.

The first evidence that synthetic Hsp90 inhibitors could also have utility in neurodegenerative disease was from recent reports by Dickey et al. who used a novel high throughput drug screening assay to show that purine-based Hsp90 inhibitors significantly reduce tau protein levels in neuroblastoma cells in vitro and induce Hsp70, Hsp40 and Hsp27 (Dickey et al., 2005). Furthermore, these compounds induced degradation of the pathogenic form of tau by a proteasome-mediated process (Dickey et al., 2006). More recently, the same group showed that one of these small-molecule inhibitors, EC102, promoted selective degradation of tau in a transgenic mouse model of Alzheimer's disease, suggesting a central role for Hsp90 in the pathogenesis (Dickey et al., 2006, *in press*). EC102 is orally bioavailable and can cross the blood-brain-barrier (BBB), unlike the ansamycin class of Hsp90 inhibitors, and also induces Hsp70 in mouse brain (Figure 5).

A second Hsp90-binding compound that induces Hsp70 is an imidazothiadiazole sulfonamide, designated AEG3482, which was originially identified as an inhibitor

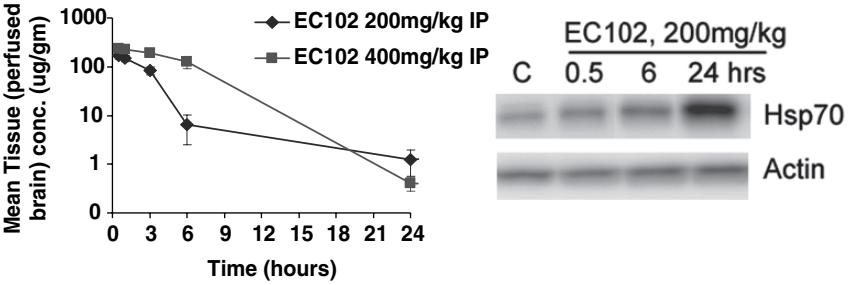


Figure 5. **EC102, a small-molecule Hsp90 inhibitor, can cross the blood-brain-barrier and induce Hsp70 in mouse brain.** EC102 dosed at 200 or 400 mg/kg IP was detected in the mouse brain by HPLC and induced Hsp70 as detected by Western blots

of JNK-dependent apoptosis of primary sympathetic neurons (Salehi et al., 2006). However, AEG3482 was unable to block JNK activity in vitro and later found to induce Hsp70 and thereby suppress JNK-mediated apoptosis. More interestingly, AEG3482 was found to bind to purified Hsp90 and is thought to induce a conformational change in Hsp90, thereby releasing HSF1 to enable transcriptional activation of Hsp70, which in turn blocks JNK activation. AEG3482 does not compete with geldanamycin to bind to the ATP-binding site of Hsp90 and neither does it cause degradation of Hsp90 client proteins. The efficacy of AEG3482 in neurodegenerative models remains to be tested.

A fascinating new finding is that Hsp90 is in an high-affinity binding, activated state in the temporal cortex of diseased Alzheimer's disease patients and not in the cerebellar cortex or in normal human brains (Dickey et al., 2007). Thus, it is possible that increased Hsp90 usage in Alzheimer's disease could provide a selective therapeutic advantage by the high-affinity binding of Hsp90 inhibitors to the diseased tissue, further suggesting that Hsp90 activity might represent a useful independent diagnostic and/or prognostic marker in neurodegenerative diseases, as has been suggested for human cancers. In the α-synuclein-induced model of neurotoxicity in flies, geldanamycin elevated Hsp70 in stressed cells but not neighbouring healthy cells (Auluck et al., 2005). Thus, data from both cancer and neurobiology fields suggest that Hsp90 inhibitors may act selectively in diseased cells, sparing their healthy counterparts.

FUTURE DIRECTIONS FOR SMALL-MOLECULE HSP90 INHIBITORS

We are presently at a very important crossroads for the development of small-molecule Hsp90 inhibitors. Several potent compounds have been synthesized by different groups in academia and biopharmaceutical companies and the first-in-class small-molecule Hsp90 inhibitor (CNF2024 from Biogen Idec) is currently in Phase I clinical trials in solid tumors and leukemias. Nevertheless, much remains to be learned before Hsp90 inhibitors can take their place in the oncologist's

pharmacopeia. Current clinical drugs may be delivered either intravenous (eg. 17-AAG) or orally (eg. CNF2024); the optimal route of administration remains to be determined in the clinic. Similarly, the importance of selectivity for the active form of Hsp90 and the most suitable client proteins to direct Hsp90 inhibitors at are still open questions. The potential of these small-molecule Hsp90 inhibitors in neurodegenerative diseases is just beginning to be explored, but the in vitro proof-of-concept studies suggest that these drugs could represent a new mechanistic approach to ameliorate neuronal cell death in several CNS disorders. For neurodegenerative disease, it is likely that induction of the heat-shock response (particularly Hsp70) will be of therapeutic value, but the role of client protein degradation is also provocative. For instance, selective degradation of mutant tau in Alzheimer's disease is induced by Hsp90 inhibitors, and analogous mutant proteins drive the familial forms of ALS and Parkinson's disease. Conversely, it is unlikely that Hsp90 activity or the heat-shock response impacts extracellular protein deposits such as amyloid plaques and the chronic, indolent nature of most neurodegenerative conditions may overtax the therapeutic index of currently available compounds. Much remains to be unraveled, but the promise of Hsp90 modulation in both cancer and CNS disorders is undeniable.

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CHAPTER 15

DRUGGING THE HSP90 MOLECULAR CHAPERONE MACHINE FOR CANCER TREATMENT

J.L. HOLMES, S.Y. SHARP AND P. WORKMAN

Signal Transduction and Molecular Pharmacology Team, Cancer Research UK Centre for Cancer Therapeutics, The Institute of Cancer Research, Haddow Laboratories, Sutton, Surrey, SM2 5NG, UK

Abstract: The ATP-dependent molecular chaperone heat shock protein 90 (HSP90) is required for the function and stability of an increasing number of oncogenic proteins. HSP90 chaperones these proteins by forming a series of multimeric complexes to facilitate client protein loading, activation and release, a process which is driven by ATP hydrolysis. Many HSP90 client proteins have been implicated in the development and progression of cancer. As a result of this, HSP90 has emerged as an exciting drug target in oncology. Of particular benefit, HSP90 inhibitors have the potential to simultaneously deplete multiple proteins involved in all the six hallmark traits of cancer. The initial approach taken to modulate HSP90 was to focus on inhibiting the ATPase activity, resulting in degradation of client proteins via the ubiquitin proteasome pathway. Several chemical classes of compounds which target the ATP binding pocket of HSP90 have shown anti-cancer activity in preclinical models. The geldanamycin derivative 17-allylamino-17-demethoxygeldanamycin (17-AAG, tanespimycin) is now undergoing phase II studies. The results from phase I trials have shown clear evidence of target modulation and an indication of clinical activity in melanoma, prostate, renal, multiple myeloma and trastuzumab-refractory breast cancers. These data have provided proof-of-principle that HSP90 is a promising drug target in cancer. A number of new synthetic small molecule inhibitors are currently undergoing development. This review will focus on the current status of the ATP-competitive HSP90 inhibitors as well as novel approaches to inhibit the HSP90 chaperone machine

Keywords: HSP90, inhibitors, chaperone

INTRODUCTION

In cancer therapeutics it is desirable to develop novel therapies which will target the hallmarks of cancer (Hanahan and Weinberg 2000). The ATP-dependent molecular chaperone HSP90 represents an exciting drug target, inhibition of which can potentially modulate all six of these hallmarks simultaneously as it interacts with multiple client proteins, for example AKT, CDK4, ERBB2 and C-RAF and many others (Maloney and Workman 2002; Workman 2004; www.picard.ch). Table 1 gives

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Table 1. Examples of HSP90 client proteins involved in the hallmark traits of cancer

Hallmark Trait	Example
Escaping apoptosis	AKT, IGF-IR
Resistance to anti-growth signals	CDK4, cyclin D
Infinite replicative potential	hTERT
Self-suffiency in growth signals	C-RAF, ERBB2, KIT
Prolonged angiogenesis	VEGF, HIF-1
Tissue invasion and metastasis	MET, MMP2

examples of client proteins involved in mediating the various hallmark traits. The advantage of this approach is that combinatorial inhibition of several oncogenic pathways in cancer cells should give a powerful anticancer effect and limit the development of resistance. In this chapter we discuss the biology of HSP90 in relation to the development of HSP90 inhibitors. In addition, we highlight alternative strategies which are being investigated to compromise the activity of the HSP90 chaperone complex.

HSP90 and the Co-Chaperone Cycle

It is widely accepted that HSP90 operates as part of a multimeric chaperone complex, as exemplified by reconstitution experiments (Dittmar and Pratt 1997). Interactions with specific co-chaperones enable HSP90 to form a dynamic chaperone complex. Important co-chaperones, which are listed in Table 2, include HOP or P60/Stil (Johnson et al. 1998), CDC37/p50 (Roe et al. 2004), HARC (Scholz et al.

Table 2. Major co-chaperones involved in the HSP90 chaperone cycle

Protein	Function
HSP90	To maintain the conformational stability and function of a range of client proteins
HSP70	Aids client protein loading onto the HSP90 chaperone complex. Also has chaperone activity independent of HSP90
HSP40	Increases the ATPase activity of HSP70
HOP	HSP90/HSP70 interacting protein – mediates the interaction between HSP90 and HSP70
AHA1	Increases the ATPase activity of HSP90
P23	Maintains the stability of HSP90 in the mature complex and acts as a client protein release factor
CDC37	Inhibits ATPase activity of HSP90 to facilitate kinase client proteins loading onto HSP90
HARC	Homolog of CDC37 – binds HSP70, HOP and immunophilins, but does not bind SRC-family kinases or C-RAF
Immunophilins	Interact with HSP90 client proteins (specifically hormone receptors) and increases ATPase activity to a limited extent

2001), P23/Sba1 (Johnson et al. 1994; Fang et al. 1998), CYP40/CPR6 (Mayr et al. 2000), CPR7 (Duina et al. 1996), and AHA1/Hch1 (Panaretou et al. 2002; Nathan et al. 1999). Each co-chaperone has a unique role in the chaperone complex and interacts with HSP90 at a specific point in the chaperone cycle (Figure 1). For example, CDC37 has been shown to facilitate kinase client protein loading in an ordered process in which a kinase-$(CDC37)_2$ complex initially binds to the HSP90 dimer via a symmetrical $(CDC37)_2$-$(Hsp90)_2$ dimer-dimer interaction (Roe et al. 2004). Conformational rearrangement of the initial complex then occurs resulting in the release of a CDC37 molecule to give an asymmetrical complex containing $(Hsp90)_2$-CDC37-kinase (Vaughan et al. 2006). P23, however, is involved in client protein release (Dittmar and Pratt 1997). Studies have shown that a minimum of HSP90, HSP70, HOP and P23 are required both for the assembly of the glucocorticoid receptor–HSP90 complex and also for the reactivation of steroid hormone receptor activity (Dittmar and Pratt 1997).

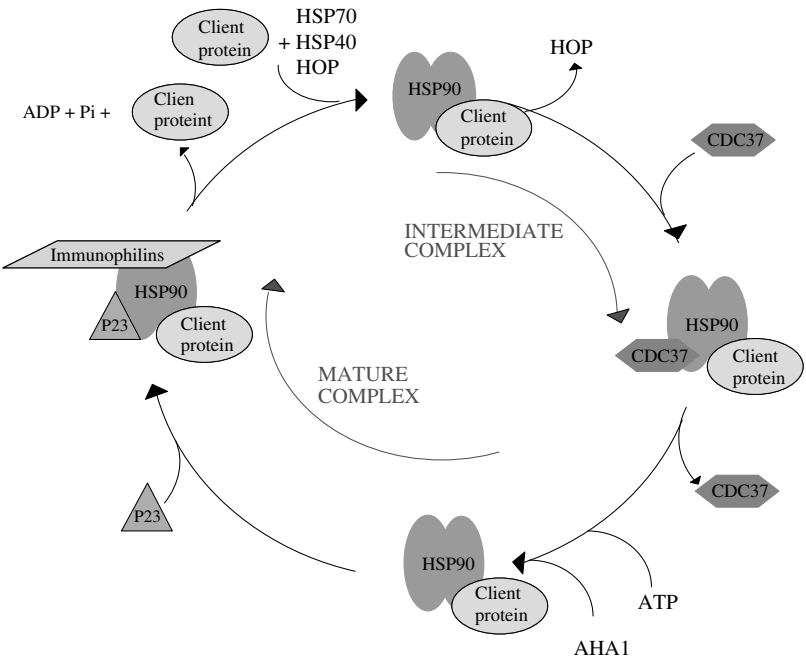


Figure 1. **Model of the HSP90 chaperone cycle**. The client protein binds to an HSP70/HSP40 complex. The TPR co-chaperone HOP facilitates its transfer onto the ADP-bound HSP90. To facilitate this process the ATPase activity of HSP90 is inhibited by HOP and CDC37. These complexes are known as intermediate complexes. When ADP is exchanged for ATP, HSP90 undergoes a conformational change which releases HSP70/HSP40 and HOP, thus allowing the ATP-dependent association of other co-chaperones, including P23 and the immunophilins to form the mature complex. The stable functional client protein is then released. Other co-chaperones involved include AHA1, which increases the ATPase activity of HSP90 and CDC37 which facilitates kinase client binding

Binding and hydrolysis of ATP is also critical for the operation of the chaperone cycle (Panaretou et al. 1998) and essential for client protein stabilization and function (Obermann et al. 1998). The ATP binding state of HSP90 determines which combination of co-chaperone binds at any given stage of the chaperone-client protein cycle. The current model of the HSP90 chaperone cycle (Figure 1) is that client proteins are loaded onto HSP90 via an interaction with HSP70, HSP40 and HOP (Pratt and Toft 1997; Hernandez et al. 2002) forming the intermediate complex. To facilitate this process the ATPase activity of HSP90 is inhibited either by HOP or by CDC37 (Roe et al. 2004).These co-chaperones are then released from the complex, allowing P23 and immunophilins to bind and form the mature complex, thus maintaining the conformation, stability and function of client proteins. Other co-chaperones such as AHA1 increase the HSP90 ATPase activity which causes a conformational change in the activation loop of HSP90 (Panaretou et al. 2002). The immunophilins also play a role in increasing the ATPase activity to a limited extent and are involved in the interaction with steroid hormone receptors (Johnson et al. 1996).

Two competing models have been proposed regarding the effect that ATP binding has on the conformation of HSP90. X-ray crystallographic studies supported a 'molecular clamp' mechanism, whereby ATP binding results in closure of a 'lid' segment and transient dimerization of the N-terminal domain of HSP90 then occurs (Prodromou et al. 2000; Pearl and Prodromou 2006). This was further confirmed by the crystal structure of yeast Hsp90 in the 'closed' conformation, with the N-terminals dimerised in the presence of the co-chaperone P23/Sba1 (Ali et al. 2006). An alternative model disputes that the N-terminals of HSP90 dimerise upon ATP binding, based on kinetic analysis of the complex upon ATP binding (McLaughlin et al. 2004). Analysis of ATP binding to human HSP90 occurred rapidly in a two-step process. The rate determining step was shown to be ATP hydrolysis as opposed to ADP dissociation. However, these studies did not reveal any biophysical evidence for the N-terminal dimerization as proposed in the yeast model (McLaughlin et al. 2004). The balance of exidence, and particularly the x-ray crystallography data, support the molecular clamp model (Pearl and Prodromou 2006).

An additional nucleotide-binding site at the C-terminal of HSP90 has also been proposed (Marcu et al. 2000a). The N- and C-terminal binding sites have distinct properties and have been reported to interact (Soti et al. 2002). The putative C-terminal binding site of HSP90 is described as a cryptic chaperone nucleotide binding site, as the N-terminal site must be occupied before the second site becomes available for binding (Soti et al. 2002).

HSP90 in Normal Cells

HSP90 is abundant in normal cells, making up approximately 1–2% of the total cellular protein. The role of HSP90 under normal conditions is to ensure the correct conformation, activation state and stability of an increasingly well-defined group of potentially metastable proteins to regulate their fate in the cell (Whitesell and

Lindquist 2005). Investigation of HSP90 homologs in various model organisms has revealed that HSP90 plays a role in development and evolution by acting as a buffer of phenotypic variations (Whitesell and Lindquist 2005). Mutations can effectively be kept functionally hidden by HSP90 until they are uncovered during periods of stress and selective pressure, for example under conditions of elevated temperature and oxidative stress (Cowen and Lindquist 2005; Rutherford and Lindquist 1998). In these situations HSP90 allows the mutated proteins to continue to interact and thereby provides the potential for survival advantage and evolutionary development.

In addition to its role under normal conditions, HSP90 is important in regulating the response to stress such as elevated temperature and oxidative stress via heat shock factor-1 (HSF-1) as discussed later.

HSP90 and Cancer

Tumour formation and progression rely on the accumulation of mutations that promote uncontrolled cell growth and division along with the ability to survive multiple cellular insults (Hanahan and Weinberg 2000). HSP90 is likely to play a role in helping the cell to support this accumulation of mutations (Whitesell and Lindquist 2005). The chaperone is unlikely to act as a conventional oncogene in its own right. There are no reported mutations of HSP90 in cancer cells. However, HSP90 has been found to be upregulated in certain cancers (Kimura et al. 1993; Conroy et al. 1998; Ciocca et al. 1993; Ralhan and Kaur 1995; Chant et al. 1995; Yufu et al. 1992). This is likely to be due to factors such as the overexpression and mutation of oncogenic proteins and also microenvironment stresses, for example hypoxia and nutrient deprivation (Mosser and Morimoto 2004). Moreover, tumours are made up of heterogeneous populations which constantly evolve under selection to survive the inhospitable environment. Both the genetic abnormalities and alterations in oncoprotein folding contribute to malignant transformation. Many oncogenic proteins have been found to be client proteins of HSP90 and hence cancer cells are highly dependent on HSP90. Activation of signalling pathways mediated by these HSP90 clients are necessary for cell proliferation, regulation of cell cycle progression, and apoptosis (Maloney et al. 2003). Additionally, mutant oncoproteins responsible for transformation often require HSP90 for maintenance of their folded, functionally active conformations (Chiosis 2006).

HSP90 is particularly attractive as a drug target in cancer due to its role in simultaneously chaperoning multiple oncogenic client proteins. Based on the model of Weinstein (2002) it has been hypothesised that cancer cells may develop ‘addiction to’ or dependence upon these multiple pathways (Workman 2004). Mutant proteins have also been shown to be more dependent on HSP90 than their wild-type counterparts, a good example being B-RAF which is frequently mutated in melanoma and other cancers (da Rocha et al. 2005; Grbovic et al. 2006). Other examples include EGFR, BCR-ABL and v-SRC. In animal models, HSP90 inhibitors have also been shown to accumulate in the tumour with little toxicity to the surrounding normal

tissues (Banerji et al. 2005b; Eiseman et al. 2005). Studies have shown that HSP90 inhibitors are able to bind more tightly to the HSP90 super-chaperone complex which predominates in cancer cells, as compared to the uncomplexed form that is present in normal cells (Kamal et al. 2003). Because multiple pathways are blocked rather than targeting a single genetic abnormality, the potential opportunities for drug resistance to develop are reduced. An additional therapeutic advantage of HSP90 inhibitors in cancer is that they may block the buffering of potentially lethal mutations, leading to a damaging effect on malignant cells.

INHIBITION OF THE HSP90 MOLECULAR CHAPERONE

The current HSP90 inhibitors can be divided into ATP competitive inhibitors and those which inhibit its activity via non-competitive mechanisms.

ATP Competitive Inhibitors

Initial efforts to develop HSP90 inhibitors have focused on ATP competitive ligands. Many of these are derivatives of natural products such as the macrocyclic antibiotic radicicol (monorden) which is isolated from the fungus *Monosporium bonorden* (Delmotte and Delmotteplaquee 1953; Figure 2). Radicicol showed potent anti-cancer properties *in vitro* and the mechanism of action of this compound was

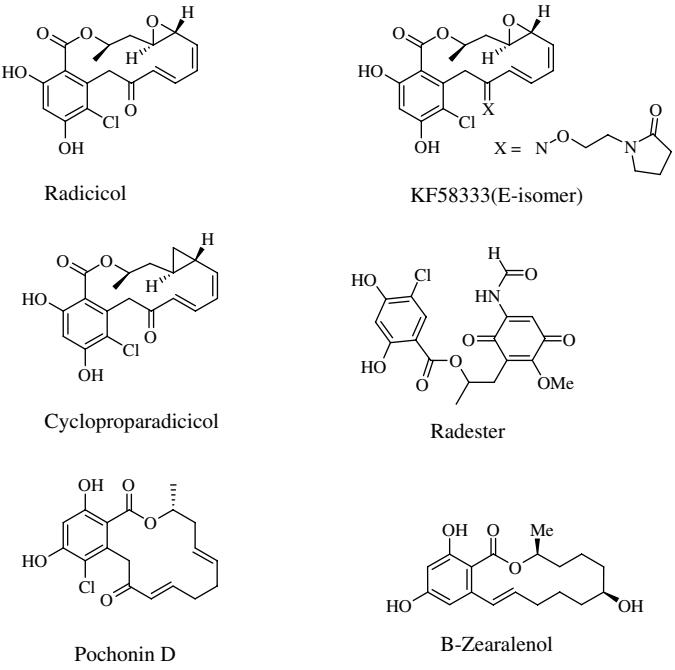


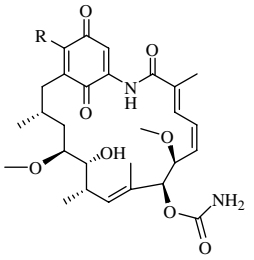
Figure 2. Chemical structures of radicicol and its derivatives

originally thought to involve inhibiting SRC (Kwon et al. 1992; Zhao et al. 1995). Later studies revealed that radicicol binds tightly to the N-terminal ATP binding site of HSP90, thereby inhibiting its essential ATPase activity (Roe et al. 1999; Schulte and Neckers 1998). This results in the degradation of client proteins such as C-RAF, CDK4 and ERBB2 and the induction of HSP70, which is now accepted to be part of the molecular signature of HSP90 inhibition. Radicicol, however, was not active *in vivo*, probably due to the instability caused by the presence of a reactive epoxide moiety and other undesirable structural features (Agatsuma et al. 2002; Soga et al. 2003). There have been attempts to remove these features from the molecule. Some success has been achieved with the semi-synthetic oxime derivatives of radicicol such as KF55823 and KF58333 which were synthesised by Kyowa Hakko Kogyo Company (Agatsuma et al. 2002; Figure 2). These oximes are more soluble and exhibit an improved pharmacokinetic profile in animal models. However, they are still at a pre-clinical stage (Ikuina et al. 2003). It has been reported that the oximes cause severe cataracts in animals (Janin 2005).

Alternative replacement strategies include substituting the epoxide with a cyclopropane as in cycloproparadicicol (Yamamoto et al. 2003; Figure 2). Several simplified radicicol analogues have also been made which revealed that the chlororesorcinol ring is crucial in retaining the activity of these compounds (Proisy et al. 2006; Atrash et al. 2006; Cooper et al. 2006). Other groups have made hybrid compounds consisting of the resorcinol from radicicol and the benzoquinone from geldanamycin. These compounds include radester (Figure 2), which has been shown to be active in cells and results in the degradation of ERBB2 and C-RAF (Shen and Blagg 2005). Analysis of the conformation-activity relationship of radicicol bound to HSP90 revealed that radicicol, in its bioactive form, binds in an L-shape conformation. Pochonin D was identified based on its ability to adopt this bioactive conformation, and was shown to be nearly as potent as radicicol at inhibiting HSP90 (Moulin et al. 2005; Figure 2).

A series of zearalenol compounds (patent WO03050295, 2003) have been developed by Biogen Idec. β-zearalenol (Figure 2) is the most potent analogue reported to date with cellular activity of 1μM in the MCF breast cancer cell line.

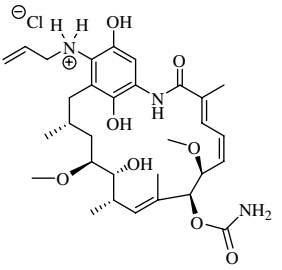
A second series of compounds derived from natural products are the benzoquinone ansamycins, which include geldanamycin (Figure 3) and herbimycin A. X-ray crystallographic studies revealed that geldanamycin binds to the ATP binding site in the N-terminal domain of HSP90 (Roe et al. 1999). This results in the proteasomal degradation of multiple oncogenic client proteins such as steroid receptors, C-RAF, AKT and BCR-ABL *in vitro* (Whitesell et al. 1994). However, metabolic instability and hepatotoxicity were reported with geldanamycin (possibly due to the benzoquinone moiety) and therefore it did not have a sufficient therapeutic window to warrant clinical development (Supko et al. 1995). Further geldanamycin analogues have been investigated. One of the most promising is the 17- allylamino derivative of geldanamycin, 17-AAG (Figure 3). This agent exhibited potent activity in cells, which may be due to accumulation of the drug in cancer cells (Chiosis et al. 2003a; Workman 2003). 17-AAG causes cell cycle arrest and



17-DMAG R = $NHCH_2CH_2N(CH_3)_2$

17-AAG R = $NHCH_2CH{=}CH_2$

Geldanamycin R = OCH_3



IPI-504

Figure 3. Chemical structures of benzoquinone ansamycin HSP90 inhibitors

apoptosis in human cancer cell lines (Hostein et al. 2001). 17-AAG is also able to induce differentiation of breast cancer cells (Munster et al. 2001) and keratinocytes (Honma et al. 2006) In addition, it exhibits anti-angiogenic and anti-invasive properties *in vitro* and *in vivo* (Sanderson et al. 2006). 17-AAG is active at well tolerated doses in a wide range of human tumour xenograft models (Kelland et al. 1999; Solit et al. 2002; Smith et al. 2005a).

After extensive pre-clinical evaluation, 17-AAG entered phase I clinical trials in both the UK and USA (Pacey et al. 2006). Analysis of the pharmacokinetic and pharmacodynamic data from the phase I trial carried out at our institution showed that the drug achieved satisfactory exposure at well tolerated doses (Banerji et al. 2005a). The molecular signature of HSP90 inhibition was also detected at 24 hours post treatment in the peripheral blood mononuclear cells and in tumour biopsies. The observed depletion of C-RAF and CDK4 together with induction of HSP70 (Banerji et al. 2005a; Banerji et al. 2005b) provide proof-of-concept for target inhibition. In addition, two patients with malignant melanoma achieved prolonged stable disease (Banerji et al. 2005a). Responses in patients with prostate and breast cancer and in multiple myeloma (Modi et al. 2006; Chanan-Khan et al. 2006) were also observed. Based on these promising phase I trial results (Pacey et al. 2006), 17-AAG is currently in phase II clinical trials in metastatic kidney, melanoma and trastuzumab-refractory advanced breast cancers (http://www.nci.nih.gov/clinicaltrials).

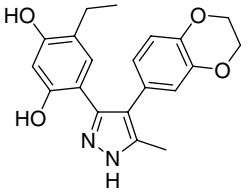
Although initial results were encouraging, several drawbacks were identified with 17-AAG, including low solubility, polymorphic metabolism by cytochrome P450 CYP3A4 (Egorin et al. 2001) and NQO1/DT-diaphorase (Kelland et al. 1999) and various toxic side effects, including hepatoxicity (Pacey et al. 2006). Due to poor solubility the original formulation of 17-AAG required a large volume of DMSO and egg phospholipids, which was unpleasant for patients. Kosan Biosciences (http://www.kosan.com) and Conforma Therapeutics, now part of Biogen Idec (http://www.biogenidec.com), have developed improved

formulations of 17-AAG (KOS953 and CNF1010, respectively) which overcame the original cumbersome formulation. A second geldanamycin analogue, 17-DMAG (alvespimycin, KOS1022; Figure 3), which is 10 times more soluble than 17-AAG was identified (Egorin et al. 2002). This drug has similar activity to 17-AAG both *in vitro* and *in vivo* (Burger et al. 2004; Smith et al. 2005b; Kaur et al. 2004) and has likewise been shown to accumulate in tumours versus normal tissues (Eiseman et al. 2005). As a result of its improved solubility and oral bioavailability, 17-DMAG has recently entered phase 1 clinical trials in the UK and USA in patients with advanced heamatologic and solid malignancies in multiple centres (http://www.clinicaltrials.gov).

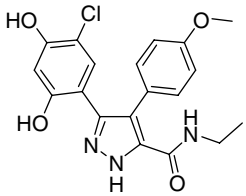
As mentioned above, the quinone moiety present in 17-AAG has been shown to have metabolic liabilities which impact on the pharmacokinetics and tumour cell sensitivity to the drug. 17-AAG is metabolised by both cytochrome P450 CYP3A4 and NQO1/DT-diaphorase (Egorin et al. 2002; Kelland et al. 1999). NQO1 activity is differentially expressed in the population due to a polymorphism in the gene that results in reduced stability and enzyme function. A correlation has been observed between cellular sensitivity to 17-AAG and NQO1 expression (Kelland et al. 1999). Cancer cells with high NQO1 activity are up to 30-fold more sensitive to 17-AAG compared to those carrying the inactivating polymorphism. NQO1 can reduce 17-AAG to the hydroquinone 17-AAGH_2, which has been shown to be a more potent inhibitor than the parental drug (Guo et al. 2005b). Infinity Pharmaceuticals (http://www.ipi.com) have developed the hydroquinone form of 17-AAG, IPI-504 (Ge et al. 2006; Figure 3) and pre-clinical studies have shown this to be an effective anticancer agent in models of haematological malignancies as well as solid tumours (Sydor et al. 2006). This drug has also been reported to be effective in combination with the proteasome inhibitor bortezomib (Velcade) in myeloma cell lines (Sydor et al. 2006). In addition, it causes depletion of KIT in imatinib-resistant gastrointestinal stromal tumours (Bauer et al. 2005). An important advantage of IPI-504 is the greater aqueous solubility of the hydroquinone compared to the parent drug 17-AAG, which allows a more favourable formulation for clinical development. Clinical trials are now underway. A potential disadvantage of IPI-504 is that it converts back to 17-AAG in the body. It may therefore exhibit toxicities associated with 17-AAG.

A further liability of 17-AAG is that it is a substrate for P-glycoprotein (MDR1) (Kelland et al. 1999), which could potentially represent a likely mechanism by which a patient could develop drug resistance.

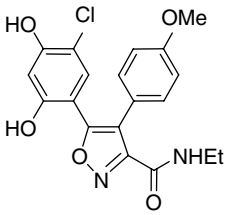
Due to the limitations associated with the natural products described above, many groups have been developing alternative ATP competitive HSP90 inhibitors. At the Cancer Research UK Centre for Cancer Therapeutics, high-throughput screening was performed in which a series of novel 3,4-diarylpyrazole resorcinol analogues were identified, as exemplified by CCT018159 (Chung et al. 2003; Figure 4). Protein X-ray crystallography studies confirmed that CCT018159 binds to the ATP binding site on the N-terminal of HSP90 (Cheung et al. 2005; Rowlands et al. 2004). This compound demonstrated comparable activity with 17-AAG against the target HSP90 but exhibited lower cellular potency. Treatment of several human cancer cell lines with



CCT018159



CCT0129397
VER-49009



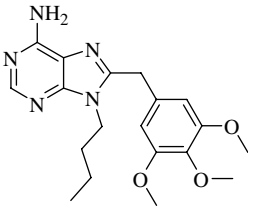
CCT0130024

VER-50589

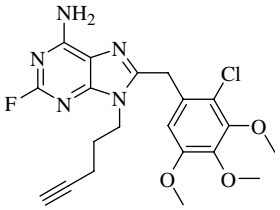
Figure 4. Chemical structures of resorcinylic pyrazole/isoxazole amide analogues of HSP90 inhibitors

CCT018159 caused depletion of client proteins such as ERBB2 and C-RAF and upregulation of HSP70 (Sharp et al. 2007a). Unlike 17-AAG, CCT018159 has good aqueous solubility and its activity and is independent of NQO1 and P-glycoprotein expression (Sharp et al. 2007a). In a subsequent collaboration with Vernalis Ltd a structure-based design approach generated a series of potent amide analogues. The pyrazole amide VER-49009 (CCT0129397; Figure 4) was identified to be very potent against the target and in cells (Dymock et al. 2005). The corresponding isoxazole VER-50589 (CCT0130024; Figure 4) exhibited the strongest binding affinity yet reported for a small molecule HSP90 inhibitor and demonstrated antitumour activity in vivo (Sharp et al. 2007b). VER-50589 represents promising advanced stage lead in this series.

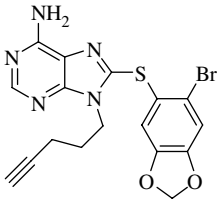
Chiosis and colleagues (2003) used a molecular modelling approach to develop a series of synthetic purine-scaffold inhibitors, as exemplified by PU3 (Chiosis et al. 2003b; Figure 5). X-ray crystallography has shown that PU3 binds to human HSP90α with the purine ring flipped in the opposite direction to ADP (Wright et al. 2004). Optimisation at the left side of the purine scaffold generated PU24FCl (Figure 5) which exhibited significantly improved solubility and also had higher affinity for HSP90 (Vilenchik et al. 2004). Focusing on the structure-activity relationship of the purine-scaffold has enabled other investigators to develop novel compounds with higher potency (Wright et al. 2004). A recent report has disclosed a series of 8-arylsulanyl, 8-sulfoxyl and 8–sulfonyl adenine derivatives of the PU-class. PU-H58 (Figure 5) has emerged as a potent purine analogue to date *in vitro* and *in vivo* (Llauger et al. 2005). Conforma Therapeutics has reported an



PU3



PU24FCl



PU-H58

Figure 5. Chemical structures of purine-based HSP90 inhibitors

orally active purine-based inhibitor, but high doses of these compounds are required for efficacy (Biamonte et al. 2006). The first purine-based HSP90 inhibitor CNF2024 has entered phase I clinical trials (Chiosis et al. 2006).

Serenex Inc. has recently reported a novel HSP90 inhibitor which was identified using a proprietary purine proteome mining technology (Steed et al. 2006). SNX-2112 acts *in vivo* as a pro-drug of SNX-5422 to facilitate oral administration and preclinical studies reported in abstract form show it to be highly potent and selective for HSP90 (Chandarlapaty et al. 2006; Steed et al. 2006).

C-Terminal Binders

The coumarin antibiotics, exemplified by novobiocin and the more potent analogue coumermycin A1 (Figure 6), are thought to bind to the C-terminus of HSP90. This disrupts the interaction of HSP90 with co-chaperones containing TPR domains which are essential for its activity (Allan et al. 2006). Novobiocin binding prevents HSP90 from undergoing the conformational changed required for ATP hydrolysis and thus acts as a non-competitive inhibitor (Marcu and Neckers 2003). Treatment *in vitro* with novobiocin results in depletion of client proteins, including C-RAF and ERBB2 (Yun et al. 2004; Marcu et al. 2000b), consistent with HSP90 inhibition. More potent analogues have been discovered from a library of novobiocin derivatives, with compound 4A (Figure 4) being the most active (Yu et al. 2005).

Cisplatin is a cytotoxic drug which reacts covalently with DNA leading to inhibition of cell division and tumour growth (Kartalou and Essigmann 2001a;

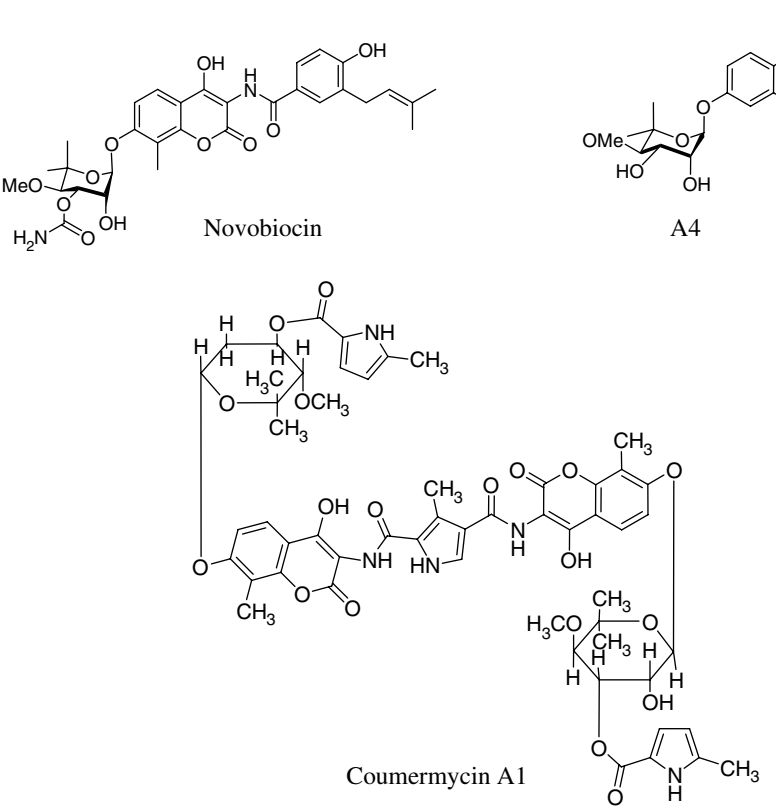
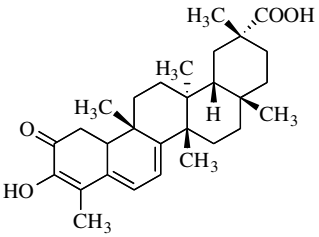


Figure 6. Chemical structures of C-terminal binding HSP90 inhibitors

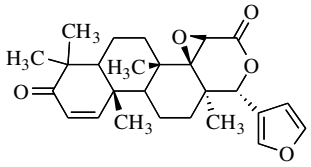
Kartalou and Essigmann 2001b). However, it has also emerged that cisplatin is able to interact with certain proteins, thereby disrupting their function. Cisplatin has been shown to bind to the C-terminal domain of HSP90 (Itoh et al. 1999) and in doing so disrupts the transcriptional activity of both the glucocorticoid and androgen receptors and their subsequent degradation by the proteasome. Other HSP90 client proteins such as C-RAF and SRC, however, are unaffected by treatment with cisplatin (Rosenhagen et al. 2003). Furthermore, cisplatin treatment does not elicit the stress response of induction of heat shock proteins in the same way as the ATP competitive inhibitors. Hence, the therapeutic significance of the effects of cisplatin on HSP90 is unclear.

Celastrol and Gedunin

Use of gene expression profiles in a 'Connectivity Map' approach has identified a group of triterpenoid natural products, celastrol and gedunin (Figure 7) that have similar expression signatures to those of HSP90 inhibitors (Hieronymus et al.



Celastrol



Gedunin

Figure 7. Chemical structures of celastrol and gedunin

2006). Treatment of cancer cells with these agents caused degradation of HSP90 client proteins including EGFR, BCR-ABL, androgen receptor and mutant FLT3. Celastrol and gedunin are able to inhibit the ATPase activity of HSP90 (Hieronymus et al. 2006); however, neither of them binds to the ATP binding site of the N-terminus of HSP90, indicating a novel mechanism of action. It has been suggested that celastrol induces a heat shock response via activation of heat shock factor-1 (HSF-1) as well as acting as a potent proteasome inhibitor, leading to the accumulation of ubiquitinated proteins (Westerheide et al. 2004). However, further work on these compounds is required to define their precise mechanism of action.

Mycograb® Human Recombinant Antibody Against HSP90

Alternatives to small molecule inhibitors include the antifungal agent Mycograb, a human recombinant antibody directed against HSP90, which has been developed by NeuTec Pharma Ltd (http://www.neutecpharma.com). The antibody binds to the middle domain of HSP90 and inhibits its activity. Although originally developed to treat systemic candidiasis in combination with the current anti-fungal drug amphotericin B, (Matthews and Burnie 2004), this agent has now entered a multi-centre phase I trial in combination with docetaxel in patients with metastatic or recurrent breast cancer (Vastag 2006). This antibody was well tolerated in the clinic (http://clinicaltrials.gov/ct/show/NCT00217815).

OTHER APPROACHES TO INHIBIT THE HSP90 CHAPERONE COMPLEX

Directly targeting HSP90 itself is not the only approach available to inhibit its activity, as illustrated in Figure 8. As discussed earlier, it has been established that HSP90 can only function efficiently as part of a multimeric complex. The series of interactions HSP90 forms with its co-chaperones during the chaperone cycle are regulated by post-translational modifications as well as ATP hydrolysis. This provides numerous points for potential intervention. Certain client proteins require their involvement of specific co-chaperones for the correct folding,

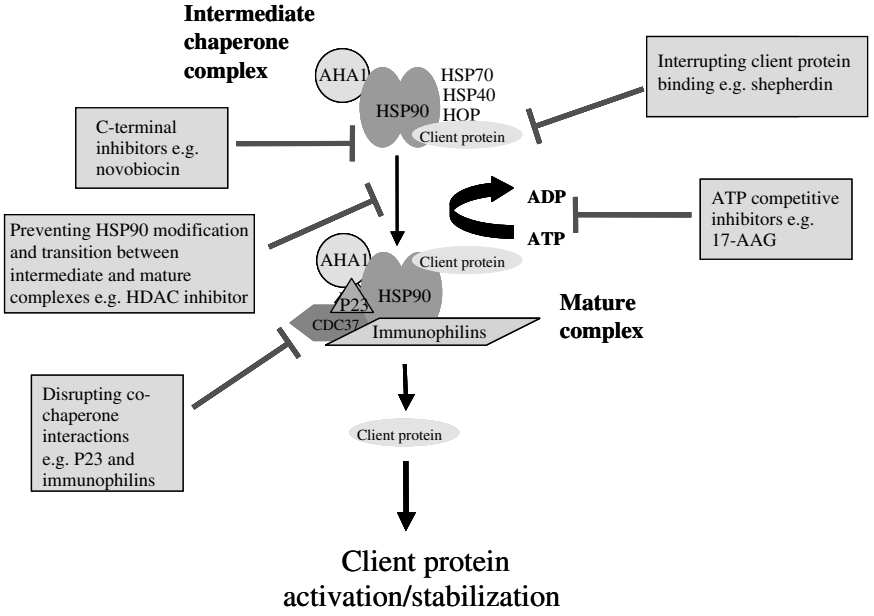


Figure 8. Schematic diagram of potential strategies for HSP90 inhibition. HSP90 forms an intermediate complex with HSP70/HSP40, HOP and other co-chaperones while in its ADP-bound form. ATP binding facilitates formation of the mature chaperone complex containing P23 and immunophilins. ATP hydrolysis enables the client protein to adopt its active conformation. A common strategy for inhibiting HSP90 is to target the N-terminal ATPase activity (e.g. 17-AAG) resulting in client protein degradation via the ubiqutin-proteasome pathway. Alternative targets include the proposed ATP binding site on the C-terminal of HSP90, preventing the dynamic post-translational modification of HSP90 and disrupting client protein or co-chaperone binding

stabilisation and activation (Bracher and Hartl 2006). Therefore by targeting specific co-chaperones it may be possible to inhibit the function of specific client proteins. Another approach is to modulate post-translational modifications of Hsp90.

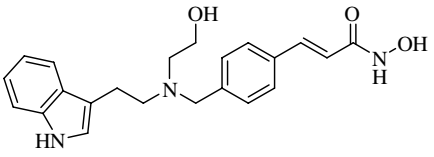
Modulating Post Translational Modifications

HSP90 activity can be regulated by a series of post-translational modifications, such as acetylation and phosphorylation. A recent report has identified the acetylation of a specific lysine residue (K294) in the middle domain of HSP90 which prevent specific co-chaperones from binding (Scroggins et al. 2007). Additionally, K294 acetylation was found to be a nucleotide-independent determinant of P23 binding to HSP90. It has therefore been proposed that the acetylation status of K294 may transmit complex allosteric information throughout the HSP90 chaperone molecule that is independent of, but perhaps synergistic with, nucleotide binding (Scroggins et al. 2007). The acetylation of HSP90 has been postulated to be involved in

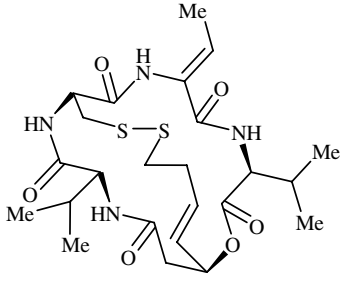
de-stabilising the P23 complex to release the client and facilitating progression of the chaperone cycle (Kovacs et al. 2005a). Detailed analysis of the effects of acetylation of residue K294 on co-chaperone interactions revealed a differential impact on the co-chaperones tested. Thus, HSP72, HOP and CHIP were less sensitive to the acetylation than AHA1 and FKBP52 (Scroggins et al. 2007).

HSP90 acetylation and deacetylation is a dynamic process within the cell. Histone deacetylase (HDAC) 6 is reported to be involved in the deacetylation of HSP90 (Kovacs et al. 2005b). It is unlikely to be the only HDAC which deacetylates HSP90 since initial reports which identified HSP90 hyperacetlylation used the HDAC inhibitor FK228, which does not inhibit HDAC6 (Furumai et al. 2002). Inhibition of histone deacetylase (HDAC) by agents such as FK228 and LAQ824 (Figure 9) cause an increase in HSP90 acetylation. This interrupts ATP binding, leading to the release of client proteins. Evidence of HSP90 inhibition, using molecular biomarkers, has been observed in both *in vitro* and phase I clinical trials (Kristeleit et al. 2004; Aoyagi and Archer 2005). Studies have shown that siRNA against HDAC6 resulted in hyperacetylation of HSP90 and disruption of the chaperone function. (Murphy et al. 2005; Bali et al. 2005). The consequences of this are that there is increased binding of HOP to HSP90 but reduced binding of P23, suggesting that the complex is stalled in the intermediate complex. This also results in decreased ATP binding and therefore reduced HSP90 activity (Yu et al. 2002). The histone acetyltransferase (HAT) responsible for HSP90 acetylation has yet to be identified.

HSP90 is also phosphorylated, which is reported to be regulated by the phosphatase PPT1 (Wandinger et al. 2006). IC101 (Figure 10), a novel cyclic

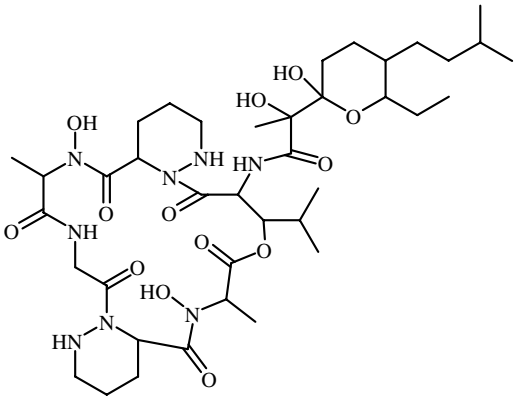


LAQ824



FK228

Figure 9. Chemical structures of HDAC inhibitors LAQ824 and FK228



IC101

Figure 10. Chemical structure of IC101

depsipeptide compound isolated from *Streptomyces albulus MJ202-72F3* (Ueno et al. 1993), has been shown to cause dephosphorylation of HSP90 on tyrosine residues (Fujiwara et al. 2004). The specific tyrosine residues affected, however, are yet to be identified. To date, no detailed pharmacological or biochemical properties of this compound have been reported. However, IC101 has been shown to induce apoptosis in several cell lines (Wandinger et al. 2006). Dephosphorylation of HSP90 by IC101 inhibits HSP90-AKT binding and results in the decreased phosphorylation and degradation of client proteins (eg C-RAF; Fujiwara et al. 2004). ATP binding to HSP90 is also inhibited by IC101, which occurs via a non-competitive mechanism. However, the mechanism by which this occurs is currently unknown. It has been suggested that the inhibition of AKT phosphorylation may trigger the apoptotic cell death seen when cells are treated with this compound (Fujiwara et al. 2004). Thus, it is possible to target HSP90 activity indirectly by modulating the post-translational regulatory mechanisms which control its activity.

Disrupting Interactions with Co-Chaperones

Recently, there has been significant interest in the effects of the interactions that HSP90 makes with its co-chaperones. Together with the biological evidence that these interactions could be useful targets for drug development, this area is likely to gain importance.

As discussed previously, each co-chaperone has a specific role in the HSP90 chaperone complex (Table 2). CDC37 is thought to be responsible for chaperoning only kinase client proteins and facilitates their loading onto the HSP90 chaperone complex (Roe et al. 2004). CDC37 is phosphorylated by casein kinase II (CKII) at Ser13 for activation (Miyata and Nishida 2005). Mutation of this site abolishes the ability of CDC37 to associate with HSP90 client proteins (Miyata and Nishida

2004). Interestingly, CDC37 is required to increase CKII activity. CDC37 and CKII are therefore thought to form a positive feedback system to regulate the activity of multiple kinases. As CDC37 is known to be upregulated in cancer (Stepanova et al. 2000) and can cause a transformed phenotype when overexpressed, targeting CKII-CDC37 could be a novel and efficient pharmacological approach (Miyata and Nishida 2004).

The co-chaperone P23 is involved in the later stages of the HSP90 co-chaperone cycle and has been implicated in endoplasmic reticulum (ER) stress-induced cell death (Rao et al. 2006). The ER is the location of protein biosynthesis and is highly sensitive to changes in its environment, such as accumulation of unfolded proteins, which results in ER stress and can trigger cell death (Schroder and Kaufman 2005). The HSP90 chaperone complex is thought to protect the cell against this ER stress-induced cell death, as the HSP90 co-chaperone P23 is cleaved close to the carboxy-terminus by caspase-3 and/or caspase-7 (Rao et al. 2006). Furthermore, inhibition of caspases 3 and/or 7 resulted in decreased cell death via this mechanism, whereas depletion of P23 by RNAi led to increased cell death, suggesting that P23 is a key modulator of ER stress-induced death. These results provide evidence that targeting P23 could be a potential therapeutic approach (Rao et al. 2006).

HOP is the adaptor protein which links the HSP70 and HSP90 complexes and has been shown to contain three distinct TPR domains (Carrigan et al. 2006). In yeast, the equivalent of HOP, known as Sti1, has been reported to play an important role in the sensitivity of cells to 17-AAG; cells lacking Sti1 have been shown to be hypersensitive to 17-AAG (Prodromou et al. 1999). There is now increasing interest in targeting the interaction between HSP90 and HOP (Richter et al. 2007). Small synthetic molecules which will selectively inhibit the interaction between HSP90 and HOP have been identified using Alpha screen technology (Richter et al. 2007). Hit compounds resulted in depletion of client proteins (eg ERBB2) in cancer cells, suggesting that these are potentially effective HSP90 inhibitors.

An alternative strategy to specifically inhibit the interaction between HSP90 and HOP is to shift the balance between the HSP90 and HSP70 chaperone complexes. Curcumin (1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is the major yellow pigment extracted from turmeric derived from the rhizome of the plant *Curcuma longa*. It is reported to cause the down regulation of HSP90 client proteins such as BCR-ABL (Wu et al. 2006) and ERBB2 (Hong et al. 1999). The depletion of these client proteins has been shown to occur only at the protein level (Wu et al. 2006), and is mediated via the ubiquitin-proteasome pathway (Jung et al. 2007). Curcumin causes a dissociation of BCR-ABL from the HSP90-P23 complexes present in the cell and increases the association of BCR-ABL with HSP90-HOP complexes, by an as yet unknown mechanism (Wu et al. 2006).

HSP90-receptor complexes typically contain one of the following co-chaperones: FK506-binding proteins 51 and 52 (FKBP51 and FKBP52 respectively), cyclophilin 40 (CYP40) and protein phosphatase 5 (PP5) (Allan et al. 2006). A common feature of these co-chaperones is the tetratricopeptide repeat (TPR) domain that forms the HSP90-binding site, linked to a peptidylprolyl isomerase (PPIase) /immunosuppressant drug-binding domain in CYP40 and the FKBPs or a protein phosphatase

domain in PP5. There is now increasing evidence that receptor function is critically dependent on which of the immunophilins is bound within the steroid receptor complexes (Ratajczak et al. 2003; Smith 2004; Riggs et al. 2004). FKBP51 and FKBP52 have been identified as potential drug targets as they have been implicated in playing a critical role in the activity of the glucocorticoid receptor (Allan et al. 2006). In a yeast model, Fkbp52 has been shown to dramatically potentiate glucocorticoid-dependent reporter gene activity through a mechanism that results in increased receptor hormone-binding affinity (Riggs et al. 2003). Co-expression of Fkbp51, however, blocked these potentiating effects of Fkbp52, which are required for the catalytic activity of Fkbp52 as well as a functional interaction of the immunophilin with HSP90 (Riggs et al. 2003). Receptor function, then, can be directly influenced by the prolyl isomerase activity of a TPR immunophilin. Therefore specific inhibitors of the assembly of TPR immunophilins into receptor complexes may be important therapeutically in hormone-dependent cancers such as breast and prostate.

Recently the co-chaperone AHA1 (Activator of HSP90 ATPase; Panaretou et al. 2002) was identified as another potential point of intervention in the HSP90 chaperone cycle. Upon binding to HSP90, AHA1 increases the ATPase activity and causes a conformational change in the catalytic loop of HSP90 by enabling the catalytically essential residues to interact with ATP (Meyer et al. 2004). AHA1 is reported to be important in the activation of both kinase client proteins such as C-RAF and AKT (Holmes et al. 2006; Richter et al. 2007) and also non-kinase client proteins, e.g. the glucocorticoid receptor (Harst et al. 2005). In addition, AHA1 has been reported to play a critical role in transporting mutated cystic fibrosis transmembrane conductance regulator (CFTR) to the cell membrane (Wang et al. 2006). Specifically, depletion of AHA1 is able to restore transport of CFTR and reverse the mutant phenotype. These data demonstrate how alteration of a single co-chaperone can have selective effects on certain HSP90 client proteins and suggest that the interaction between HSP90 and AHA1 could be targeted therapeutically.

HSP70 is an HSP90 co-chaperone which interacts with HSP90 via the adaptor protein HOP. Levels of HSP70 are reported to be elevated in many human tumour cells (Calderwood et al. 2006; Mosser and Morimoto 2004) and this co-chaperone is thought to play a role in tumourigenesis. Treatment with cytotoxic agents such as paclitaxel, doxorubicin and gemcitabine has also been shown to induce expression of HSP70 which reduces the sensitivity of cells to these agents (Jaattela et al. 1998; Kwak et al. 1998; Sliutz et al. 1996). HSP70 has anti-apoptotic capabilities, which include its ability to prevent the loss of mitochondrial membrane potential in response to apoptotic stimuli to, block the translation of pro-apoptotic factors to the mitochondria and to modulate several stages of the pro-apoptotic JNK pathway (Mosser and Morimoto 2004). As a result of these findings HSP70 has emerged as a novel drug target. There is currently one HSP70-interacting compound which is undergoing clinical trials as an immunosuppresent agent, 15-deoxyspergualine (15-DSG; Figure 11; Tanabe et al 2000). This agent has been found to interact with the C-terminal EEVD motif of the constitutively expressed isoform, HSC70 (Nadler et al. 1998) and to cause a mild increase in ATPase activity

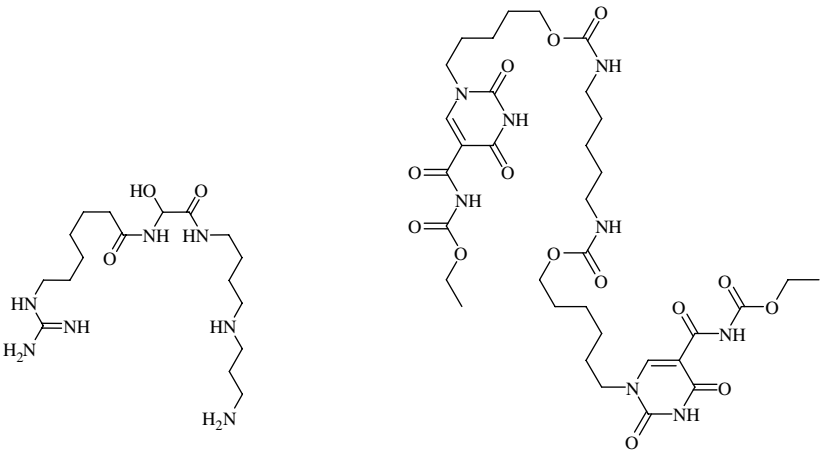


Figure 11. Chemical structures of HSP70 interacting agents

(Nadeau et al. 1994). However, the mechanism by which this occurs is currently unknown (Fewell et al. 2001).

An *in silico* screen of 15-DSG analogues identified NSC630668-R/1 (R/1; Figure 11) which inhibits both the endogenous and HSP40 stimulated ATPase activity of yeast Hsp70 (Fewell et al. 2001). R/1 is thought to act as a peptide mimetic by interacting with the peptide-binding domain of HSC70 which leads to its inactivation. Two R/1 structurally related compounds (MAL3-39 and MAL3-101) have been identified to interfere with the ability of HSP40 to enhance HSP70-mediated ATP hydrolsis but have no effect on the endogenous HSC70 ATPase activity (Fewell et al. 2004).

The ATPase activity of HSP70 can be modulated by 3'-sulfogalatolipids containing either a ceramide or a glycerolipid (Mamelak et al. 2001). These agents bind to the HSP70 ATPase domain and inhibit both the endogenous and stimulated ATPase activity of HSP70.

Peptide aptamers, which act as substrate mimics, have also been used as an alternative approach. One such molecule, ADD70, was designed based on the regions of the HSP70 substrate apoptosis-inducing factor (AIF) which are required for binding to HSP70 (Schmitt et al. 2006). This construct has been reported not to be cytotoxic, but does have anti-cancer activity which is proposed to occur via a cell-cell specific immune response mediated by CD8+ T-cells (Schmitt et al. 2006). ADD70 has also been shown to exhibit chemosensitising properties both *in vitro* and *in vivo* (Schmitt et al. 2006). Analysis of the interaction between ADD70 and HSP70 revealed selectivity for the inducible isoform of HSP72 and not the constitutive HSC70 (Schmitt et al. 2006). Selectivity of this nature could be a great asset to this agent as it is often the inducible form of HSP70 which is upregulated in cancer, whereas it is relatively undetectable in normal cells (Schmitt et al. 2006).

Therefore this approach may achieve cancer versus normal cell selectivity and offers promising therapeutic potential.

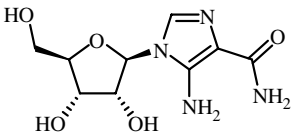
Interrupting Client Protein Binding

An alternative approach has been investigated whereby the activity of HSP90 is unaffected but client proteins are prevented from binding to the complex. A cell permeable peptidomimetic referred to as shepherdin was modelled on the structure of the binding interface between HSP90 and the antiapoptotic and mitotic regulator survivin (Plescia et al. 2005). This peptide is able to interact with residues in the ATP binding pocket of HSP90, which destabilises the client proteins such as survivin, AKT and CDK4 and induced massive cell death in tumour cells by apoptosis and non-apoptotic mechanisms (Plescia et al. 2005). Interestingly, the levels of HSP90 or HSP70 expression were not affected. Shepherdin has been shown to be selective for tumour cells over normal cells, with no effect on viability or colony formation in non-transformed cells (Plescia et al. 2005). Studies have shown that Shepherdin induced dramatic death of acute myeloid leukaemia cells *in vitro*. In studies with human tumour xenografts in mice this peptide was well tolerated with no toxicity and depletion of survivin and AKT was detected, together with inhibition of tumor growth (Gyurkocza et al. 2006).

Based on the chemical and conformation properties of shepherdin bound to the N-terminal domain of HSP90, a novel non-peptidic small-molecule inhibitor, 5-aminoimidazole-4-carboxamide-1-β-Dribofuranoside (AICAR; Figure 12) was designed using a structure and dynamics based computational approach (Meli et al. 2006). AICAR causes client protein degradation *in vivo* and exhibits antiproliferative and pro-apoptotic activity in a number of tumor cell lines, but not non-tumorigenic cells (Meli et al. 2006). This molecule represents a novel antagonist of HSP90 developed using an innovative approach.

Targeting HSF-1

HSF-1 is the master regulator of heat shock induced protein expression, a mechanism which evolved to protect cells from environmental and physiological stress (Young et al. 2004). Normally HSF-1 is found in the cytoplasm in complex with HSP90



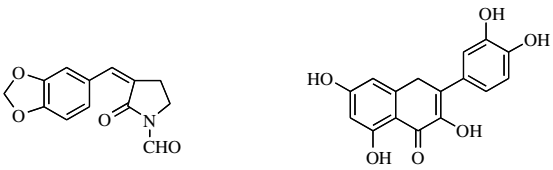
AICAR

Figure 12. Chemical structure of AICAR

(Westerheide and Morimoto 2005). When cells are stressed, HSF-1 trimerises, is phosphorylated at serine residues and translocates to the nucleus (Whitesell et al. 2003). It then binds to the heat shock element (HSE) regions in the promoters of *HSP70* and other heat shock genes, causing increased expression of HSP70 (Hahn et al. 2004). Knockdown of HSF-1 by siRNA has shown that depletion of this target alone has no significant effect on either cell proliferation or morphology of the cells, nor does it affect the sensitivity of cells to cisplatin or elevated temperatures (Rossi et al. 2006). However, depletion of HSF-1 in several cancer cell lines successfully attenuated the induction of anti-apoptotic heat shock proteins HSP72 and HSP27 when the cells were stressed. This led to a dramatic increase in sensitivity to hyperthermochemotherapy with a significant increase in apoptotic cell death (Rossi et al. 2006). Proteasome inhibitors have also been reported to induce the expression of heat shock proteins (Kim et al. 1999) due to the elevated intracellular concentrations of misfolded proteins, which cause HSF-1 to dissociate from the repressive complex with HSP90 (Westerheide and Morimoto 2005).

Two compounds have been identified which inhibit the heat shock response, namely the flavonoid quercetin (3,3',4',5,7-pentahydroxyflavone; Figure 13) and the benzylidene lactam KNK437 (Figure 13). The precise targets and mechanisms of action of these compounds have not been fully characterised. Quercetin has been shown to reduce HSF-1 DNA binding, hyperphopshorylation and HSP72 expression (Nagai et al. 1995; Yokota et al. 2000). KNK437 inhibits HSP70 induction and thermotolerance. When cells were treated with KNK437 in combination with 17-AAG, increased apoptosis and loss of clonogenic survival is observed (Guo et al. 2005a). Neither quercetin or KNK437, however, are potent enough for clinical use as HSF-1 modulators.

Two novel inhibitors of HSF-1, NZ28 and emunin, have recently been identified by a high-throughput screen. These agents inhibit the induction of heat shock response mediated through HSF-1 (Zaarur et al. 2006). However, the inhibition of HSF-1 activity was slight following treatment with either compound and it was therefore proposed that their predominant mechanism of action was at the post-transcriptional level (Zaarur et al. 2006). For a recent, detailed review of this area see Powers and Workman 2007.



KNK437 Quercetin

Figure 13. Chemical structures of HSF-1 inhibitors

COMBINATION OF HSP90 INHIBITORS WITH OTHER AGENTS

HSP90 and Cytotoxic Agents

Many cancers such as ovarian, breast and melanomas have an activated phosphatidylinositol-3-kinase (PI3K)/AKT pathway which has been implicated in tumour proliferation, survival and the development of resistance to current treatments (Vivanco and Sawyers 2002). AKT is a client protein of HSP90 and 17-AAG treatment results in inhibition of AKT activation and expression in tumours (Basso et al. 2002). It has been reported that inhibition of the PI3K/AKT pathway can sensitise cells with activated AKT to cytotoxic drugs (Bhattacharya et al. 2006). The HSP90 inhibitor 17-AAG has been shown to potentiate the effects of paxlitaxel in cells with high levels of activated AKT (Sain et al. 2006). The observed synergy appears to be schedule-dependent and occurs when the drugs are given concomitantly. Altering the sequence of exposure to the drug reduces the synergistic effects observed (Sain et al. 2006; Munster et al. 2001). The precise mechanism behind this synergistic interaction is currently still unclear.

Various pragmatic combinations of 17-AAG plus a range of cytotoxic agents are being evaluated pre-clinically and clinically. 17-AAG is currently in clinical trials in combination with docetaxel in hormone refractory prostate cancer (protocol ID MSKCC-03006), cytarabine in refractory leukaemia (protocol ID MAYO-MC0313), irintecan in advanced metastatic cancer (protocol ID MSKCC-IRB-05017) and cisplatin and gemcitabine in advanced metastatic cancer (protocol ID MAYO-MC0111). Initial results from clinical studies of the cisplatin, 17-AAG, gemcitabine combination indicated activity with this regimen, however, there were problems with toxicity (Haluska et al. 2004).

HSP90 and Proteasome Inhibitors

Inhibition of HSP90 by the current inhibitors such as 17-AAG and radicicol results in the recruitment of ubiquitin-ligases to the HSP90 chaperone complex and the degradation of the client proteins via the proteasome (Connell et al. 2001). There are agents available, such as bortezomib, which specifically inhibit this proteasomal degradation of proteins. Bortezomib has been approved for the treatment of multiple myeloma. The combination of these two classes of drugs was hypothesised to cause accumulation of unfolded, aggregated, ubiquitinated proteins in the cell which could result in increased cell death (Zaarur et al. 2006). Studies have demonstrated that the combination 17-AAG and bortezomib had a significantly greater effect on cell proliferation than either drug alone (Mimnaugh et al. 2004). Based on these findings clinical trials combining these two drugs were initiated. Dose escalation is currently ongoing in the phase I trials of this combination. Initial reports indicate that there have been no additive toxicity or pharmacokinetic interactions to date, and similar levels of 20S proteasome inhibition are being achieved both in the combination and in the bortezomib alone arm of the trial (Chanan-Khan et al. 2006).

HSP90 and Topoisomerase II Inhibitors

Topoisomerase II plays a crucial role in mitosis and meiosis during chromosome condensation and segregation and is a well established target for cancer therapy. Topoisomerase II has been reported to form a complex with HSP90; however when HSP90 activity is inhibited by either 17-AAG or radicicol there is an increase in topoisomerase II activity and no degradation of the protein (Barker et al. 2006a). Nevertheless, an increase in cell death is seen (Barker et al. 2006b). It is proposed that this is due to a novel mechanism in which the HSP90 inhibitor interrupts the HSP90-topoisomerase II complex resulting in the formation of an increased number of cleavable complexes, ultimately resulting in a rise in DNA damage and a subsequent increase in cell death. There are currently no clinical trials of this combination.

HSP90 and HDAC Inhibitors

Combination of the HDAC inhibitor LBH589 with 17-AAG *in vitro* has resulted in synergistic effects that are associated with mitochondrial damage, caspase activation and apoptosis in the cell line models tested, particularly in leukaemic cell lines (George et al. 2005). Investigations into the mechanism by which HDAC inhibitors affect HSP90 response suggest that the synergistic interaction observed could be due to more profound inhibition and disruption of the chaperone association of HSP90 with its client proteins resulting in increased polyubiquitylation and proteasomal degradation (George et al. 2005). It is, however, important to note that HDAC inhibitors like LAQ824 not only cause the hyperacetylation of HSP90 (see earlier) but also affect histones and other proteins such tubulin (Hubbert et al. 2002). Therefore these factors may play an important role in the increased effectiveness of combinations of HSP90 and HDAC inhibitors. In contrast, we have demonstrated an antagonistic interaction between 17-AAG and the HDAC inhibitor trichostatin A (TSA) in a human ovarian cancer cell line (Maloney et al. 2007). Another supporting study has shown that geldanamycin inhibits TSA-induced cell death and histone H4 hyperacetylation in COS-7 cells (Huang et al. 2002). These contradictory results may indicate that the functional link between HSP90, chromatin regulation and gene transcription could influence the outcome of such combinations and may be cell type dependent. Recent gene expression microarray and proteomic analysis of the effects of 17-AAG treatment in human ovarian cancer cells identified changes in the expression of several genes and proteins involved in chromatin modifications including acetylation and methylation. Proteins affected included the histone acetyltransferase HAT-1, the arginine methyltransferase PRMT5 and the heterochromatin protein HP1(Maloney et al. 2007). PRMT5 was reported to be a novel HSP90 binding partner and potential client protein (Maloney et al. 2007). In addition, the activity of a lysine-specific histone methyltransferase SMYD3 has also been reported to be increased by HSP90 (Hamamoto et al. 2004). Together these findings further strengthen the links between HSP90 and post-translational chromatin modifying enzymes and supports the rationale of targeting both classes of proteins in combination.

CLINICAL DEVELOPMENT

HSP90 inhibitors are thought to have considerable potential for cancer treatment. The first-in-class HSP90 inhibitor 17-AAG entered clinical trials in 1999 in patients with advanced metastatic cancer. The results from the trials have been summarised elsewhere (Pacey et al. 2006). In the phase I trials, 17-AAG gave a tolerable toxicity profile and the molecular signature of HSP90 inhibition was demonstrated in peripheral blood mononuclear cells and tumour biopsies (Banerji et al. 2005a; Solit et al. 2004; Erlichman et al. 2004; Grem et al. 2005). Stable disease in two patients with malignant melanoma was reported (Banerji et al. 2005a). Phase I trials carried out elsewhere also reported stable disease in renal, prostate and breast cancer (Solit et al. 2004). 17-AAG is now in phase II evaluation as a single agent, as well as in phase I clinical trials in combination with drugs such as docetaxel, paclitaxel, SAHA, bortezomib and imitanib (www.nci.nih.gov/clinicaltrials).

However, toxicities observed with 17-AAG include liver transaminitis, diarrhoea, nausea and vomiting. The cumbersome formulation containing large volume of DMSO may have contributed to some of these side-effects. Improved formulations of 17-AAG, such as the cremaphore-based KOS953 (developed by Kosan Pharmaceuticals) and another involving an oil-in-water emulsion (developed by Conforma Therapeutics) are being evaluated in the clinic. The more water soluble and orally bioavailable geldanamycin derivative, 17-DMAG has entered phase I clinical trials as an intravenous and oral drug in haematologic cancers and solid tumours/breast with trastuzumab.

The water soluble hydroquinone derivative of 17-AAG, IPI-504 (Infinity Pharmaceuticals), is in phase I clinical trials in multiple myeloma and gastrointestinal stromal cancers (Dimetri et al, ASCO abstract, 2007).

The first purine-scaffold compound from Conforma Therapeutics, CNF2024, has entered phase I clinical trials in solid tumours (Chiosis et al. 2006). It will be interesting to determine whether this non-quinone agent will be devoid of liver toxicity.

The antibody Mycograb (developed by NeuTec/Manchester University, UK) is currently in Phase II clinical trials for invasive candidiasis (Vastag 2006).

The novel small molecule HSP90 inhibitors of different structural classes, discussed earlier, are in preclinical development. One example is the pyrazoleisozole resorcinol series developed by our institution in collaboration with vernalis and now licensed to Novartis (McDonald et al 2006) Several other companies (e.g. Astex, Serenex, Biotica) have also developed HSP90 inhibitors. Many of these compounds are anticipated to enter clinical trials in the near future.

CHALLENGES AND FUTURE PERSPECTIVES

HSP90 is an exciting new target with both pre-clinical and clinical evidence indicating that inhibition could deliver potential patient benefit. HSP90 functions as part of a multi-chaperone complex that requires many co-chaperones for activity. HSP90 and the co-chaperones are regulated by ATP hydrolysis and post-translational modifications (Pearl and Prodromou 2006). The HSP90 chaperone

cycle is a complex drug target with multiple potential sites for intervention. Targeting the ATP binding site of HSP90 has already proved to be an effective strategy to disrupt the activity of this chaperone machine. This provides a broad spectrum approach in which multiple oncogenic proteins are simultaneously inhibited. The advantages of HSP90 family ATPase inhibitors are that they should provide a combinatorial effect on multiple oncogenic client proteins and pathways, as well as modulating all of the hallmark traits of cancer cells (Workman 2004). This is likely to mean that drug resistance is relatively difficult to develop. Despite the promise of this approach, there may be situations in which it would be preferable to specifically target a particular subgroup of HSP90 client proteins. This could be achieved by focusing on other aspects of the chaperone complex, including the targeting of individual co-chaperones such as HSP72, AHA1, P23, CDC37 or HOP. A more selective approach may limit side effects and potentially increase the therapeutic window available for HSP90 inhibitors.

One of the major mechanisms for therapeutic selectivity of HSP90 is the greater dependence on the HSP90 complex of mutated oncogenic client proteins compared to wild-type (da Rocha et al. 2005; Grbovic et al. 2006). This can lead to the exploitation of 'addiction' to particular oncogenes. In some tumours, combinatorial effects on multiple client proteins may be important. The selectivity for cancer cells over normal cells of HSP90 ATPase inhibitors in the stress response also needs to be considered. Cancer cells have been shown to be under great stress due to hypoxia and nutrient deficiency and are therefore thought to be more reliant on HSP90 than normal cells which are not in a stressed state (Whitesell and Lindquist 2005). Evidence for cancer cell selectivity would need to be obtained for alternative approaches to intervening in the chaperone cycle.

The full extent of the potential of the current HSP90 inhibitors will only become clear over the next five years. Concerns have been raised that inhibiting HSP90 may be detrimental to patients with some cancers. The ability of HSP90 to buffer mutations is well documented (Rutherford and Lindquist 1998). In late stage cancers which are carrying a higher mutational load than early stage cancer it has been suggested that HSP90 inhibitors may in fact in some cases accelerate tumour progression (Richter et al. 2007). Investigations of 17-AAG in a model of breast cancer metastasis to bone revealed that the HSP90 inhibitor 17-AAG enhanced the incidence of bone metastasis and osteolytic lesions *in vivo* (Price et al. 2005). On the other hand, a reduction in lymph node metastasis was seen in an prostate orthotopic model (Sanderson et al. 2006). However, the phase I and II clinical trials of 17-AAG have not shown any indication of enhanced metastasis, with no drug-related disease progression reported. Stable disease and some responses have been reported in melanoma, prostate, renal, multiple myeloma and breast cancers.

As more HSP90 inhibitors enter the clinic in the coming years it will be a very exciting time for this field. Drugging not only the HSP90 ATP binding site but also other features of the HSP90 chaperone complex will enable the full therapeutic potential of inhibiting the HSP90 chaperone complex to be revealed.

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CHAPTER 16

CDC37 AND PROTEIN KINASE FOLDING

ROBERT MATTS[1] AND AVROM J. CAPLAN[2,*]

[1]*Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK 74078, Oklahoma, USA*

[2]*Department of Pharmacology and Biological Chemistry, Mount Sinai School of Medicine, New York, NY 10029, USA*

Abstract: Cdc37 is a molecular chaperone that collaborates with Hsp90 to fold protein kinases and other clients including transcription factors. Cdc37 function in protein kinase folding is dependent on direct interaction between the chaperone and the N-lobe of the kinase catalytic domain. In addition, Cdc37 can inhibit the ATPase activity of Hsp90 that is thought to promote assembly of the kinase client with both chaperone proteins. Treatment of cells with the Hsp90 inhibitor, geldanamycin, inhibits assembly of Hsp90:kinase compelxes event although it does not promote disassembly of Hsp90:Cdc37 complexes. Cdc37 interaction with its kinase clients is dependent of phosphorylation at its N-terminus by casein kinase II. Cdc37 is highly expressed in cancer cells and tissues and can promote tumorigenesis when overexpressed. This is correlated with increased levels of Cdk4 and Cdk4:cyclin D complexes that promote cell cycle progression. The chapter focuses on structure function relationships between Cdc37, Hsp90 and their kinase clients. The role of Cdc37 in promoting tumorigenesis is also discussed.

Keywords: Molecular chaperone, protein kinase, Cdc37, Hsp90, geldanamycin

INTRODUCTION

Cancer cells are characterized by unregulated cell division that is promoted by protein kinases and other signaling molecules. Protein kinases in particular are seen as promising targets of chemotherapy with more than 40 different compounds in development or in clinical trials. Each of these compounds is targeted to a specific kinase, although it is common for more than one kinase to be inhibited due to the high sequence similarity between different kinase family members. By

*Corresponding author: Department of Pharmacology and Biological Chemistry, Box 1603, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, Tel: 212-241 6563, Fax: 212-996 7214, e-mail: avrom.caplan@mssm.edu

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contrast, drugs used to inhibit the molecular chaperone Hsp90 are currently in clinical trials due to their ability to promote degradation of many kinases (Whitesell and Lindquist, 2005) (Also See in this volume: *Neckers*, Chapter 12, *Whitesell*, Chapter 13; *Kamal et al*, Chapter 14, *Holmes*, Chapter 16). This is based on the need for protein kinases to be folded in association with Hsp90 and Cdc37, a relatively kinase-specific molecular chaperone (MacLean and Picard, 2003; Pearl, 2005). In this chapter, we will review the current studies of Cdc37 in the folding of kinase and some non-kinase clients, and address how its function may differ in cancer cells from healthy tissues.

The connection between Cdc37 and the cell cycle was first described when a mutant yeast strain was found to contain a cell division cycle phenotype. This mutant, termed Cdc37 was found to arrest in the G1 stage of the cell cycle and to have genetic interactions with the Cdc28 protein kinase, which controls cell cycle progression (Reed, 1980a, b). The function of Cdc37 as a molecular chaperone and co-chaperone of Hsp90 was demonstrated more than ten years after this initial characterization. The kinase-specific nature of Cdc37 was initially observed in experiments that studied the assembly of nuclear receptors and tyrosine kinases in rabbit reticulocyte lysates (Stancato et al., 1993). In these studies, Cdc37 (or p50 as it was also called), was found to associate with a kinase but not with glucocorticoid receptors. This view of Cdc37 as a kinase-specific chaperone pervades the literature to this day, but it is also not correct. Androgen receptor, a close relative of the glucocorticoid receptor, interacts with Cdc37 and requires function Cdc37 for proper signaling (Fliss et al., 1997a; Rao et al., 2001). Other non-kinase proteins that interact with Cdc37 include a viral reverse transcriptase, the transcription factor MyoD and nitric oxide synthase (Harris et al., 2006; Yun and Matts, 2005).

The extent to which Cdc37 interacts with the protein kinome has been established from yeast genetic studies and also from insights into the structural basis for Cdc37 interactions with protein kinases. Genetic studies examined how mutation in Cdc37 affected 65 different yeast kinases, accounting for over 50% of the yeast kinome (Mandal et al, submitted). In these studies, steady state analysis of kinase levels in strains expressing a mutant form of Cdc37 was measured by Western blotting. The results demonstrated that over 75% of these kinases were present at reduced levels due to rapid degradation by the proteasome shortly after synthesis. These results show that Cdc37 functions to protect most newly made protein kinases from degradation shortly after synthesis. This function is consistent with the finding that Cdc37 protects unfolded ß-galactosidase from misfolding and maintaining it in a folding-competent conformation (Kimura et al., 1997a). As described below in more detail, Cdc37 also functions to promote kinase folding post-translationally in association with Hsp90. In addition to this proteomic approach described above, several studies have concluded that Cdc37 interacts with highly conserved sequence motifs present on most protein kinases, suggesting that the chaperone has a general role in kinome biogenesis. This will be described in more detail below, but together, these approaches suggest that Cdc37 has a general role in biogenesis of the eukaryotic protein kinome.

CDC37 STRUCTURE AND FUNCTION

Hsp90 and Cdc37 function as partners that facilitate the folding of their cognate clients. Cdc37 is made up of three domains (Shao et al., 2003a) (Figure 1). Proteolytic fingerprinting of purified recombinant Cdc37 with trypsin suggested that human Cdc37 is composed of an N-terminal domain that spans amino acids 1-127, a middle domain that spans amino acids 128-282, and a C-terminal domain spanning amino acids 283-378. Another study carried out with the non-specific protease, Subtilisin, indicated that Cdc37s central domain spans amino acid residues 147-276 (Zhang et al., 2004). Both studies demonstrated that Cdc37' central domain was highly resistant to proteolysis, indicating that this domain is stable and highly structured (Shao et al., 2003a; Zhang et al., 2004). Additional evidence suggest that the region that spans residues 128 to 147 may represent a "subdomain" of Cdc37, as its presence or absence in deletion constructs of Cdc37 alter both the kinase-binding and Hsp90-binding properties of these mutant constructs (Shao et al., 2003a), to be discussed in more detail below).

Several studies (Grammatikakis et al., 1999; Lee et al., 2002; Scholz et al., 2000; Shao et al., 2001; Shao et al., 2003a; Shao et al., 2003b) have demonstrated that the N-terminal domain of Cdc37 binds client (e.g., kinase), while its middle domain binds Hsp90. The function of Cdc37s C-terminal domain is unknown. Genetic studies suggest that only the activity of Cdc37s kinase-binding domain is required for viability of yeast (Lee et al., 2002). However, while viable, these yeast strains grow slowly at 30°C and are temperature sensitive for growth at 37°C

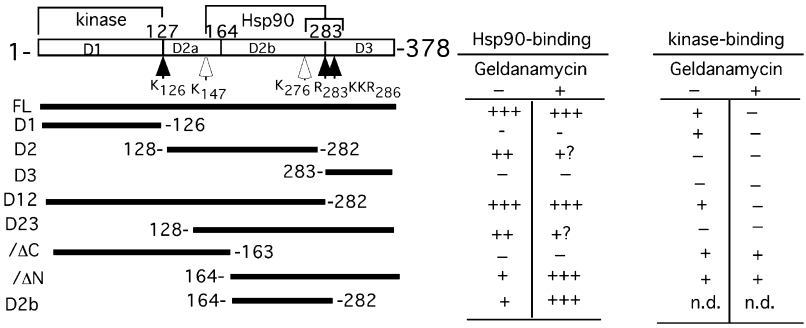

Figure 1. **Domain structure of Cdc37 and binding properties of Cdc37 deletion mutant constructs.** The upper left hand corner of the figure a schematic of the domain structure of Cdc37 as described in Shao et al (Shao et al., 2003a). The closed and open arrows indicate sites that are readily cleaved by trypsin (Shao et al., 2003a) and by Subtilisin (Zhang et al., 2004), respectively, which have been used to map the junctions between the N-terminal kinase-binding domain (kinase) the the middle Hsp90-binding domain (Hsp90). Under the domain diagram is a summary of the Cdc37 deletion constructs used to map the kinase binding and Hsp90 binding activity of the domains, as described in Shao et al (Shao et al., 2003a). The table to the right summarizes the Hsp90 and kinase binding activity of the domains in the presence or absence of geldanamycin. For Hsp90: +++, wild type binding; ++, binding 50-75% wild type levels; +, binding reduced by more than 66%; +?; binding reduced in some, but not all experiments; -, no binding. For kinase: + binding; -; no binding; n.d., not determined.

(Lee et al., 2002). Inclusion of Cdc37's central domain restores wild type growth at all temperatures. Thus, in yeast the C-terminal domain appears to be completely dispensable for Cdc37 function.

Homologues of Cdc37 are present in the genomes of protista, fungi, and animals, but are absent in genomes of eubacteria and archibactera. No Cdc37 homologue has been identified in genomes of plants, suggesting that Cdc37 may have arose after plants diverged from the evolutionary precursor to fungi and animals. Cdc37's first 40 amino acids are the most highly conserved residues in the protein from yeast to human, suggesting strong evolutionary pressure to conserve amino acid residues critical for Cdc37's kinase binding function (Bandhakavi et al., 2003; Fliss et al., 1997b; Shao et al., 2003b). Genetic and biochemical studies have demonstrated that several conserved residues at the N-terminus of Cdc37 and phosphorylation of a conserved Ser by casein kinase II (S13 in vertebrate Cdc37, Ser14 and Ser17 in yeast) are critical for Cdc37's high-affinity kinase binding (Bandhakavi et al., 2003; Miyata and Nishida, 2004; Shao et al., 2003a; Shao et al., 2003b). However, the biochemical and structural basis for the recognition of protein kinases by Hsp90 and Cdc37 remains to be fully elucidated.

It is also noteworthy that a mutation in the third domain of Drosophila Cdc37 (W338C), impairs signaling via the sevenless receptor tyrosine kinase and results in lethality at the pupal stage (Cutforth and Rubin, 1994). Thus, in multi-cellular organisms the C-terminal domain appears to have an uncharacterized essential function, at least at some critical stage of development. Sequence alignments of representative Cdc37s from C. elegans to human, indicate that within a shared core of 60 amino acids within the C-terminal domain, 20 residues are invariant and 17 residues represent conservative substitutions. The question as to the nature of the function of this domain is thus intriguing.

THE ATPASE CYCLE OF HSP90

Hsp90's reaction cycle involves its switching between different conformations that are enforced by the binding and hydrolysis of ATP (reviewed in (Pearl and Prodromou, 2002; Pearl and Prodromou, 2006; Picard, 2002; Pratt and Toft, 2003) & (Hartson et al., 1999; Shao et al., 2003a; Soti et al., 2001; Yun et al., 2004)) (also See: *Pratt et al*, Chapter 1). Hsp90 contains two nucleotide binding sites: a ATP-binding Bergerat fold within its N-terminal domain (Prodromou et al., 1997; Stebbins et al., 1997), and a nucleotide binding site in its C-terminal domain (Garnier et al., 2002; Marcu et al., 2000; Yun et al., 2004). Hsp90's C-terminal domain is also responsible for Hsp90's stable dimerization (Yamada et al., 2003), and contains sequences that regulate ATP hydrolysis by Hsp90's N-terminal domain (Chadli et al., 2000; Owen et al., 2002; Richter et al., 2001; Wegele et al., 2003). Hsp90's N-terminal, central and C-terminal domains have all been implicated in the binding of protein clients (Meyer et al., 2003; Scheibel et al., 1998; Yamada et al., 2003).

The current model for Hsp's reaction cycle is based primarily from results from studies on the Hsp90-dependent reconstitution of SHR function Currently Hsp90 is

proposed to function through a molecular "clamp-like" mechanism (reviewed in in (Pearl and Prodromou, 2002; Pearl and Prodromou, 2006; Picard, 2002; Pratt and Toft, 2003)), which is now supported by the recently published structures of dimeric Hsp90 co-crystallized with p23 (Ali et al., 2006) and the structure of the E. coli homologue of Hsp90, HtpG (Shiau et al., 2006). "Early" chaperone complexes are formed upon the binding of SHR to Hsp70 in a ATP-dependent reaction that requires client interaction with Hsp40. "Intermediate" complexes are then formed through the binding of HOP [Hsp70/Hsp90-organizing protein], which functions to tether "early" client/chaperone complexes to Hsp90 to form a single complex (Johnson et al., 1998). The N-terminal domains in the Hsp90 dimer associate upon binding ATP, effectively capturing the bound client, and positioning catalytic residues present in Hsp90's middle domain proximal to the γ-phosphate of the bound ATP (Meyer et al., 2003; Meyer et al., 2004; Wegele et al., 2003). Thus, N-terminal clamping enhances Hsp90's ATPase activity (Grenert et al., 1999; Prodromou et al., 2000; Richter et al., 2001; Wegele et al., 2003; Weikl et al., 2000), as does the presence of client substrate (McLaughlin et al., 2002). ATP-hydrolysis is ultimately required for the release of substrate, as nonhydrolyzable ATP analogs inhibit this process (Young and Hartl, 2000), as does the binding of molybdate. Hsp90's C-terminal nucleotide-binding site is opened upon "clamping" of its N-terminal domains (Soti et al., 2001). "Late" Hsp90/client complexes are formed upon the dissociation of Hsp70 and HOP and the binding of p23 (Felts and Toft, 2003), and other Hsp90-associated proteins (e.g., the tetratricopeptide-repeat (TPR) domain-containing co-chaperones, protein phosphatase 5, and the high Mr immunophillins, FKBPs and cyclophilin-40s (Riggs et al., 2004)). Binding of ATP to Hsp90's C-terminal site destabilizes the binding of nucleotide to the N-terminal domain (Soti et al., 2001), and appears to stimulate the release of bound client (Yun et al., 2004). Hsp90 subsequently relaxes to its default conformation. However, a recent report suggests that binding of novobiocin to the C-terminal nucleotide binding site causes the C-terminal domains of the Hsp90 dimer to dissociate (Allan et al., 2006), which supports a potential "DNA-gyrase" type reaction mechanism for client release.

The Hsp90-specific inhibitors geldanamycin and radicicol bind to Hsp90's N-terminal ATP binding site, preventing the binding of ATP, N-terminal domain "clamping", and the binding of p23, resulting in the accumulation of "intermediate" complexes (Johnson and Toft, 1995; Schulte et al., 1999; Smith et al., 1995; Sullivan et al., 1997). Novobiocin and its derivatives bind to Hsp90's C-terminal nucleotide binding site, and inhibit Hsp90 function by promoting Hsp90 to adopt a conformation from which client proteins are released (Allan et al., 2006; Marcu et al., 2000; Yun et al., 2004). Molybdate, which is postulated to act as a mimic of the γ-phosphate of ATP, also inhibits Hsp90 function (Hartson et al., 1999; Johnson and Toft, 1995; Sullivan et al., 2002). The binding of molybdate to Hsp90 "freezes" Hsp90 in its high-affinity client binding state, resulting in the formation of Hsp90-client complexes that are stable in buffer containing high salt concentrations (Hartson et al., 2000; Hartson et al., 1999; Prince and Matts, 2004; Scroggins et al., 2003).

REGULATION OF HSP90'S ATPASE CYCLE

Hsp90-associated co-chaperones modulate the ATPase cycle of Hsp90. The association of HOP with Hsp90 inhibits the ATP binding (reviewed in in (Pearl and Prodromou, 2002; Pearl and Prodromou, 2006; Picard, 2002; Pratt and Toft, 2003) & (Hernandez et al., 2002; Prodromou et al., 1999; Siligardi et al., 2004)). The presence of Hsp70/Hsp40/client complexes reverses this inhibition, with HOP facilitating the ATP-dependent assembly of Hsp90-HOP-Hsp70 complexes and the transfer of bound client from Hsp70 to Hsp90 ("intermediate" Hsp90-client complexes) (Hernandez et al., 2002; Kanelakis et al., 2002; Morishima et al., 2000). During this "loading-phase" HOP suppresses the hydrolysis of ATP by Hsp90. The presence of bound client induces a conformational transition in Hsp90 that drives N-terminal dimerization, traps the nucleotide and commits Hsp90 to ATP hydrolysis (McLaughlin et al., 2002).

Like HOP, Cdc37 has been reported to suppress Hsp90's ATPase activity (Roe et al., 2004; Siligardi et al., 2002), and animal Cdc37 has been proposed to act in a manner similar to HOP during the "loading phase" during which kinase initially interacts with the chaperone complex. However, unlike HOP, Cdc37 is found in both naïve Hsp90/Cdc37 complexes lacking client and late complexes formed between Hsp90 and protein kinases (Hartson et al., 2000; Shao et al., 2001; Shao et al., 2003a; Shao et al., 2003b). Thus, it is unclear at this time whether naïve Hsp90/Cdc37 complexes represent "early" complexes formed prior to loading of the Hsp90/Cdc37 chaperone machine with client kinase, or a complex formed after the release of kinase client at the end of Hsp90/Cdc37's reaction cycle. This uncertainty arises from the observations that Hsp90 forms complexes with client kinase that lack Cdc37 in the presence of the Hsp90-specific inhibitor geldanamycin. However, geldanamycin has no effect on the stability of Hsp90/Cdc37 complexes in the absence of client (Shao et al., 2003a).

Recently, Hsp90-dependent activation of purified recombinant Chk1 kinase *in vitro* was demonstrated to minimally require Hsp40, Hsp70, Hsp90 and Cdc37 phosphorylated on S13 by casein kinase 2 (Arlander et al., 2005). However, while Hop was not detected to be stably associated with complexes formed between Hsp90 and Chk1, HOP markedly stimulated the efficiency of Chk1 activation when added to the minimal system (Arlander et al., 2005). The notion that HOP may play a role in Hsp90/Cdc37 dependent kinase maturation is supported by the observation that Sti1 (the yeast HOP homologue) interacts with Cdc37 in yeast (Abbas-Terki et al., 2002) and over-expression Cdc37 in suppresses defects in cell growth and kinase maturation in yeast strains carrying a delection Sti1 (Lee et al., 2002). Furthermore, neither Hsp90 nor Cdc37 maintained a stable interaction with the protein kinase Ste11 in yeast deleted for Sti1 (Lee et al., 2004). However, overexpression of Cdc37 suppressed this defect and led to stable complex formation between Hsp90 and Ste11 even in the absence of Sti1. These findings show some functional overlap in Sti1 and Cdc37 function in helping to load kinase clients onto Hsp90. Since Sti1 and Cdc37 have a physical interaction even in the absence of Hsp90 they probably act together for kinase loading in a complex that includes Hsp70, at least in yeast.

The role of p23 kinase biogenesis is less certain. Although, p23 was not required to reconstitute Chk1 activity in vitro (Arlander et al., 2005), p23 has been found to be present in Hsp90/Cdc37 complexes form between the Hsp90-dependent kinases Lck (the Src-family lymphoid cell kinase) and HRI (the heme-regulated eIF2α kinase) in reticulocyte lysate (Hartson et al., 2000; Scroggins et al., 2003; Xu et al., 1997; Yun et al., 2004). In yeast, p23 (Sba1) was not required for the activity of Ste11 or v-Src kinases, although it did play a role in the biogenesis of heterologously expressed PKR (Donze et al., 2001) and in the response to amino acid starvation mediated by Gcn2 (Donze and Picard, 1999).

INTERACTION OF CSP37 WITH HSP90

The crystal structure of Cdc37's middle and C-terminal domain bound to Hsp90's N-terminal domain (Roe et al., 2004), together with the recently solved crystal structure of a dimer of full length Hsp90 in a complex with p23 in which its N-terminal domains of the Hsp90 are "clamped" (Ali et al., 2006), confirms the mechanism by which Cdc37 inhibits Hsp90's ATPase activity (Siligardi et al., 2002; Zhang et al., 2004). The binding of Cdc37 to Hsp90's N-terminal domain blocks access of catalytic residues in Hsp90's middle domain (Arg380) to ATP's γ-phosphate (Roe et al., 2004). In addition, Cdc37 also inserts the side chain of Arg167 into Hsp90's ATP binding pocket, which forms an ion pair with a Glu residue that is involved in catalysis of ATP hydrolysis (Meyer et al., 2003; Meyer et al., 2004; Roe et al., 2004). There is also a considerable change in the conformation of the peptide backbone that extends the α-helix from residues 101–109 by approximately two turns to its N-terminus (93–109), and the α-helix from 86–92 by one turn to its C-terminus (Roe et al., 2004). These changes induced by Cdc37 binding result in the opening of the lid of the ATP binding pocket causing the side chain of a Lys98 (which interacts with the β-phosphate of bound nucleotide in Hsp90's closed conformation) to flip 12 Å away and form an ion pair with a Glu residue in the first strand of β-sheet in Hsp90's N-terminal domain. In this conformation, the nucleotide binding site in the Hsp90/Cdc37 remains fully accessible to nucleotide or the binding of inhibitors (e.g., geldanamycin).

Of major interest is that Cdc37 interacts directly with Q119 and F120 within the invariant G-Q-F-G-V-G sequence that makes up the P-loop that interacts with the γ-phosphate of ATP (Ali et al., 2006; Roe et al., 2004). It is noteworthy that Minami and coworkers identified a consensus binding sequence for their N-terminally truncated Cdc37 mutant of G-X-F-G, with Q being in the X position in several of the peptides identified (Terasawa et al., 2006b). These results raise the question of whether Minami and co-workers simply identified the major Hsp90 interaction site for Cdc37, or whether its P-loop binding specificity also represents a potential binding site for the G-box (P-loop) of certain protein kinases.

The crystal structures of the Hsp90/Cdc37 and full length Hsp90 dimer with bound p23 indicates that the Hsp90/Cdc37 structure is incompatible with "clamping" of Hsp90's N-terminal domain, such that Cdc37 must clearly be ejected from

its site of interaction for clamping of Hsp90's N-terminus to occur (Ali et al., 2006; Roe et al., 2004). In addition, the "lid" region of Hsp90, which contains all the major amino acid residues with which Cdc37 interacts (Roe et al., 2004), swings nearly 180° upon lid closure, resulting in ATP's γ-phosphate becoming cradled in the P-loop at the C-terminal hinge of the lid (Ali et al., 2006). This conformational change buries Cdc37's binding site within the interface between the Hsp90's N-terminal and middle domains (Ali et al., 2006). Of interest is the question of whether dissociation of Cdc37 is coupled in some manner to the repositioning of Hsp90's lid and P-loop residues.

Additional studies indicate that Cdc37 is in equilibrium between a monomeric and dimeric form. Two studies have reported the Kd for this equilibrium to be around ~ 5–10 μM (Roe et al., 2004) and 80 μM (Zhang et al., 2004), respectively. Furthermore, while one study reported that the C-terminal Hsp90 binding domain of Cdc37 retains its ability to dimerize, albeit with a much lower affinity (Kd ~ 170 μM, (Siligardi et al., 2002)), another study reported that the C-terminal domain of Cdc37 was monomeric (Zhang et al., 2004). The major differences between these two studies is likely due to the Cdc37 constructs utilized in the studies: the construct that dimerized with high affinity contained residues 138–378 of Cdc37, while the other study utilized the central Cdc37 domain comprised of residues 147–276 (Siligardi et al., 2002; Zhang et al., 2004). However, the two studies are in agreement that two molecules of Cdc37 (or their Hsp90-binding domain construct of Cdc37) bind per Hsp90 dimer.

The binding constant of full length Cdc37 for Hsp90 has been reported to be between 1.46 to 3.8 μM (Roe et al., 2004; Zhang et al., 2004). The Cdc37/138–378 construct was observed to bind to the N-terminal nucleotide binding domain of Hsp90 (Nsp90NT: residues 1–222) and full length Hsp90 with similar affinities (Kd = 1.80 and 1.46 μM, respectively, (Roe et al., 2004)). Similarly, the Cdc37/147– 276 bound both full length Hsp90 and Hsp90NT (Zhang et al., 2004). However, it is noteworthy that full length Cdc37 cannot bind Hsp90NT, and that the minimal Hsp90 construct that binds full length Cdc37 is the Hsp90NT plus Hsp90's charged linker region (residues 1–261) (Zhang et al., 2004). Coincidentally, the charged linker region of Hsp90 evolved with the advent cytoplasmic forms of eukaryotic Hsp90, and Cdc37 appears to have evolved with the emergence of eukaryotic cells.

KINASE RECOGNITION BY HSP90 AND CDC37

Using sequence alignments and the crystal structures of protein kinases as a guide, regions have been defined within the catalytic domains of HRI (Scroggins et al., 2003), Lck (Prince and Matts, 2004; Scroggins et al., 2003), and Cdk2 (Prince et al., 2005) that must be present for the binding by Hsp90 and Cdc37, and the minimal constructs of the catalytic domains of Lck and Cdk2 that are required to trigger ATP-dependent high affinity binding of Hs90. Hsp90 was found to interact motifs contained within both the N-terminal (NL) and C-terminal (CL) lobes of the catalytic domain of kinases, while Cdc37 appears to only recognize kinase features

present in the NL of kinases (Prince and Matts, 2005; Prince et al., 2005; Scroggins et al., 2003). However, only constructs of the catalytic domain containing a complete N- or C-terminal lobe and part of the adjacent lobe bound to Hsp90 and Cdc37 in salt-stable complexes independent of molybdate (e.g., triggered Hsp90 nucleotide-dependent conformation switching) (Prince and Matts, 2004; Prince et al., 2005). Furthermore, the results suggest that the portion of the second domain that is required to trigger conformational switching represent a stabilized section of super-secondary structure, as deletion of residues that immediately follow the αE helix of Lck and Cdk2, which appear to cap and stabilize the helix, fail to trigger conformational switching of Hsp90 (Prince and Matts, 2004; Prince et al., 2005). Deletion of the G-box or the first three-strands of β-sheet in Lck had no effect on its binding to Hsp90 and Cdc37, while deletion of the αC-helix resulted in both the loss in the ability of the kinase construct to induce Hsp90 conformational switching and Cdc37 binding (Prince and Matts, 2004). However, in agreement with similar studies carried out with Cdk4, the G-box of Cdk2 was required for Cdc37 binding (Prince et al., 2005). The studies carried out with Cdk4 kinase also corroborate the importance of the αC-helix and the loop that connects it to the fourth strand of β-sheet in the kinase NL for kinase recognition by Hsp90/Cdc37 (Zhao et al., 2004b). Thus, while it is clear that the G-box is required for Cdc37 recognition of certain kinases, the work with the Lck kinase indicates that the G-box is not universally required for Cdc37 binding.

Additionally, other evidence also suggest that the loop connecting the αC-helix to the β4-strand of the NL is one of the primary determinants for recognition of kinases by Hsp90 (Citri et al., 2006; Xu et al., 2005). This hypothesis is based on results from experiments with ErbB1 and ErbB2 chimeras containing the loop region of the other homologue (Xu et al., 2005). The results suggest that the hydrophilic, negatively charged patch formed by ErbB1 loop region stabilizes the kinase resulting in its Hsp90-independent function after its initial Hsp90-dependent maturation, while the hydrophobic nature of ErbB2's loop destabilizes its structure requiring its constitutive interaction with Hsp90 and Cdc37 to maintain its stability and function after its maturation (Xu et al., 2005). Sequence analysis of loop regions present in Hsp90-dependent and independent protein kinases by Yarden and co-workers appear to support the hypothesis that the surface electrostatics determined by the hydrophilic/hydrophobic nature of the loop impacts on the binding of Hsp90/Cdc37 to kinases (Citri et al., 2006). These results raise the additional issue of whether Hsp90/Cdc37 "recognize" nascent kinases and mature, but unstable kinases in the same manner, as both ErbB1 and ErbB2 require Hsp90 for their initial folding (Sakagami et al., 1999; Xu et al., 2001; Xu et al., 2002).

The question of what is "recognized" by Cdc37 and Hsp90 has been further explored in utilizing the Hsp90-independent c-Jun N-terminal kinases (JNK) (Prince and Matts, 2005). To investigate the question of why some protein kinases are Hsp90- and Cdc37-dependent for their function, while others are not, the structural motifs within JNKs that confer or defer Hsp90 and Cdc37 interaction were examined (Prince and Matts, 2005). Studies on the Cdk2 kinase indicated that

an antibody directed against sequences in the C-terminus of its αC-helix blocked Hsp90 and Cdc37 binding, suggesting that it may represent a recognition motif for Hsp90/Cdc37 binding or the binding of the antibody to the sequence destabilized the structural motif with which Hsp90 and Cdc37 interact (Prince et al., 2005). However, contrary to expectations only Hsp90 bound JNK when its C-terminal non-catalytic structural motif was deleted, a deletion that would cause exposure of the αC-helix and the loop that connects it to the β4-strand on the surface of the kinase (Prince and Matts, 2005). Furthermore, both Hsp90 and Cdc37 recognized structural features that were exposed or destabilized upon deletion of JNK1's N-terminal non-catalytic structural motif, which would expose the surface of the NL's five stranded β-sheet (Prince and Matts, 2005).

Low resolution cryo-electron microscopy images of Hsp90 and Cdc37 support the hypothesis that Hsp90 interacts with elements present in both the NL and CL of kinase catalytic domains and that Cdc37 N-terminal domain recognizes features present in the NLs of kinases (Vaughan et al., 2006). The NL and CL of Cdk4 appears to interact with the N-terminal and middle domains, respectively, of one of the Hsp90 subunits within the Hsp90 dimer. The middle domain of the single Cdc37 molecule present in the complex appears to interact with the N-terminal domain of the second Hsp90 subunit, and it is positioned between the two N-terminal domains of the Hsp90 dimer, such that the two domains are not in direct contact. The middle domain of Cdc37 is thus positioned to be adjacent to the NL of the kinase bound to N-terminal domain of the opposing subunit in the Hsp90 dimer. An "extra" area of electron density that is postulated to be the N-terminal domain of Cdc37 is position over the NL of the bound Cdk4, and no interaction of Cdc37 with the CL is apparent (Vaughan et al., 2006). The complex was isolated from Sf9 cells in the presence of molybdate to stabilize the complex during purification. The question arises as to what stage in the Hsp90/Cdc37/kinase reaction cycle does this complex represent.

The work cited above has led to a model in which Cdc37 interacts solely with structural features present in the N-terminal portion of the catalytic domain of kinases (Prince and Matts, 2004, 2005; Prince et al., 2005; Scroggins et al., 2003; Zhao et al., 2004b). This model is based on observations that the N-terminal lobe (NL) of kinases is both necessary and sufficient for Cdc37 to be stably associated with Hsp90-NL complexes, and no Cdc37 was found to be associated with complexes form between Hsp90 and the C-terminal lobe (CL) of the catalytic domain of kinases. However, Minami and co-workers have recently reported reported that N-terminal domain of Cdc37 has kinase binding activity that interacts with the CL of Raf-1 (Terasawa et al., 2006a). Furthermore, they have reported that an N-terminally truncated mutant of Cdc37 containing residues 181-378 binds kinase (Terasawa et al., 2006b), via recognition of the G-box (P-loop) motif present in the N-lobe (NL) of the catalytic domains of protein kinases (Terasawa and Minami, 2005). While Shao et al., have observed that a mutant construct of Cdc37 containing residues 164-378 binds kinase (Cdc37/ΔN, (Shao et al., 2003a)), the construct also bound Hsp90, leading them to conclude that the kinase-binding activity of the Cdc37/ΔN mutant was most likely mediated indirectly through its

ability to bind Hsp90. In addition, Cdc37 constructs containing residues 128-378 and 128-282 (the central Hsp90 binding domain of Cdc37) were observed to bind Hsp90, but not kinase (Shao et al., 2003a). The basis for discrepancies between the conclusions drawn from these pieces of work, which have utilized different Cdc37 constructs and protein kinases, requires additional investigation.

STRUCTURAL ANALYSIS OF PROTEIN KINASE CONFORMATIONAL CHANGES ASSOCIATED WITH TRANSITIONS IN ACTIVATION STATE

The discussion above indicates that it is unclear at this time whether the loop region that follows the αC-helix of kinases represents a Hsp90/Cdc37 recognition motif, or whether it acts with varying efficiencies to stabilize kinase structures, which if not stabilized leads to constitutive or conditional interaction of the kinase with Hsp90. Changes in Hsp90/Cdc37 binding to mutant kinases have been observed to occur when the mutation does not alter the primary amino acid sequence of the catalytic domain. For example, wild type Lck becomes Hsp90-independent after its initial folding, and geldanamycin does not accelerate its turnover (Bijlmakers and Marsh, 2000; Hartson et al., 1996; Hartson et al., 1998; Yorgin et al., 2000). On the other hand, Lck that carries a mutation that changes the regulatory tyrosine residue in its C-terminal tail (Y505) to phenylalanine, is dependent upon Hsp90/Cdc37 to maintain its function after its initial maturation, and geldanamycin destabilizes Lck/Y505F and accelerates its turnover (Bijlmakers and Marsh, 2000; Hartson et al., 1998). The mutation of the regulatory 499 tyrosine in the related kinase Hck has a similar effect on its stability (Scholz et al., 2000; Scholz et al., 2001).

The crystal structures of Hck (Sicheri et al., 1997) and c-Src (Williams et al., 1997) indicate that their phosphorylated C-terminal tails bind to phosphotyrosine binding sites in their SH2 domains. This interaction fixes the orientation of the NL and CL of the kinases through "lynch pinning" the C-terminus of the kinase to its N-terminal SH2 domain, causing the linker region between the SH2 domain and the catalytic domain to form stabilizing interactions with its SH3 domain and NL of the kinase (Sicheri et al., 1997; Williams et al., 1997). Y to F mutations in the C-terminal regulatory tail Src-family kinases cause the kinases to become constitutively active and transforming, but unstable. However, it should be noted that addition of the high affinity Src-family kinase inhibitor PP2 (Kd = 5 nM) stabilizes Lck/Y505F in cells treated with geldanamycin (Giannini and Bijlmakers, 2004). Thus, thermodynamic stability of the catalytic domain of protein kinases, rather than any primary sequence motif per se, may be what determines the Hsp90/Cdc37 dependence of a protein kinase.

Structural analyses carried out by Williams *et al* of conformational changes that occur when protein kinases transition between their inactive and active conformations give additional insights into what principles may govern the Hsp90/Cdc37 dependence of protein kinases (Williams et al., 1997; Williams et al., 1998). Other than changes in the conformation of the activation loop, the conformation of the

CL of kinases shows little if any change in the crystal structures of the active and inactive forms (Williams et al., 1997; Williams et al., 1998). On the other hand, the NL of the kinases examined appear to undergo conformational changes between their inactive and active states that occurred about a fulcrum or pivot point that is centered upon the loop extending from the αC-helix and the strand that connects the NL to the CL (Williams et al., 1997; Williams et al., 1998). The loop extending from the αC-helix interacts tightly with residues in the CL anchoring its position within the NL. Superimposition of residues within the NL of the inactive and active forms of the kinases indicated that the β-sheets underwent little change in conformation, and that the major changes occurred in the conformation of the αC-helix whose position was somewhat randomly disposed (Williams et al., 1997; Williams et al., 1998). Because the loop region following the αC-helix is anchored, they concluded that the αC-helix, and in particular structures at the N-terminus of the helix, must bear the brunt of the conformational strain that occurs as kinases transition between their inactive and active state (Williams et al., 1997; Williams et al., 1998). It is of interest to note that the N-terminal residues that make up the αC-helix of the Hsp90-dependent kinases Cdk2 (Schulze-Gahmen et al., 1996) and Akt (Yang et al., 2002a; Yang et al., 2002b) are intrinsically unstructured in their inactive forms and become structured upon the binding of cyclin (Jeffrey et al., 1995) or phosphorylation of S473 (Yang et al., 2002a; Yang et al., 2002b)., respectively, which leads to the activation of these kinases.

In combination, these analyses suggest a model in which Hsp90 and Cdc37 each recognize distinct features within the catalytic domains of kinases. Hsp90 appears to bind to the back side of the catalytic domains of kinases recognizing features in both the the NL and CL. In addition, Hsp90 may facilitate the folding of intrinsically unstructured regions (e.g., the αC-helix) whose subsequent stability is modulated by additional tertiary interactions within the kinase fold (e.g., the nature of the αC-β4 loop, or interactions with other regulatory domains or motifs). The observation that binding of Cdc37 to JNK was induced by deletion of the first two strands of β-sheet (Prince and Matts, 2005), suggests a role for Cdc37 in stabilizing the β-sheet structure of the N-terminal lobe, as these two structural β-strands interact with all five β-strands within the NL of the catalytic domain.

MODULATION OF CDC37'S KINASE-BINDING AND HSP90-BINDING

As indicated above, Cdc37 is made up of three distinguishable domains (Figure 1). Evidence published by Shao et al (Shao et al., 2003a) suggests that amino acids present in the N-terminal region of Cdc37's [residues126–163, referred to as D2a (Shao et al., 2003a)] may function as an allosteric switch that modulates both the kinase binding potential of Cdc37's N-terminal domain (D1) and the Hsp90 binding potential of its central domain (referred to as D2b). The crystal structure of Cdc37 bound to the N-terminus of Hsp90 indicates resides (148–286) in the middle domain of Cdc37 binds Hsp90 (Roe et al., 2004). In the absence of D2a, the binding of the Cdc37 construct containing residues 164–282 (unpublished results) or 164–378

(a.k.a. Cdc37/ΔN in (Shao et al., 2003a)) to Hsp90 is reduced by ~70% compared to wtCdc37 (Shao et al., 2003a). However, the binding of these Cdc37 constructs to Hsp90 was comparable to wtCdc37 levels in the present of geldanamycin.

In comparison, the binding of Cdc37 middle domain constructs containing residues 128–163 [Cdc37/128–282 (D2) or Cdc37/128–378 (D23), (Shao et al., 2003a)] to Hsp90 was marginally reduced relative to wtCdc37, and geldanamycin either slightly reduce or had little effect on the binding of these constructs to Hsp90. While novobiocin has no effect on the binding of the Cdc37/128–378 (D23) to Hsp90, novobiocin induces some change in the conformation of complexes formed between the Cdc37/164–378 construct and Hsp90 that leads to the sequestration of its His-tag, as complexes containing Hsp90 and the Cdc37/164–378 construct could be immunoadsorbed with anti-Hsp90 antibodies, but not with anti-His-tag antibodies in the presence of novobiocin (Shao et al., 2003a). However, novobiocin had no effect on the co-adsorption of Hsp90 with His-tagged Cdc37/128–282 (D2) or Cdc37/128–378 (D23) constructs (Shao et al., 2003a).

Additional data suggest that D2a modulates the interactions of Cdc37 with Hsp90 in response to Hsp90's nucleotide-induced conformational switching. wtCdc37 does not interact with kinase or Hsp90-kinase complexes in the presence of geldanamycin, even though naïve Hsp90/Cdc37 complexes are stable in the presence of geldanamycin (Hartson et al., 2000). In contrast, the Cdc37/164–378 was detected in Hsp90-kinase complexes, even in the presence of geldanamycin (Shao et al., 2003a), suggesting that D2a modulates the interactions of Cdc37 with Hsp90 in response to nucleotide-modluated conformational switching induced by kinase binding. This hypothesis is consistent with the observations of Zhang *et al* that the binding of full length Cdc37 to the N-terminal lobe of Hsp90 requires the presence of Hsp90's charged linker (Zhang et al., 2004), while Cdc37 from which the N-terminus has been deleted (Cdc37c, a mutant containing residues 148–378) binds avidly to an N-terminal construct of Hsp90 from which the charge linker had been deleted. These observations led the authors to conclude that sequences N-terminal to Cdc37's Hsp90 binding domain suppress the Hsp90 binding potential of Cdc37 in the absence of Hsp90's charge linker region (Zhang et al., 2004). Zhang *et al* also noted that the binding of geldanamycin causes a compaction of Hsp90's tertiary structure relative to the nucleotide-free and nucleotide bound forms of Hsp90 (Zhang et al., 2004). This observation supports the conclusion that the "default" conformation and geldanamycin-bound conformation of Hsp90 are not equivalent, as the Cdc37/164–378 construct binds more avidly to the geldanamycin-bound Hsp90 (Shao et al., 2003a). Furthermore, Scheibel and co-workers also have reported that the charged region connecting the N-terminal and middle domains of Hsp90 plays an important role in regulating chaperone function of Hsp90 (Scheibel et al., 1999).

The presence or absence of D2a also affects the kinase-binding activity of Cdc37 constructs. Cdc37 constructs containing residues 1–163 (a.k.a. Cdc37/ΔC in (Shao et al., 2003a)) binds kinase independent of Hsp90 in the presence or absence of geldanamycin (Grammatikakis et al., 1999; Scholz et al., 2000; Shao et al., 2001;

Shao et al., 2003a). However, the capacity of Cdc37/1–128 (D1) to bind kinase is inhibited by geldanamycin (Shao et al., 2003a). Thus, in the context of full length Cdc37, a conformation must exist in which the both the kinase binding potential and Hsp90 binding potential of Cdc37 is repressed (e.g., the conformation of Cdc37 that dissociates from Hsp90-kinase complexes containing bound geldanamycin). The observations discussed above suggest that the region contain amino acids 128–163 plays a role in regulating the kinase- and Hsp90-binding activities of Cdc37 in response to nucleotide-mediated conformational switching of Hsp90.

CDC37 AND CANCER

Cdc37 is an essential gene that is required for folding of protein kinases. It is no surprise, therefore, that it has important roles to play in tumorigenesis. The evidence for this comes from studies showing that Cdc37 is upregulated in cancer cells compared with normal tissue. In healthy prostate tissue, for example, Cdc37 is barely detectable, but it is readily observed in prostate tumor tissue (Stepanova et al., 2000b). Importantly, increased levels of Cdc37 were also observed in premalignant lesions of the prostate known as prostatic intraepithelial neoplasms. This finding supports the notion that Cdc37 is important at the earliest stages of tumorigenesis. In healthy tissues, Cdc37 can be found co-expressed with cyclin D in proliferating tissues. In breast, Cdc37 expression follows that of cyclin D1, both of which are absent in epithelial cells of virgin mice but present in epithelium of pregnant animals. Cdc37 is also found to highly expressed in proliferative tissues of the embryo and in adults, the proliferative zones of the small intestine and stomach similarly have high expression of cyclin D1 and Cdc37. Other tissues where Cdc37 expression is high include the thymus, spleen. It is also expressed in some post-mitotic tissues including brain (Perdew et al., 1997; Stepanova et al., 1996).

Overexpression of Cdc37 in primary human prostate cells is sufficient to stimulate proliferation and lead to increased activity of Raf-1. The levels of Cdk4 were also increased and $p16^{INK4a}$ levels decreased. By contrast, overexpression of a dominant negative form of Cdc37 (lacking the Hsp90 binding domain) led to growth arrest and apoptosis (Schwarze et al., 2003). In transgenic animals, overexpressed Cdc37 is sufficient to induce mammary tumor formation and hyperplasia in the prostate. In animals that co-overexpress c-myc or cyclin D and Cdc37, there is a marked increase in the rate of tumor formation and the types of tumors that form compared with animals that have only one gene overexpressed (Stepanova et al., 2000a). These data suggest some form of collaboration between c-myc or cyclin D with Cdc37. For c-myc/cyclin D and Cdc37 this collaboration probably manifests in increased Cdk4 activation and subsequent Rb inactivation. In addition, Cdc37 can compete with p16 for binding to Cdk4, providing yet another example of how increased levels of Cdc37 may lead to Cdk4 activation outside of the normal controls that constrain its activity (Schwarze et al., 2003; Zhao et al., 2004a). These findings correlate with increased levels of Cdk4:Cdc37 complexes compared to Cdk4:p16 complexes in hepatocellular carcinoma. Other examples of Cdc37

upregulation in cancer also point to a protein kinase-dependent mechanism for its role in tumorigenesis. Anaplastic large cell lymphomas (ALCL) can be divided into two groups based on those that express the NPM-ALK fusion kinase and those that do not. In those expressing NPM-ALK, there is also upregulation of Cdc37, whereas no enrichment of Cdc37 levels was observed in ALCL cells lacking this kinase (Thompson et al., 2005).

Anecdotal evidence also connects CK2 and Cdc37 in cancers. Cdc37's kinase binding activity requires its phosphorylation by CK2 (Bandhakavi et al., 2003; Miyata and Nishida, 2004; Shao et al., 2003b). Elevating Cdc37 enhances CK2 activity (Bandhakavi et al., 2003; Kimura et al., 1997b) by an apparent feed-forward mechanism (Bandhakavi et al., 2003). CK2 activity is elevated in cancer cells, including leukaemias (Pinna and Meggio, 1997; Xu et al., 1999) and CK2 transgenic mice develop lymphomas (Seldin and Leder, 1995). CK2-driven lymphomagenesis is enhanced by cooperation with c-myc and pharmacologic inhibition of CK2 activity in cell lines established from CK2α transgenic T cell lymphomas reduces their proliferation (Channavajhala and Seldin, 2002).

The above studies show convincingly that Cdc37 is important for tumorigenesis, yet little is known about how its expression is regulated. Cdc37 is not stress inducible like other molecular chaperones, nor is it stress repressible. It therefore remains to be established how it becomes induced in tumor cells.

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CHAPTER 17

THE ELEVATED LEVELS OF HEAT SHOCK PROTEINS IN CANCER: A SUITABLE CASE FOR TREATMENT?

STUART K. CALDERWOOD[1,3,*], DANIEL R. CIOCCA[2], PHILLIP J. GRAY JNR[1], NAVA ZAARUR[3], STAN LEPCHAMMER[1] AND MICHAEL Y. SHERMAN[3]

[1]*Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA*
[2]*Institute of experimental Medicine and Biology of Cuyo (CRICYT-CONICET) and Argentine Foundation for Cancer Research (FAIC), Mendoza, Argentina*
[3]*Boston University Schools of Medicine, Boston, MA 02118, USA*

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INTRODUCTION

While the current approaches to cancer therapy, which rely heavily on modalities such as surgery, chemotherapy and radiotherapy are making incremental inroads into the cure of many cancers, it is clear that more effective and specific agents are needed if a major impact is to be made. The way forward thus seems to be the development of more effective and less toxic approaches. The preceding 30 years have seen the major advances in determining the molecular modifications within cells that lead to cancer. This has led to the development of more rational, molecular-based approaches to treatment. Small molecule inhibitors are being designed to target the nodal steps in carcinogenesis, using either random screening approaches or powerful *in silico* screening based on ultrastructural information that is rapidly accumulating on cancer proteins. This is best characterized in the development of the kinase inhibitor imatinib/Gleevac that blocks the action of the oncogenic BCR-ABL kinase in a subset of chronic myelogenous leukemias (Buchdunger et al., 2001). Patients can be selected for treatment based on screens for BCR-ABL activity and results have been spectacular in terms of tumor response (Buchdunger et al., 2001). However, targeting an individual protein in cancer, in which rapid evolution towards a drug resistant state

*Corresponding author: BIDMC, 21-27 Burlington Avenue, 553B, Boston MA 02215, Tel: 617 632 0628, e-mail: scalderw@bidmc.harvard.edu

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is the norm is prone to ultimate failure and recurrence occurs regularly in CML patients treated with imatinib/Gleevac (O'Hare et al., 2006; Sawyers, 2005). As we shall discuss below, Hsp inhibitors may be able to both answer the call for a specific agent and outflank the ability of tumors to evolve resistant drug traits.

Heat shock proteins (Hsp) have begun to emerge in recent years as legitimate targets for in cancer therapy (Calderwood et al., 2006; Neckers, 2002; Workman, 2004) (Also See: *Ciocca et al*, Chapter 2, this volume). These proteins are close relatives of the molecular chaperones that mediate protein quality control in all organisms (Mayer and Bukau, 2005; Neckers and Ivy, 2003; Wegele et al., 2004). The Hsp are strongly induced when protein structure is destroyed during thermal or oxidative stress (Lindquist and Craig, 1988). Hsp production in stress increases to dominate cellular gene expression and large amounts of the proteins are made; the extraordinarily high concentrations of Hsp synthesized probably reflect the fact that Hsps interact stoichiometrically with many protein substrates and may remain bound to them for prolonged periods (Wegele et al., 2004).

Recent studies, carried out on cancers of diverse morphologies indicate that Hsp expression is elevated and, at these abundant concentrations the Hsp play significant roles in the emergence of cancer cells and their resistance to therapy (Figure 1)

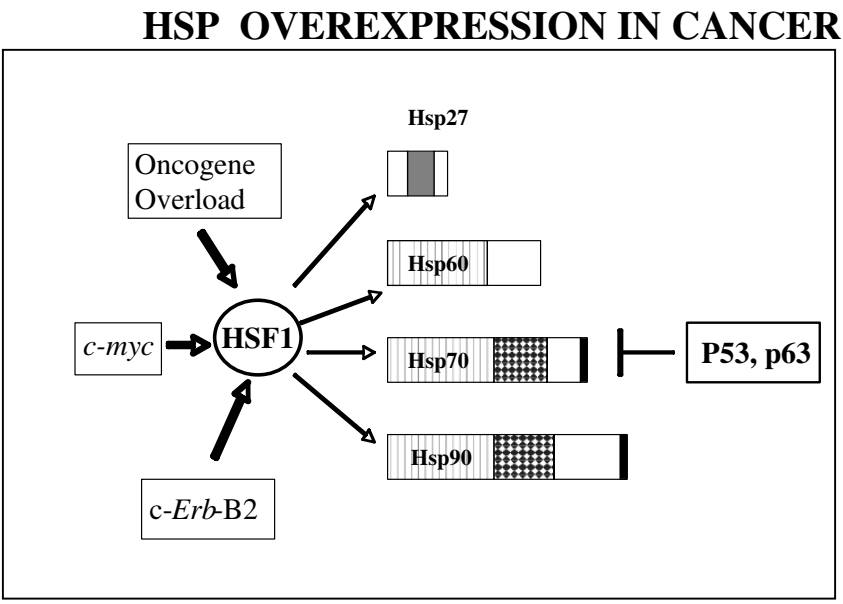

Figure 1. Heat shock protein overexpression in cancer. Hsp27, Hsp60, Hsp70 and Hsp90 overexpression in cancer may be due to a number of factors including positive activation due to increases in Hsp substrates due to "oncogene overload" and activation by oncoproteins such as c-Erb-B2 and c-Myc. In addition, Hsp70 is repressed by p53 and p63 and inactivation of these proteins during cancer leads to Hsp increases due to the relief of such repression. Many of the Hsp contain ATPase domains (▥) and substrate binding domains (◩). Hsp27 does not contain an ATPase domain, in common with other small Hsp, but contains a conserved α-crystallin domain (■)

(Ciocca and Calderwood, 2005; Nylandsted et al., 2000; Rohde et al., 2005; van 't Veer et al., 2002). The Hsp are the products of a number of gene families named for their approximate Mr as Hsp 10, 27, 40, 60, 70, 90, and 110 (Bukau and Horwich, 1998; Lindquist and Craig, 1988). (Tang et al., 2005). These proteins carry out protein folding when assembled into "chaperone machines," high Mr complexes formed from multimeric self-association as in HSP27 and HSP60, or within multi-protein complexes as with HSP70 and HSP90 (Netzer and Hartl, 1998; Pratt et al., 2004) (Figure 2). Many of the Hsp use the energy of ATP hydrolysis to release from refolded cellular substrates using intrinsic ATPase domains (Bukau and Horwich, 1998). Early in the course of evolution, the *hsp* genes developed the capacity for massive induction by environmental insult and in all species, exposure to stress coordinately induces the *hsp* gene families and inhibits the pathways of programmed cell death (PCD) (Figure 1) (Georgopolis and Welch, 1993). Cytoprotection involves the aggregate effects of multiple mechanisms, which include the inhibition by Hsp of protein aggregate formation and direct blockage of the death pathways at multiple sites (Beere, 2001; Cashikar et al., 2005; Li and Werb, 1982). This stress response is regulated by the heat shock transcription factor family (*hsf*), of which HSF1 is

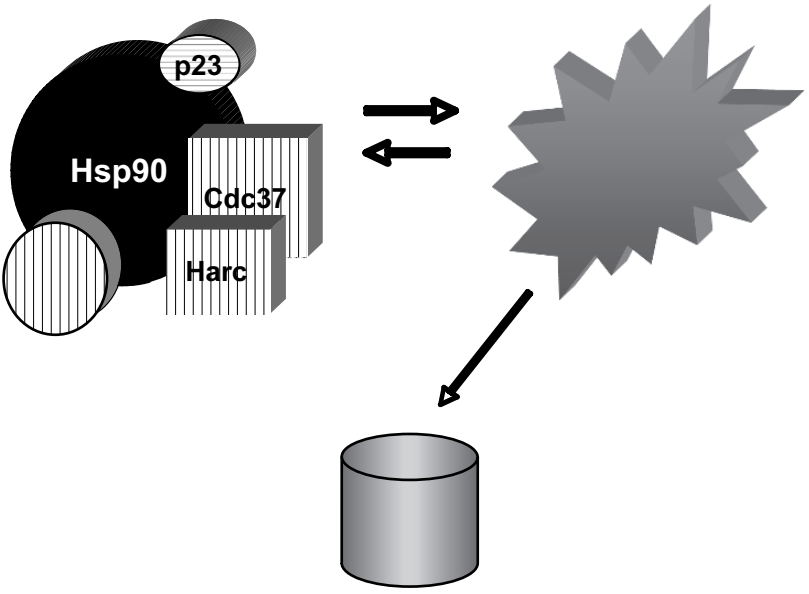

Figure 2. Hsp90 chaperone machines. Hsp90 is found in a heterogeneous series of high molecular weight complexes with co-chaperones such as p23, FKBP52, FKBP52, Cyp-40, Cdc37 and Harc. These complexes chaperone the overexpressed and mutated proteins that accumulate in cancer cells and mediate tumorigenesis. Hsp90 chaperone complexes preventin oncoproteins from aggregating in the cell. Inhibition of Hsp90 leads to denaturation of the oncoproteins and their degradation by the proteasome. Hsp90 and its co-chaperones thus play a permissive role in the formation and progression of cancer

the dominant factor (Wu, 1995). HSF1 contributes to transcriptional regulation of each family of Hsp through interaction with the heat shock elements (HSE) in their promoters and is also elevated in cancer [7,66].

MECHANISMS OF HEAT SHOCK PROTEIN ELEVATION IN CANCER

It seems unlikely that malignant progression directly mimics the stresses such as heat shock and oxidative stress that lead to Hsp transcription. However, one mechanism that could be involved in Hsp elevation in cancer is the proliferation of Hsp substrates that may ensue from oncogene mutation and overexpression that accompanies tumorigenesis (Kamal et al., 2003) (Also See: *Neckers*, Chapter 12, *Whitesell & McLellan*, Chapter 13, *Kamal et al*, Chapter 14, *Holmes et al*, Chapter 15 in this volume). Such substrates are known to be chaperoned by Hsp90 and perhaps other members of the Hsp cohort (Figure 2) (Kamal et al., 2003). A related pathway by which Hsp are induced involves a feedback response to Hsp sequestration by substrates; thus when Hsp bind to denatured proteins in stressed cells HSF1 is de-repressed, switched on and more Hsp are synthesized (Zou et al., 1998). "Oncoprotein overload" may thus de-repress HSF1 and drive Hsp synthesis (See *Calderwood et al*, Chapter 3). However, in addition to this mechanism, elevated Hsp gene transcription in cancer is coupled to some of the primary signal transduction pathways of malignant transformation (Calderwood et al., 2006). A major mechanism for HSP regulation in normal cells involves the tumor repressor p53 and the structurally related protein p63, which repress *hsp70* transcription through their influence on binding sites for the transcription factor NF-Y present on a number of HSP promoters (Wu et al., 2005). During the transformation of many cell types, p53 undergoes inactivating mutation or allelic loss, highly penetrant genetic changes associated with over 45% of cancers at many organ sites (Calderwood et al., 2006). p53 inactivation relieves the repression of *hsp* promoters and leads to enhanced *hsp70* transcription through both loss of *hsp70* promoter repression and a gain-of–function *trans* activation mechanism (Agoff et al., 1993; Ghioni et al., 2002; Madden et al., 1997; Tsutsumi-Ishii et al., 1995). Alterations in p63 are closely associated with Hsp70 expression in cancer and expression of an alternative splicing isoform, ΔNp63α which acts as a dominant negative inhibitor of p63, upregulates Hsp70 and Hsp40 levels in head and neck cancer (Wu et al., 2005). In addition to reversal of repression by p53, Hsp are also induced in cancer can through positive effects on transcription through the signaling circuitry of the cell stress pathway, involving activation of the HSE elements present in all *hsp* promoters and enhanced *hsp* mRNA stability, translation and protein stability (Georgopolis and Welch, 1993; Lindquist and Craig, 1988; Wu, 1995). Khaleque et al have shown that the tumorigenic factor heregulin-β1 binds to the surface of breast cancer cells and causes increased Hsp60, Hsp70 and Hsp90 expression, an effect associated with enhanced survival and transformation in an *hsf1* gene dependent manner [66]. Heregulin-β1 activates HSF1 through activation of proto-oncogene HER2 and

phosphatidylinositol-3 kinase (PI-3 kinase) at the cell surface and activation of hsp genes through promoter HSE elements [66]. As PI-3 kinase is a key factor in many cancers, particularly through its activation by PTEN mutation and induction of c-Myc, this may be an important mechanism for HSP elevation in cancer. In addition c-Myc which is activated by heregulin and HER2 positively regulates Hsp transcription through a mechanism involving HSE (Taira et al., 1999). In the case of HSP90, c-Myc activates the Hsp90A promoter and inhibition Hsp90A synthesis decreases the transforming effects of c-Myc expression (Teng et al., 2004). HSF1 also plays a number of additional roles in the malignant phenotype, including the override of cell cycle checkpoints leading to tumor aneuploidy and enhanced metastasis which may involve non-Hsp dependent effects of HSF1 (Hoang et al., 2000; Wang et al., 2004). HSF1 is activated by cancer treatment with Hsp90 inhibitors and proteosomal inhibitors and these off-target effects of the drugs may result in treatment resistance through enhanced HSP expression (Mathew et al., 1998; Workman, 2004). Coupling of Hsp27 expression to HSF1 is less tight than for the other Hsp. Indeed Hsp27 expression in breast cancer appears to involve another transcription factor Brn3b that is itself increased in breast carcinomata (See: *Budram-Mahadeo & Heads*, Chapter 5).

Intuition would also suggest to us that Hsp expression is induced by the microenvironmental stresses, including hypoxia and hypoglycemia imposed by the deteriorating tumor milieu as tumors grow (Folkman, 2002). However little information is available to encourage this suggestion and the published data indicate that another transcriptional stress response, the unfolded protein response rather than Hsp expression is activated by changes in the tumor milieu (such as hypoxia and hypoglycemia) and growth of tumor cells as xenografts leads to the inhibition rather than enhanced expression of Hsp in cells growing in tumors (Tang et al., 2005).

APPROACHES TO TARGETING TUMOR HSP IN CANCER THERAPY

The functions of Hsp in growth and survival of cancer cells may potentially be targeted by inhibitors either (a) individually by inhibitors of the molecular chaperone functions of Hsps or (b) compounds that target the whole Hsp cohort by blocking upstream activation of Hsp expression.

Targeting of Individual Hsp

Hsp90

The effects of the Hsp on anabolic pathways leading to tumor progression, their *self-sufficiency in growth signals* – are mediated largely through HSP90 (Calderwood et al., 2006). This molecular chaperone plays an essential role in stabilizing the fragile structures of many of the receptors, protein kinases and transcription factors that lie along the pathways of normal cellular growth as well as malignant transformation (Buchner, 1999) (See: *Pratt et al*, Chapter 1). Pharmacological targeting of HSP90 leads to the degradation of such proteins, inhibition of tumor growth

through G_1 arrest and morphological and functional differentiation (See: *Neckers*, Chapter 12, *Whitesell & McLellan*, Chapter 13, *Kamal et al*, Chapter 14, *Workman*, Chapter 15 in this volume). The activity of Hsp90 involves at least three functional main domains, including an N-terminal ATPase domain, an internal substrate binding domain and a C-terminal TPR domain protein binding motif which mediates interaction with other proteins (Figure 1) (Buchner, 1999; Neckers and Ivy, 2003). Hsp90 carries out its functions within the cell by catalyzing the folding of its substrates (often referred to as "clients"), a wide range of signaling molecules and oncogenes, in cooperation with Hsp70, then maintaining the client in an active, protected form in cooperation with a group of "co-chaperones"; these include the immunophilins FKBP51, FKBP52, and Cyp40, the p23 protein and with the cell cycle active proteins cdc37 and Harc (Figure 2) (Cheung-Flynn et al., 2005; Pearl, 2005; Roiniotis et al., 2005). Hsp90 activity has been effectively targeted in a range of cancers using derivatives of two families of compounds base on the *ansamycin* geldanomycin and the *coumarin* family drug novobiocin (Neckers, 2002; Neckers and Lee, 2003; Sharp and Workman, 2006). (Also See: *Neckers*, Chapter 12, *Whitesell & McLellan*, Chapter 13, *Kamal et al*, Chapter 14, *Workman*, Chapter 15). The selectivity of the ansamycin class of hsp90 inhibitors for tumor cells is partially due to their far greater concentration of Hsp90 substrates, the mutated and overexpressed oncoproteins that permit tumor development (Kamal et al., 2003). Due to the evident effectiveness of this approach, in cancer treatment other aspects of Hsp90 function have not found a great deal of attention with regard to inhibitory drugs. However, it is apparent that Hsp90 function requires the C-terminal EEVD motif in order to bind to the scaffold protein Hop and cooperate with Hsp70 in folding cascades, as well as the group of co-chaperones mentioned above as well as the functioning of its substrate binding domain (Frydman and Hohfeld, 1997). The EEVD motif is one of the targets of the deoxyspergualin family of compounds that also interact with Hsp70 and are in use as immunosuppresants in transplant studies (Brodsky, 1999; Fewell et al., 2004). As mentioned, a number of co-chaperones also play roles in Hsp90-client interactions (Figure 2) (See *Pratt et al*, Chapter1. *Matts & Caplan*, Chapter 16). Notable among these is Cdc37, a co-chaperone required specifically for interactions with protein kinase clients and which possesses the properties of an oncogene. As Cdc37 is overexpressed in many prostate cancers this protein could provide an attractive alternative approach to the direct targeting of Hsp90 in drug development (Grammatikakis et al., 1999; Schwarze et al., 2003). In addition, Cdc37 possesses the independent transforming properties of an oncogene over and above its role an Hsp90 co-chaperone (Schwarze et al., 2003). These alternative targets may be exploited in future studies.

Hsp27 and 70 are inhibitors of programmed cell death

Hsp70 and Hsp27, although not mediators of proliferation are elevated in a wide spectrum of human cancers and their role in tumorigenesis involves their abilities to *block programmed cell death* (PCD) and *replicative senescence*, two other steps in tumor progression (See; *Brunet et al*, Chapter 11). At elevated levels, these Hsp

are powerful inhibitors of stress-mediated cell killing in all cellular organisms and mediate a profoundly resilient state (Gerner and Schneider, 1975). They have been shown to play an essential role in the survival of a number of human cancer cells and inactivation or knockdown, particularly of Hsp72 or Hsp27 leads to the spontaneous activation of PCD which is not observed in normal tissues of origin (Beere, 2001). Multiple PCD pathways must be inhibited to permit tumor progression (Nylandsted et al., 2000; Paul et al., 2002; Tenniswood et al., 1992). The enhanced activity of a number of oncogenes during cell transformation, most notably c-Myc and Ras, induce PCD through death pathways mediated by p53 and Bcl-2 family proteins, and these pathways must in turn be subverted to permit the emergence of cancer cells (Hanahan and Weinberg, 2000; Nelson and White, 2004). The relationship of HSP27 and HSP70 to the p53 and Bcl-2 pathways is however currently not clear although each can function independently in countering death signals (Beere, 2001; Jaattela, 2004). Convincing evidence for the inhibition of caspase-dependent apoptosis has been shown when the expression of either HSP70 or HSP27 is elevated (Beere, 2001). Notable molecular targets for HSP27 or HSP70 within the caspase dependent apoptosis include c-*jun* kinase, apaf-1 and caspase 8 (Beere, 2001) (See; *Brunet et al*, Chapter 11). Hsp70 has also been implicated in other mechanisms of cell killing addition to caspase-dependent apoptosis, and Hsp70 overexpression inhibits a death pathway involving cell digestion by lysosomally-derived cathepsins (Nylandsted et al., 2004).

To escape from replicative senescence and undergo unlimited growth, tumor cells are also required to bypass "crisis" at which point the telomeres on chromosomes have shortened sufficiently to prevent successful future cell divisions (Campisi, 2005). p53 sensitive expression of the enzyme telomerase in tumor cells is sufficient to bypass crisis and permit unlimited growth in some cells (Campisi, 2005) and Hsp90 has been shown to be essential for telomerase stability further indicating the importance of Hsp90 in transformation (Workman, 2004). Recent studies also indicate that Hsp70.2, a non-stress inducible Hsp70 normally expressed during spermatogenesis, is expressed to high level in breast cancer and inhibits the onset of senescence (Rohde et al., 2005). Hsp70.2 functions to antagonize the engagement of the senescence pathways by decreasing activity of both p53 (and p21) dependent and independent mechanisms of senescence (Rohde et al., 2005). Another member of the Hsp70 family, the mitochondrial protein Hsp75 or *mortalin* plays an analogous role in countering replicative senescence and increasing the number of cell divisions in transformed cells and senescence can be induced by mortalin knockdown (Wadhwa et al., 2002). Mortalin functions at least in part by inhibiting p53 activity (Proskuryakov et al., 2003; Wadhwa et al., 2002) (Also See: *Wadhwa*, Chapter 7). Hsp70 and Hsp27 family proteins are thus highly versatile cytoprotective molecules that may foster the emerging malignant cell and permit it to evade both fast (PCD) and slow (senescence) pathways of cell inactivation that limit the tumorigenic phenotype. Consistent with this, elevated expression of both Hsp27 and Hsp70 are correlated with chemotherapy resistance (Vargas-Roig et al., 1998).

Towards Hsp70-based drugs

Although the exact *modus operandum* of Hsp70 in cancer is not entirely clear, its influence on transformation almost certainly involves its molecular chaperone activities. Hsp70 is essentially a polypeptide binding ATPase, which binds to and manipulates its substrates in a cycle of reactions indicated in cartoon form in Figure 3. Hsp70 is less discriminating than Hsp90 in its interactions, and its substrates are not referred to as "clients". Hsp70 is a classical allosterric molecule and occupation of its peptide binding pocket stimulates ATPase activity while subsequent ATP binding triggers substrate release from the adjacent peptide binding domain (Figure 3) (Bukau et al., 2006). The tertiary structure of Hsc70, a close homolog to Hsp70 has recently been solved and this information may permit rational approaches to hsp70 drug design (Jiang et al., 2005; Sousa and Lafer, 2006). These biochemical interactions are aided by co-chaperones including DNA-J family proteins such as Hsp40 that stimulate substrate binding and ATP hydrolysis and a range of nuclear

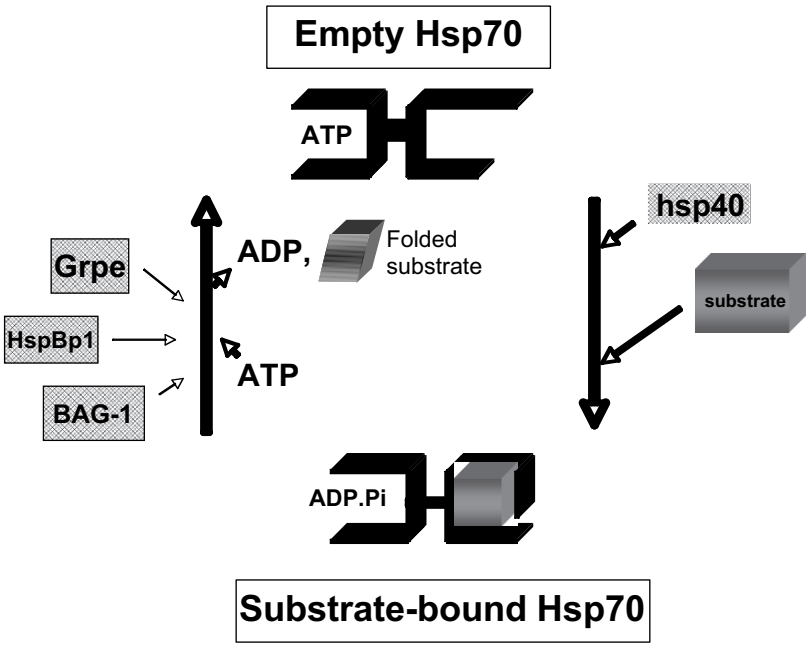

Figure 3. The Hsp70 cycle. Hsp70 cycles between an "empty", ATP bound form which is poised to interact with substrate and a substrate bound form associated with ADP which holds avidly to target polypeptides. Substrate selection occurs with the cooperation of Hsp40 which catalyzes the ATPase activity of Hsp70 and permits substrate binding. Hsp70 is an allosteric molecule and the exchange of ATP for ADP.Pi within the N-terminal ATPase domain is "sensed" by the substrate binding domain which then closes and binds avidly to the substrate. To release the substrate, a number of nucleotide exchange proteins, including BAG-1, HspBP1 and Grpe catalyze ATP-ADP exchange, resulting in opening of the substrate domain and substrate release. Refolding of some substrates may involve the mechanical interactions that accompany opening and closing of the substrate domain

exchange factors such as Grpe, BAG-1 and HSPBP1 that stimulate ADP release and ATP binding (Figure 3) (Bukau et al., 2006).

In addition Hsp70 also contains a C-terminal EEVD domain essential for chaperone function and interaction with other TPR domain proteins such as the scaffold protein Hop that permit coupling to Hsp90 and the ubiquitin E3 ligase CHIP (Qian et al., 2006). There appear therefore to be a range of structures and interactions that could potentially be targeted by small molecules. One class of drugs that has been examined is 15-deoxyspergualin and derivatives that target protein binding to TPR domain proteins (Brodsky, 1999). The drugs inhibit Hsp70/Hsp40 stimulation of ATPase activity in the micromolar range (Fewell et al., 2004). These effects are not observed in the ER Hsp70 homolog BiP/Grp78 as might be predicted as this protein contains a C-terminal ER retention sequence in the place of the EEVD motif (Fewell et al., 2004). However the specificity of such drugs might be questioned as TPR domains occur in a range of proteins and for instance 15-deoxyspergualin inhibits Hsp90 (D'Andrea and Regan, 2003). Another approach to Hsp70 inhibition might be to block its inhibitory interaction with key proteins involved in the apoptotic cascade such as apoptotic protease activation -1 (APAF-1) and apoptosis inducing factor (AIF) (Garrido et al., 2006). Garrido et al (Brunet et al, Chapter 11) have shown that expression of the key polypeptide sequence involved in Hsp70-AIF interaction can inhibit tumorigenicity of a range of cancers and their response to cytotoxic drugs (Schmitt et al., 2006). "Substrate choking" may therefore be a viable approach to Hsp70 inhibition. Lastly of course, Hsp70 contains an essential ATPase domain and drugs that bind into the groove of the ATPase domain and inhibit ATP binding or nucleotide exchange may have the potential to be used in cancer therapy (Figure 3). One drawback to targeting Hsp70 is that its close homolog, heat shock cognate 70 (HSC70) bears strong structural resemblance (80% homology to HSPA1A) is essential for survival of normal cells (Rohde et al., 2005).

Hsp27

Hsp27, the protein product of the *HSPB1* gene is a member of the small Hsp family (Didelot et al., 2006). Hsp27 is the most abundant of the Hsp and is overexpressed in a wide range of cancer cells (Ciocca and Calderwood, 2005; Ciocca et al., 1993; Tang et al., 2005). During the stress response Hsp27 undergoes activation at the posttranslational level through the p38/MAPKAPK2 pathway and is massively induced at the transcriptional level, and both levels of activation may participate in activation in cancer (Didelot et al., 2006). In contrast to Hsp70 and Hsp90, hsp27 does not contain an ATPase domain (Figure 3) and interacts with its substrates (aggregated proteins) through formation of multimeric complexes (Arrigo, 2005). The elevated Hsp27 levels in cancer mediates cytoprotection which may involve its binding to the apoptotic inducer cytochrome C and has also been linked to increased cell migration and metastasis (Didelot et al., 2006; Garrido et al., 2006) (Also See: *Arrigo*, Chapter 4, *Brunet et al*, Chapter 11). Targeting HSP27 with

small interfering RNA approaches promotes death both alone and in combination with proteasome inhibitors (Chauhan et al., 2003).

Drugging the Heat Shock Response

An elegant solution to the problem of elevated Hsp expression would be to inhibit HSF1 and thus downstream transcription of the *hsp* genes. Indeed HSF1 knockdown by RNA interference blocks Hsp expression in a number of cancer cell lines (Rossi et al., 2006; Zaarur et al., 2006). One approach to HSF1 inhibition that has been employed was to use isoflavonoid drugs such as quercitin or genistein that inhibit the tyrosine phosphorylation step upstream of HSF1 activation (Asea et al., 2001; Lepchammer et al., 2002). It was shown that quercitin increases the rate of apoptosis in prostate carcinoma cells in *vitro* and is an effective inhibitor of growth of mouse xenografts of human prostate carcinoma such as PC-3 and DU145 cells (Asea et al., 2001; Jones et al., 2004). Quercitin was particularly effective when combined with elevated temperatures (hyperthermia) (Asea et al., 2001). Hyperthermia inactivates cells in part by sequestering the Hsp in protein aggregates and combination with small molecule inhibitors of HSF1 is thus a promising approach to cancer therapy. In addition, recent studies have identified novel HSF1 inhibitors including stresgenin, KNK437 and *triptolide*, a diterpene triepoxide derived from plants as which antagonized *trans* activation of hsp promoters (Akagawa et al., 1999; Ohnishi et al., 2004; Westerheide et al., 2006). However, little is currently known regarding their effectiveness as stand-alone agents in cancer therapy.

We have attempted to develop compounds based on ability to inhibit induction of the heat shock response and inducible molecular chaperone activity (Zaarur et al., 2006). The heat shock response results in the induction of resistance to stress-induced death (thermotolerance) that is reflected at the molecular level in resistance to denaturation of heat sensitive proteins (Schlessinger, 1994). The heat shock response is largely mediated by the massive Hsp increases induced by stress (Li and Werb, 1982). In a high through put screen carried out on a diverse chemical library, of thermotolerance inhibitors, we found that approximately 8% of compounds were able to block the protection from death and protein folding associated with the heat shock response (S. Lepchammer, M.Y. Sherman and S.K. Calderwood, unpublished data). Among these compounds are, presumably, inhibitors of chaperone function and Hsp expression. We have further gone on to examine compounds that inhibit induction of molecular chaperone activity by inhibiting Hsp gene expression (Zaarur et al., 2006). We have found that a class of drugs epitomized by a compound that we refer to as emunin can strongly inhibit Hsp70 expression in a wide range of cancer cells. Curiously, these compounds appear to function largely at the post-mRNA level and do not markedly alter Hsp70 regulation at the levels of transcription or mRNA accumulation (Zaarur et al., 2006). Hsp70 translation differs from many normal mRNAs in employing CAP-independent mechanisms and future studies will explore whether these are involved in the activities of the emunin class of drugs (Rubtsova et al., 2003).

HSF1 ACTIVATION AND HEAT SHOCK PROTEINS AS COMPLICATIONS OF TREATMENT

It is apparent that several of the novel approaches to cancer treatment can lead to the activation of HSF1 and the powerful synthesis of Hsp. These include inhibitors of the proteasome such as velcade, and hsp90 inhibitors such as geldanomycin and 17-AAG (Sharp and Workman, 2006; Zaarur et al., 2006). These effects may therefore counteract many of the effects of treatment through synthesis of Hsp90 itself and the expression of the anti-death protein Hsp72. We have shown that inhibition of Hsp expression induced by each of these agents using emunin leads to marked sensitization of cancer cells to treatment (Zaarur et al., 2006). HSF1 induction may have additional deleterious effects that are independent of Hsp expression as we have shown that the factor associates with and activates the pro-metastatic protein gene co-repressor MTA1 in breast cancer, an effect that may mediate its known pro-metastatic effects (Hoang et al., 2000). HSF1 may also participate in progression of gastrointestinal cancers by repressing the promoter of the XAF1 gene. XAF1 is a suppressor of XIAP, a caspase 3 inhibitory protein and loss of XAF1 after HSF1 overexpression thus leads to protection from caspase dependent apoptosis (Wang et al., 2006). Elevation of HSF1 by prolonged treatment with drugs or during malignant transformation may thus be implicated in tumorigenesis through multiple mechanisms, including expression of anti-apoptotic proteins Hsp70 and XIAP and repression of ERE regulated genes including pro-apoptotic c-*Myc* (Khaleque et al., 2005; Wang et al., 2004); (Khaleque et al, in press). In addition, HSF1 is implicated in the *multiple drug resistance* phenotype and resistance to doxorubicin though its role in activating the multidrug resistance gene 1 (MDR-1). This appears to be an effect independent of its normal transcriptional functions and occurs in the absence of Hsp expression (Tchenio et al., 2006). Targeting HSF1 may therefore take on greater urgency.

CONCLUSION

In conclusion therefore heat shock proteins are overexpressed in many cancers and play key roles during malignant transformation in chaperoning growth promoting oncoproteins and inhibiting the pathways of senescence and death. Development of inhibitory compounds targeting the Hsp has begun particularly for hsp90 for which effective compounds are already available. Development of agents targeting Hsp27 and hsp70 is soon anticipated.

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CHAPTER 18

HSP70-BASED ANTICANCER VACCINES: CHAPERONING THE IMMUNE RESPONSE

JIANLIN GONG[1], JIMMY R. THERIAULT[2]
AND STUART K. CALDERWOOD[1,2]

[1]*Department of Medicine, Boston University School of Medicine, Boston, MA 02118, USA*
[2]*Molecular and Cellular Radiation Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215*

INTRODUCTION: EXTRACELLULAR HSP70 AND ITS ROLE IN ANTI-TUMOR IMMUNITY

The *hsp70* gene family encode molecular chaperones intrinsic to cellular life and essential for cell survival (Georgopolis and Welch, 1993; Lindquist and Craig, 1988). The essential intracellular functions of the *hsp70* genes are in *protein folding* and *protection from cell death pathways*. Each Hsp70 family member contains two major functional domains, a C-terminal peptide binding domain and an N-terminal ATPase domain that controls the opening and closing of the peptide binding domain (see review (Bukau and Horwich, 1998; Bukau et al., 2006)). These two domains mediate the immune properties of Hsp70, permitting the acquisition of cellular antigens and their delivery to immune effector cells (Calderwood et al., 2005b; Noessner et al., 2002; Srivastava and Amato, 2001). There are at least 12 members of the human *hsp70* gene family members, including proteins expressed in cytoplasm, endoplasmic reticulum and mitochondria (Bukau and Horwich, 1998; Lindquist and Craig, 1988; Tang et al., 2005). In addition, cells possess both constitutive cytoplasmic Hsp70 members (such as human HSC73) and members induced by stress (HSP72) (Schlessinger, 1994; Tang et al., 2005). *Hsp70* becomes dysregulated in cancer and the elevated Hsp70 levels that ensue protect emerging cancer cells from the triggering of programmed cell death that accompanies many of the steps in transformation, while also creating an opportunity for immune attack (Calderwood et al., 2007; Calderwood et al., 2006; Ciocca and Calderwood, 2005; Clark and Menoret, 2001; Cornford et al., 2000; Nylandsted et al., 2000; Tang et al., 2005) (Also see *Ciocca et al*, Chapter 2, this volume). Many studies now suggest that Hsp70, through its ability to bind peptide antigens is a major factor linking the tumor phenotype, with its accompanying expression of mutated and overexpressed

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oncoproteins, to immunity (Also see: *Bonorino and Souza*, Chapter 10). A pro-immune function for Hsp70 family members is also inferred from the fact that the immunosuppressive drug 15-deoxyspergualin binds with high affinity to Hsp70 proteins (Nadler et al., 1992). In addition, expression of Hsp70 in the execution stages of tumor cell killing directly stimulates tumor immunogenicity (Daniels et al., 2004; Melcher et al., 1998).

ROLE OF HSP70 FAMILY MEMBERS IN ANTIGEN PROCESSING AND CROSS PRESENTATION

Mice and human subjects are able to mount a T cell mediated response to cancer based on their recognition of epitopes from mutated proteins, the products of overexpressed oncoproteins or ectopically expressed developmental genes (Pardoll, 2003). However, it is evident that most tumors of non-viral origin are not rejected and that tumor cells often evolve a range of strategies that lead to suppression of the immune responses, including complete ablation of MHC class I gene expression, that may be stimulated (Chouaib et al., 2002; Moller and Hammerling, 1992; Pardoll, 2003). In addition, as tumors cells with intact MHC class I molecules (MHC I) present tumor antigens to antigen presenting cells (APC) in the absence of a co-stimulatory molecule, tolerance to tumor antigens is a frequent occurrence (Zou, 2006). However, approaches to enhancing the immunogenicity of human cancer have been demonstrated (Pardoll, 2003). Hsp70 and other molecular chaperones offer a novel approach to enhancing tumor immunogenicity as they are powerful multi-functional agents in tumor immunotherapy with (1) talents as immune adjuvants for activating APC and breaking tolerance to tumor antigens and (2) as antigen chaperones which can deliver tumor antigens to APC (Belli et al., 2002; Calderwood, 2005b; Daniels et al., 2004; Huang et al., 2003) (Daniels et al., 2004; Melcher et al., 1998; Srivastava, 2002; Srivastava and Amato, 2001).

The immunological properties of Hsp70 derive initially from their roles in antigen processing. Tumor antigens are peptides originating in the damaged, denatured or superfluous intracellular proteins that are degraded through the ubiquitin proteosome pathway to small peptides (Goldberg et al., 2002; Rock et al., 2002). A fraction of the peptides released from the proteasome are then used for immune surveillance (Goldberg et al., 2002; Gromme and Neefjes, 2002; Rock et al., 2002). Cytoplasmic peptides are taken up into the endoplasmic reticulum (ER) through the ABC family transport system that involves the *transporters associated with antigen processing* (TAP); TAP1 and TAP2 form a complex in the ER membrane that transports peptides and delivers them to major histocompatibility class I (MHC I) protein complexes (Schumacher et al., 1994; Shepherd et al., 1993). Peptides of suitable size and sequence are then bound by MHC I, transported via the vesicular system and displayed on the cell surface where they are subject to surveillance by the T cell receptors of $CD8^+$ lymphocytes (Neefjes et al., 1993). Such cell surface display of antigens on MHC class I molecules permits identification of non-self foreign antigens in microorganism-infected cells, which are then targeted for lysis by CTLs.

However, this pathway can also be used to detect malignant cells if tumor antigens can be discriminated against normal self antigens (Germain, 1994; Monaco, 1992; Noessner et al., 2002; Topalian et al., 1994). Hsp70 family members participate in antigen processing through their ability to bind peptides using their polypeptide binding domain (Bukau and Horwich, 1998; Flaherty et al., 1990; Flynn et al., 1989; Wilbanks et al., 1995). It has been assumed that Hsp70 might bind peptides released into the cytoplasm from the proteasome in a similar way to their acquisition by the TAP1/TAP2 complex. A number of studies have addressed the peptide sequence binding preferences of Hsp70 and indicated a similar peptide sequence preference as for MHC I, with roles for hydrophobic and basic amino acids (Flynn et al., 1989; Fourie et al., 1994; Gragerov and Gottesman, 1994; Wu and Wang, 1999). It is not known whether Hsp70 binds preferentially to any class of intracellular peptides although in an *in vivo* proteomic study conducted by Grossmann *et al* Hsp70 binds to peptides 8-26 amino acids in length (Grossmann et al., 2004). Hsp70 interacts selectively with a 5 amino acid core sequence containing some acidic residues (Grossmann et al., 2004).

In addition, Hsc73 takes part in the processing of external antigens in the lysosome proximal endosome compartments of APC and is thus involved in peptide loading of MHC class II and presentation to $CD4^+$ T lymphocytes (Panjwani et al., 1999). Hsc73 co-associates with MHC II in spherical organelles in macrophages and participates in presentation of external antigens to MHC class II restricted T lymphocytes (Panjwani et al., 1999). A role for hsc73 in protecting peptides generated by proteolysis in MHC II containing endosomes similar to the role proposed for the Hsp70 family in chaperoning cytoplasmic peptides has been suggested (Agarraberes et al., 1997; Chiang et al., 1989; Cuervo and Dice, 1998). The presence of hsc73 in clathrin coated pits present in endosomes and lysosomes is well established and hsc73 has been shown to participate in targeting proteins for degradation through recognition of a consensus sequence (KFERQ) (Agarraberes et al., 1997; Chiang et al., 1989; Cuervo and Dice, 1998). A common theme for Hsp70, of mobilizing target proteins towards the sites of degradation while sparing a fraction of the partially digested peptide for immune surveillance is indicated for the main pathways of protein degradation in (1) the cytoplasm and (2) lysosome. As the lysosomal pathway can be stimulated by serum starvation of tissue culture cells, it may thus play a role in generation of tumor immunity in nutritionally-deprived tumor cells, that could potentially be captured in the production of anti-tumor Hsp70 based vaccines (Cuervo and Dice, 1998).

Hsp72 and hsc73 thus have ready access to tumor antigens from both the intracellular and external processing pathways. These proteins however, devoid of a leader sequence for secretion would seem doomed to remain trapped along with their cargo in the cytoplasm of the tumor. Hsp70 can however, escape from the cytoplasm as indicated by its appearance in the serum of many patients (Pockley, 2003). It has also been shown that Hsp70 is released from tumor cells undergoing necrosis and such Hsp70 is highly immunogenic (Mambula and Calderwood, 2006a; Srivastava, 2003) (Daniels et al., 2004). Hsp70 can also be secreted from intact tumor cells

and other cell types by a non-classical pathway involving lysosomal endosomes (Mambula and Calderwood, 2006b). Hsp70, with or without tumor antigen cargo, does therefore have access to the extracellular spaces, the bloodstream and targets in the immune system.

INTERACTION OF HSP70-PC WITH IMMUNE CELLS

Extracellular Hsp70-peptide complexes (hsp70.PC) can therefore exit their home cells and interact with the host immune system. Interactions have been shown between Hsp70 and cell surfaces of macrophages, B cells, dendritic cells, natural killer cells and T lymphocytes (Srivastava, 2002). As dendritic cells (DC) are the most potent antigen processing cells (APC) in tumor immunity, we will concentrate mostly on Hsp70.PC interaction with this cell type (Larsson et al., 2001). In order for DC to stimulate a potent T cell response, at least two signaling events need to occur. These include (1) the acquisition of antigen by DC, presentation on cell surface MHC class I molecules and binding of MHC class I-antigen complexes to the T cell receptors of $CD8^+$ T cells and (2), the transmission of a second signal that leads to induction of co-stimulatory molecules such as B7 and CD40 that bind counter-receptors or ligand on the T cells and lead to a robust immune response (Figure 1).

Hsp70 (green symbol) complexed to antigenic peptide (red rectangle) binds to receptor R1 on APC which triggers internalization of the complex. A series of intracellular interactions next leads to hsp70.PC dissocation from R1, peptide dissociation from Hsp70 and with or without further protealytic processing, peptide binding to MHC class I and presentation on the cell surface (blue rectangle). The MHCI-peptide complex is then recognized by T cell receptors on $CD8^+$ T lymphocytes, leading to T cell activation. Full activation of APC to a form that

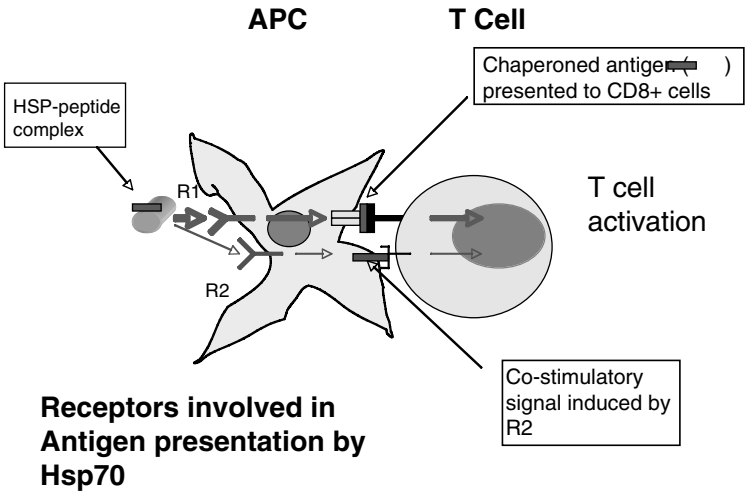

Figure 1. Mechanisms of T cell activation by hsp70 peptide complexes

can interact effectively with T cells and lead to killing of target cells requires the receipt of a second signal through receptor R2. Occupation of R2 by Hsp70 leads to the expression of co-stimulatory molecules (dark blue symbol) which migrate to the APC surface adjacent to MHCI-peptide complexes and interact with counter receptors on the T cell surface.

These second, MHC1-independent signals may be provided by adjuvants that stimulate innate immunity or by helper T cells (Engleman et al., 2004). Hsp however have been proposed to offer "one-stop shopping" as regards tumor antigen presentation, providing antigen portage plus the conferring of the second signal (Srivastava and Amato, 2001). Several Hsp including Hsp70, Hsp90, gp96 and calreticulin have been proposed to confer (1) the ability of cell extracts to cross prime $CD8^+$ T cells; this group of stress proteins is evidently necessary and sufficient for this purpose (Binder and Srivastava, 2005). Cross priming involves hsp70.PC binding, internalization and delivery of the peptide to MHC class I molecules to form MHCI-peptide complexes expressed on the APC surface (Figure 1). Previous studies indicated that Hsp-mediated uptake of tumor antigens occurs through receptor-mediated endocytosis because of the low (nanomolar) concentrations of Hsp involved and the saturability of the process, a property of binding to surfaces with limited numbers of high affinity receptors (Singh-Jasuja et al., 2000a; Singh-Jasuja et al., 2000b). Hsp60, Hsp70, gp96, and hsp110 appear to be able to (2) directly confer a second, adjuvant-like signal and lead to expression of inflammatory cytokines (IL-1β, TNFα, IL-6, IL-12) and co-stimulatory molecules (Calderwood et al., 2005a). Whether these two effects are achieved through binding to one receptor or multiple cell surface receptors is not clear. A major emphasis has therefore been placed on identifying Hsp70 receptors on the APC cell surface. Four main classes of cell surface structure have been proposed as Hsp receptors, including: (i) the CDI4/TLR 2/4 complex known to play a key role in innate immunity, (ii) the CD91 receptor involved in internalizing a number of extracellular proteins (iii) the tumor necrosis factor receptor family protein CD40, and (iv) members of the C-type lectin/Scavenger Receptor (SR) families (Asea et al., 2000b; Becker et al., 2002; Binder et al., 2000; Delneste et al., 2002; Srivastava, 2002). We have carefully evaluated these receptor types by cloning expression vectors encoding each receptor into CHO cells (normally null for Hsp70 binding activity) as described (Thériault et al., 2006; Theriault et al., 2005). The results of these experiments are summarized in Table 1.

Our current findings indicate that Hsp70 binds with relatively high avidity to the SR members LOX-1, SREC-1 and FEEL-1 and to the c-type lectins NKG2D and NKG2A (Thériault et al., 2006; Theriault et al., 2005). We observed minimal binding to c-type lectins DC-SIGN, Dectin1, CLEC-1 and CLEC2 (Table 1). In addition, we were, under the conditions of the assay unable to detect high avidity binding to CD14, TLR2, TLR4, CD91 or CD40, cell surface proteins that were previously reported as Hsp70 receptors (Table 1) (Thériault et al., 2006; Theriault et al., 2005). Nonetheless, there is little doubt that most of these receptors play key roles in the immune effects of Hsp70 (Becker et al., 2002; Srivastava, 2002). These considerations therefore imply the possibilities of (1) kinetically rapid but

Table 1. Candidate receptors for HSP7

Receptor	Type	cells	HSP70 binds?
TLR2	Signaling	APC etc	–
TLR4	Signaling	APC etc	–
CD14	Signaling	APC etc	–
CD40	Signaling	APC	–
CD91	adaptor protein	many	–
LOX-1	scavenger	APC, endo	+
DC-SIGN	scavenger	APC	–
Dectin 1	scavenger	APC	–
CLEC-1	scavenger	APC	–
CLEC2	scavenger	APC	–
SREC-1	scavenger	APC	++
FEEL-1	scavenger	?	++
NKG2A	CTL	NK	++
NKG2C	CTL	NK	++
NKG2D	CTL	NK, T cell	++

Binding to receptors was assayed in CHO transfectants with two exceptions, which are DC-SIGN, which was in K-562 (also HSP binding null) and the TLR, which were in HEK293. Binding is indicated by +, lack of binding by –

reversible Hsp70-receptor binding not detected by the equilibrum binding assay or (2) hierarchical roles for the receptors with a primary receptor such as SR providing a platform for secondary, tertiary etc receptors that may mediate signaling. An illustration of hierarchical regulation of signaling receptors after SR occupation by ligand is that SREC-1 can act in combination with TLR2 to mediate signaling in response to E. *coli* membrane protein OMPA (Jeannin et al., 2005).

UPTAKE OF HSP70-PEPTIDE COMPLEXES BY APC, ACTIVATION OF TRANSMEMBRANE SIGNALING CASCADES AND PRESENTATION OF PEPTIDES TO CELL SURFACE MHC MOLECULES

Binding of Hsp70 is succeeded by the activation of transmembrane signaling mechanisms, internalization and the delivery of the peptide cargo of Hsp70 to MHC molecules.

Hsp70-Induced APC Maturation and Pro-Inflammatory Signaling

Hsp70 can initiate a potent innate immune/inflammatory response resulting in APC maturation (Noessner et al., 2002) (Also See: *Tamura et al*, Chapter 20). APC maturation requires the activation of a gene expression program including co-stimulatory molecules such as CD40, OX40L, B7.1 and B7.2 on the cell surface in order to interact more efficiently with T cells (Figure 2) (Ohashi and DeFranco, 2002). Indeed, a key functional role for CD40 in breaking tolerance in an

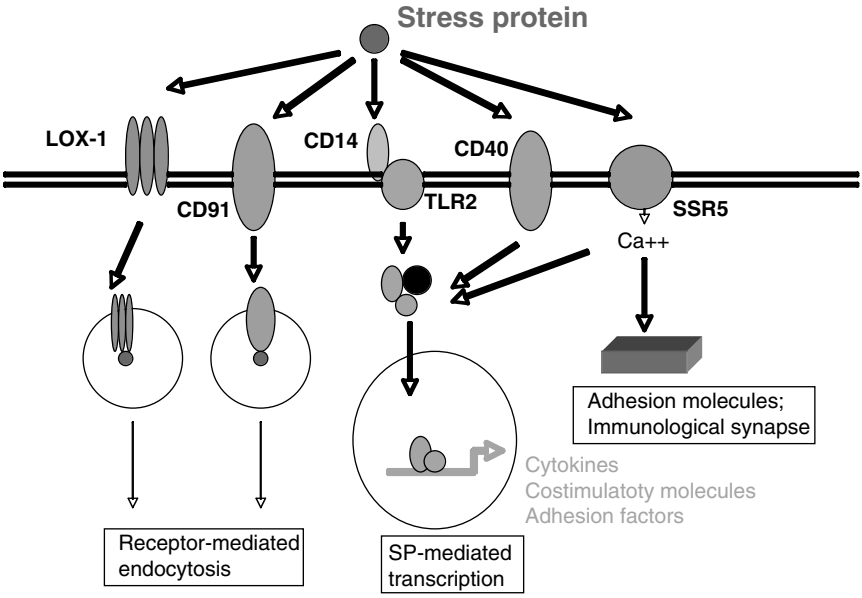

Figure 2. **Hsp70 Receptors**. Receptors known to interact with Hsp70 are shown. These can be divided into known internalizing receptors such as the scavenger receptor LOX-1 and CD91, and receptors with powerful transmembrane signaling activity such as TLR2, CD40 and SSR5. Receptors may act in parallel as shown here or may be organized into hierarchical interaction patterns. The SR can form large structures on the cell surface which could act as platforms for coordinating Hsp70 mediated signaling

autoimmune form of diabetes by Hsp70 has been demonstrated (Millar et al., 2003). It seems likely that Hsp70-induced DC maturation involves CD40 up-regulation and probably requires activation of the transcription factor NF-kB (Pulendran, 2004).

Most of the genes involved in DC maturation require the activation of NF-kB and therefore receptors that are involved in DC maturation by Hsp70 likely cause downstream activation of NF-kB (Figure 2) (Pulendran, 2004). Some intriguing studies have shown that extracellular Hsp70 induces NF-kB through the activation of the CD14/TLR signaling pathway in a CD14-dependent manner in APC (Asea et al., 2000b; Vabulas et al., 2002). This results in the expression of TNF-α, IL-1β and IL-6 as well as co-stimulatory molecules (Asea et al., 2000b; Vabulas et al., 2002). Although the role of this pathway in the innate immune response to molecular chaperones is not clear it can be activated equally effectively by Hsp70.PC and free Hsp70 (Asea et al., 2000a). However, this effect does not seem to be direct since we could find no evidence for direct binding of extracellular Hsp70 to TLR 2/4 or CD14 (Theriault et al., 2005). Some skepticism has been directed at the role of CD14 or TLR due to potential of Hsp70 contamination by endotoxin in these effects (Gao and Tsan, 2003). However, the evidence for Hsp activation of TLR signaling now seems almost overwhelming (Asea et al., 2002;

Hasan et al., 2005; Quintana and Cohen, 2005; Takenaka et al., 2004). In addition, other Hsp70 receptors are coupled to NFκB and LOX-1 has recently been shown to activate the NF-kB pathway in endothelial cells, ligand binding of CD40 can lead to NFκB activation in DC as can binding to SREC-1 (Cominacini et al., 2000; Jeannin et al., 2005; Ouaaz et al., 2002). Oxidized-LDL can also induce CD40 expression through LOX-1 suggesting a putative role of LOX-1 in APC maturation (Delneste et al., 2002; Li et al., 2003). Recent studies also show that mycobacterial hsp70 binds to the CCR5 chemokine receptors on DC promoting DC aggregation, immune synapse formation between DC and T cells and enhanced immune responses (Floto et al., 2006). It is not clear whether human Hsp70 binds to CCR5 although experiments showing increased Ca^{++} flux after Hsp70 binding suggest such and interaction as chemokine receptors signal through calcium transients (Asea et al., 2000b).

Hsp70-PC Internalization by APC and Peptide Association with Cell Surface MHC Molecules

Following receptor-induced Hsp70 internalization by APC, bound peptides may proceed through a number of potential pathways to be released from Hsp70 binding and re-presented on the cell surface by major histocompatibility complex (MHC). Hsp70.PC could be taken up by the endocytic pathway and presented to MHC class II molecules by the standard extracellular pathway of antigen presentation. In addition, Hsp70 can also present peptides to MHC class I molecules by cross-presentation (Baker-LePain et al., 2003; Srivastava, 2002). While little work has been carried out on Hsp70, Gp96 has been shown to be rapidly internalized after interacting with surface receptor(s) in a early endosomal compartment (Arnold-Schild et al., 1999; Berwin and Nicchitta, 2001). Endocytosed Gp96 co-localizes with FcR and MHC class I but not with other receptors such as CD91, transferrin, rab5a or lysosomal markers such as LAMP-1 or LAMP-2 (Berwin and Nicchitta, 2001; Singh-Jasuja et al., 2000a). It has been shown recently that Hsp70.PC are rapidly internalized by each of the SR family members LOX-1, SREC-1 and FEEL-1, strongly suggesting a role for these structures in pathways involving Hsp.PC uptake and cross-priming (Figure 2) (Thériault et al., 2006).

As mentioned above, heat shock proteins may also bind to other immune cells in addition to APC. For instance hsp60 acts as a co-stimulator of regulatory T cells by interacting with TLR2 (Zanin-Zhorov et al., 2006) and can also interact with TLR4 in B cells (Cohen-Sfady et al., 2005). These interactions which are anti-inflammatory in nature may be involved in regulating response to Hsp and in preventing autoimmunity in vaccine treated patients (Daniels et al., 2004). In addition, NK cells associate with Hsp70 on the surface of tumor cells and mediates tumor cell killing (Multhoff, 2002; Multhoff and Hightower, 1996). We have recently found that Hsp70 binds to the NK receptors NKG2A and NKG2D and these interactions could be involved in NK killing of Hsp70 expressing tumor cells (Thériault et al., 2006).

STRATEGIES FOR HSP70 BASED VACCINE DESIGN

The major pioneer in molecular chaperone-based anticancer vaccine design has been Pramod Srivastava who has prepared autologous vaccines in mice and in human patients with the direct aim of targeting the unique, often mutated antigens that characterize each individual neoplasm (Belli et al., 2002; Mazzaferro et al., 2003; Srivastava, 2002; Srivastava, 2003; Srivastava, 2000). In this approach, Hsp70.PC are isolated from the patients' tumors by affinity chromatography using ATP-agarose and formulations of Hsp70 applied in a multi-dose regimen. The aim is for Hsp70.PC to be cross-presented by the patient's APC and for the unique mixture of peptides from the individual tumor to induce immunity. These studies build on the pioneering work of Srivastava and Old who showed that experimentally induced cancers of inbred mice and rats elicit stronger individually tumor specific immunity (which is not tumor-type specific) (Srivastava and Old, 1988). These studies are in Phase III trials although current results suggest few major differences between controls and vaccine treated patients (Srivastava, 2006). Success may be limited by the amount of tumor available after resection as response to treatment evidently is related to the number of treatments with Hsp vaccine (Srivastava, 2006). Other limiting factors may be structural and relate to the ability of individual Hsps to bind peptides, their peptide repertoire and ability to induce a co-stimulatory response. For instance *hsp70* gene family member Hsp110 has a greatly enhanced ability to bind peptides compared with other Hsps, can bind avidly to large polypeptides and is a superior agent in cancer vaccine production than smaller Hsp (Manjili et al., 2003; Segal et al., 2006). Recent studies have suggested that larger polypeptides are superior to smaller peptides in inducing immunity (Rock et al., 2005; Shen and Rock, 2006).

Another approach may be to use combinations of chaperones with the hope of increasing the repertoire of peptides presented to APC. Indeed the studies of Binder and Srivastava (2005) indicate that Hsp70, gp96, hsp90 and calreticulin carry between them the whole of the intracellular peptide repertoire required for cross priming T cells against ovalbumin or β-galactosidase. An approach using what are described as "chaperone rich lysates" has been described in which tumor cell lysates are partially purified by iso-electric focusing to enrich the above chaperones (Zeng et al., 2006). Such preparations are effective against mouse tumors in prophylactic context. Curiously, although the lysates are prepared under highly denaturing conditions for IEF using molar concentrations of urea, the preparations evidently retain peptides and are able to induce antigen specific responses (Zeng et al., 2006). These studies are supported by a recent publication showing that the effectiveness of an Hsp70-based vaccine derived from tumor-DC fusion cells was partially dependent on co-isolation of Hsp90 (Enomoto et al., 2006). Use of multichaperone formulations may thus be indicated. This study also showed that gentle and rapid isolation of the hsp70 is optimal for retaining hsp70-peptide interactions and immunity and that peptides are lost when Hsp70 purification by affinity chromatography is employed (Enomoto et al., 2006). These studies used MUC1, a non-mutated antigen whose expression and processing is altered in cancer. Mice expressing

MUC1 became tolerant to this antigen while the Hsp70-based vaccine was able to overcome tolerance and cause tumor rejection, suggesting that non-mutated antigens may be targeted by Hsp70-based vaccines (Enomoto et al., 2006). The effectiveness of the Hsp70-based vaccine derived from tumor-DC fusion cells was dependent on stimulation of innate immunity and was ineffective in MyD88 knockout cells further indicating a role for TLR.

Other approaches emphasize the inflammatory nature of Hsp70. An approach devised by Vile *et al* involves targeted killing of normal melanocytes overexpressing HSP70 to generate an antigen-specific $CD8^+$ T lymphocyte response against established melanoma (Calderwood, 2005a; Daniels et al., 2004). The rationale behind this unorthodox approach is that the majority of peptides that are presented by melanoma cells and recognized by T cells from patients arise from developmental proteins that are also expressed in normal melanocytes (Engelhard et al., 2002). The existence of shared antigens between the proliferating melanocytes and the melanoma cells suggested that if a $CD8^+$ T cell response could be generated against the dying melanocytes it could also target the tumor cells (Daniels et al., 2004). This approach requires the specific targeting of proliferating melanocytes using local expression of a cell suicide gene (HSVtk) under the control of a promoter from the *tyrosinase* gene that is specifically active in this cell population. For tumor rejection, the critical requirement was that killing take place in melanocytes engineered to overexpress HSP70 (Daniels et al., 2004). Cell death in the sacrificed population leads to the extracellular release of the HSP70, modulation of DC function, and generation of a $CD8^+$ T cell response against melanocytic TSTA that eradicates primary and metastatic melanoma (Daniels et al., 2004). The preclinical studies in mice show the feasibility of this approach and its potential for translation for clinical treatment of malignant melanoma (Daniels et al., 2004). One further question is whether such an approach could be generalized to the treatment of other tumors at different sites. This approach would require the targeted sacrifice of a population of normal cells with an antigenic repertoire similar to the tumor under treatment. A related approach, involves local killing of tumor cells with forced overexpression of Hsp70, obviating the need to find a related normal tissue for priming antitumor immunity (Huang et al., 2003; Todryk et al., 1999). Of course, the question of potential autoimmune destruction of normal tissues, especially as this approach depends on the generation of $CD8^+$ T lymphocytes directed against antigens common to normal cells and tumor cells is a concern with this approach (Daniels et al., 2004). The targeting of the small fraction of proliferating melanocytes may however limit the extent of normal cell targets. In addition, the studies of Vile *et al* show that the effects of the treatment are self-regulatory, through the activation of $CD4^+CD25^+$ lymphocytes, which inhibited the activity of tumor-specific $CD8^+$ T lymphocytes and rapidly attenuated the response (Daniels et al., 2004). It is interesting to speculate that this may be due to delayed triggering of the anti-inflammatory effects of heat shock proteins which can activate the $CD4^+CD25^+$ population of lymphocytes (Chandawarkar et al., 1999; Quintana et al., 2004).

The outcome of immunotherapy approaches involving Hsp may therefore depend on the relative potencies of their pro-immune and anti-inflammatory effects.

CONCLUSION

Anti-cancer vaccines prepared using Hsp70 as a primary ingredient are beginning to show promise in animal studies and clinical trials, although it is clear that further advances may be required to increase their effectiveness. These advances depend partially on increasing our understanding of the immunobiological properties of Hsp70. The potential of the Hsp as vaccines depends on their ability to function both in cross-priming of T cells and as immunological adjuvants. Understanding of these effecst has been delayed by the open question of the key receptors that mediate Hsp interaction with immune cells. However, discovery of the SR as primary Hsp70 receptors and TLR and CCR5 as signaling receptors may lead the way for rapid development of this approach.

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CHAPTER 19

PILOTING OF EXOGENOUS ANTIGEN INTO CROSS-PRESENTATION PATHWAY BY HEAT SHOCK PROTEINS

YASUAKI TAMURA, GORO KUTOMI, JUN OURA, TOSHIHIKO TORIGOE AND NORIYUKI SATO

Department of Pathology, Sapporo Medical University, School of Medicine, South 1 West 16, Cho-ku, Sapporo 060-8556, Japan

Abstract: Recent evidences have been indicating that heat shock proteins (HSPs) play an important role as a "danger signal" in the extracellular milieu on behalf of immune surveillance. Above all, Hsp70, gp96 and Hsp90 have been shown to elicit intriguing efficient CTL responses by so called "cross-presentation" with yet entirely unknown mechanism. Here, we discuss that the immunologic roles of HSPs, particularly Hsp90, in the MHC class I-restricted cross-presentation by bone marrow-derived dendritic cells (DCs). We show that Hsp90-peptide complex enters the endocytic pathway via putative Hsp90 receptor and associated peptide might be transferred onto endosomal MHC class I molecules. Moreover, we show that immunization with Hsp90-peptide complex efficiently elicits CTL responses and antitumor effect. Interestingly, this presentation is TAP-independent, but rather follows endocytic pathway. Meanwhile, when Hsp90-whole protein (OVA) antigen complex was pulsed to DCs, this protein antigen could enter at least in part via TAP-dependent pathway to the ER, and finally was presented to MHC class I molecules. However, OVA alone without Hsp90 could not enter into this pathway, but rather into MHC class II pathway.

Here we discuss novel insights into the immunologic role of Hsp90 in cross-presentation of antigens, efficient induction of MHC class I-restricted CTL responses, and application to peptide/protein antigen-based immunotherapy of cancers

Keywords: Heat shock protein, antigen presentation

INTRODUCTION: HSP-MEDIATED CROSS-PRESENTATION BY ANTIGEN-PRESENTING CELLS

It has been well demonstrated that immunization with tumor-derived HSPs or HSP complexed with an antigen peptide elicits tumor- or antigen-specific $CD8^+$ T cell responses (also See: *Bonorino & Souza*, Chapter 10, *Gong & Calderwood*, Chapter 18, this volume). Above all, Hsp70- and gp96-antigen complexes are well-studied and have been shown to be immunogenic and potent in stimulating the

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generation of tumor-specific CTLs. Hsp70- and gp96-based vaccines have been tested in early-phase clinical trials in solid tumors as well as in lymphoma and leukemias; all showed minimal toxicity and potential efficacy (Castelli et al., 2004; Mazzaferro et al., 2003; Rivoltini et al., 2003). Phase III clinical trials using tumor-derived Hsp70 and gp96 as vaccines are ongoing for melanoma and renal cell carcinoma.

The ability of HSPs to facilitate the cross-presentation of MHC class I-restricted epitopes and to prime $CD8^+$ T cell effector responses is well established (Delneste, 2004; Doody et al., 2004). Although, immunized HSPs are exogenous antigens, these HSP-antigen complexes can gain access to the class I antigen presentation pathway, resulting in cross-presentation. The immune response has been attributed to the ability of HSPs to form stable complexes with tumor-derived antigenic peptides, thereby facilitating the cross-presentation of MHC class I-restricted epitopes and priming of $CD8^+$ T cell responses.

Dendritic cells (DCs) are main conductor for efficient cross-presentation. Recent reports have shown that antigen-presenting cells (APCs) such as dendritic cells can internalize HSPs by receptor-mediated endocytosis and direct chaperoned proteins/peptides into the intracellular pathway for MHC class I-restricted presentation to $CD8^+$ T cells, concomitant with the induction of dendritic cell maturation and cytokine secretion. In fact, some HSP receptors expressed on APCs have recently been identified. CD91 (Binder et al., 2000), LOX-1 (Delneste et al., 2002), CD40 (Becker et al., 2002) and SR-A(Berwin et al., 2003) have been proved to be common receptors for HSPs. However, the underlying mechanism for efficient cross-presentation, in particular, how the HSP-antigen complex can enter the MHC class I pathway, remains unclear.

Furthermore, recent studies have also shown that HSP-peptide complexes can also lead to antigen presentation on MHC class II molecules, thus activating $CD4^+$ T cells (Haug et al., 2005; SenGupta et al., 2004). Therefore, it is possible that uptake of HSP-peptides complexes leads to antigen presentation on both MHC class I and class II molecules on dendritic cells, thus activating $CD8^+$ CTL as well as $CD4^+$ T cells. However, Shild et al. have reported that, although antigen peptides chaperoned by gp96 can be presented in the context of both MHC class I and class II molecules, immunization with gp96 elicits CD8-biased T cell responses (Ramirez et al., 2005). Doody et al have also demonstrated same results (Doody et al., 2004). Therefore, it is essential to know the effects of the HSP-antigen complex on tumor antigen presentation via class I and class II pathways in vivo because such knowledge is crucial for the development of effective HSP-based immunotherapies, especially in the case of protein antigens in association with HSPs.

In contrast to Hsp70 and gp96, the role of Hsp90 in cross-presentation remains unclear. Hsp90 is the most abundant protein in the cytoplasm; therefore it is assumed that Hsp90 also plays an important role in cross-presentation. However, it is not clear whether Hsp90 is involved in tumor immunity. Udono reported, for the first time, that immunization with Hsp90 purified from tumor elicited tumor-specific CTL responses (Udono and Srivastava, 1994). Very recently, Kunisawa and Shastri reported that Hsp90 chaperoned C-terminal flanking antigenic peptides

(Kunisawa and Shastri, 2006). These results have led to much interest in the importance of Hsp90 in antigen presentation. Taking these facts into consideration, it is conceivable that Hsp90 does participate in antigen presentation and possibly cross-presentation. In this chapter, we will discuss the mechanism of HSP-mediated cross-presentation and the players involved in this intriguing immune response.

HSPS ACT AS DANGER SIGNALS TO THE IMMUNE SYSTEM THROUGH HSP RECEPTORS EXPRESSED ON APCS

It is suggested that extracellular HSPs act as "danger signals" to the immune system in the case of life-threatening events (Figure 1).

When viral infection or tumor cell damage occurs, cell-associated antigens, such as HSPs-antigen complexes are released into the extracellular milieu, APCs such as dendritic cells and macrophages, detect the signals through certain receptors, resulting in intracellular signaling (Basu et al., 2000). HSP receptors are divided into 2 categories, one is toll-like receptor (TLR) for mainly signaling for DC maturation and activation, and the other is endocytic receptors for cross-presentation. TLR-2 has been shown to be the receptor for Hsp70 (Asea et al., 2002). TLR-4 is the receptor for Hsp70 (Asea et al., 2002; Vabulas et al., 2002). However, doubts were raised as to what extent this effect was due to lipopolysaccharide contaminations of the

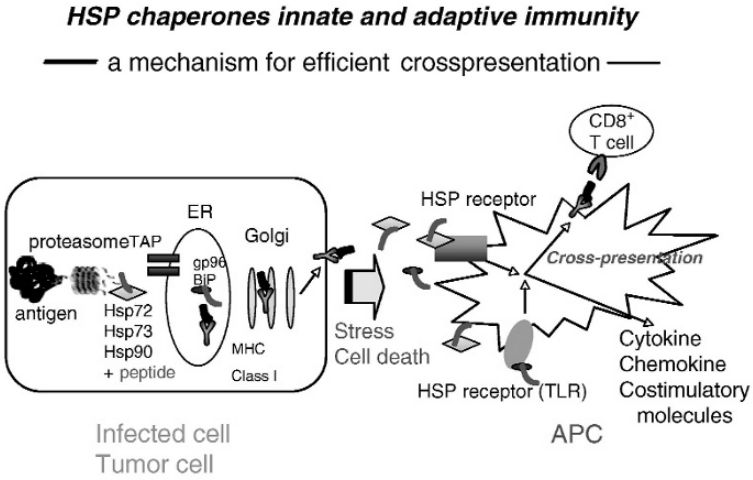

Figure 1. **Role of extracellular HSP-peptide complexes as danger signals.** HSP-antigen complexes are acquired by bone marrow-derived antigen presenting cells (APCs) and are cross-presented to cytotoxic T lymphocytes (CTLs). HSP-antigen complexes bind to Toll-like receptor (TLR) and induce maturation and activation of dendritic cells (DCs). On the other hand, HSP-antigen complexes also bind to HSP receptor, such as CD91, LOX-1, SR-A, on the DCs, followed by endocytosis. Internalized HSP-antigen complexes are shuttled into MHC class I pathway and induce antigen-specific CTL response. The HSP-antigen complexes elicit both innate and adaptive immunity simultaneously, indicating that HSP-peptide complexes act as effective danger signals

HSP preparations. It is required re-examination for this phenomenon using HSPs, nominally endotoxin-free. In contrast, HSP-specific endocytic receptors expressed on the APCs, including CD91 for gp96, Hsp70 and Hsp90 (Basu et al., 2001; Binder et al., 2000), SR-A for gp96 and calreticulin(Berwin et al., 2003), LOX-1 for Hsp70 (Delneste et al., 2002), and CD40 for Hsp70 (Becker et al., 2002), were identified. However, it is still unknown how HSP-antigen complexes are transported and where HSP releases chaperoned antigens. What is the fate of HSPs after endocytosis? What actors are responsible for translocating the antigen from the endosome to cytosol? Additional studies will be required to understand these unsolved issues.

THE ROADS TO MHC CLASS I PRESENTATION

MHC class I molecules principally present peptides derived from endogenous protein to cytotoxic T cells. However, in certain antigen-presenting cells, peptides derived from exogenous antigens also are presented by MHC class I molecules. At least four independent pathways of protein processing and subsequent peptide presentation by class I molecules have been described. The dominant pathway uses endogenously synthesized proteins that have been processed in the cytosol by proteasomes. Peptides are transferred by transporter-associated antigen processing (TAP) to the ER where they bind in the grooves of nascent MHC class I molecules. The peptide/class I MHC heavy chain/β_2M complex is then transported via the Golgi apparatus to the cell surface. In second and third pathways, exogenous antigens are internalized and processed into peptides that are transported to the ER to bind MHC class I. One is the cytosolic leak of internalized antigens by phagocytosis, macropinocytosis, and enocytosis, resulting in degradation by proteasomes (Hotta et al., 2006; Rodriguez et al., 1999). Degraded peptides are then transported through TAP into the ER. However, the mechanism for the translocation of exogenous antigens to cytosol and players involved in this translocation remain unknown. Another pathway is via an ER-phagosome fusion. When exogenous antigens are engulfed into phagosomes, the phagosomal membrane and ER membrane fuse with each other, forming ER-phagosome compartments (Guermonprez et al., 2003; Houde et al., 2003). These ER-phagosome fusion compartments involve TAP molecules and proteasomes outside of the membrane (Ackerman et al., 2003). Phagocytosed antigens are pumped out the ER-phagosome fusion compartment through sec61. This is called the ER-associated degradation (ERAD) mechanism. Then proteasomes, which are attached to the outer face of the membrane of ER-phagosome fusion, degrade antigens into peptides, followed by entry into the ER again through TAP molecules, and the resulting antigen peptides bind to MHC class I molecules (Ackerman et al., 2005; Ackerman et al., 2006). In contrast, at least some exogenous peptides or proteins appear to reach MHC class I through a pathway completely independent of the ER. In this fourth pathway, endosomal processing and endocytosed class I MHC molecules may be involved. MHC class I molecules have been shown to internalize from the cell surface in T cells, B cells, fibroblasts, and macrophages. Recycling of endocytosed class I MHC molecules back to the

cell surface has also been observed (Gromme et al., 1999). The endocytosis and recycling of class I MHC may facilitate peptide exchange, allowing class I MHC molecules to bind multiple peptides in one lifetime. Antigen presentation mediated by the three types of pathways mentioned above is called cross-presentation, allows display of exogenous antigens in the context of MHC class I molecules. This is particularly important in host defense against infectious diseases and cancers that cannot access the classical pathway for MHC class I presentation.

Internalization of exogenous antigens may allow cell fragments, intracellular pathogens and proteins to be degraded in the endocytic pathway by mechanisms involving reduction, unfolding and lysosomal proteolysis. Such a process could contribute to cross-presentation by facilitating the exchange of previously loaded MHC class I-associated peptides for newly generated peptides derived from exogenous proteins. MHC class I internalization from the cell surface is regulated via monoubiquitination of a conserved lysine in the MHC class I cytoplasmic tail. Lysine modification also regulates the inclusion of MHC class I molecules into multivesicular bodies, where they colocalize with MHC class II on the membranes of the internal vesicles. In a number of recent studies, MHC class I molecules were detected within all of the described endocytic compartments (Kleijmeer et al., 2001). Early endosomes, characterized by the presence of transferring receptor, were shown to contain 17% of class I heavy chain (HC) within endocytic compartments. The multivesicular late endosomes (LEs) and lysosomes, identified by the presence of LAMP-2 and CD63, contained 56% and 27% of endocytic class I HC, respectively. Importantly, β_2M could also be detected within each of these endocytic compartments, suggesting the possibility that stable MHC class I complexes exist in these compartments. Cycloheximide treatment, which blocks the biosynthetic pathway of MHC I, indicated that the class I present in the endocytic compartments was derived from the cell surface. We have confirmed the presence of MHC class I in the endocytic compartments of murine bone marrow-derived DCs. Furthermore, as described recently, we have demonstrated that co-localization of the receptor-mediated endocytosed exogenous Hsp70/90-antigen complex with MHC class I in the early endosomes of the DCs. These observations suggest that antigens derived from the exogenous Hsp70/90 may be loaded onto recycling MHC class I, after which the MHC class I/peptide complex is transported to the cell surface.

Considering the significance of the HSP-peptide complex in cross-presentation, this model suggests that a ‘pre-processed antigen’ would be required as it would be inefficient for a whole protein to be degraded non-specifically within the endocytic compartments by resident catabolic enzymes. Not only would this be a slow process, it would be by chance alone that an appropriate peptide capable of binding the MHC class I groove would be generated. The notion of such preprocessed antigens fits well with a role for HSPs as chaperones for peptide antigens. Proteins synthesized within the cell would be processed within the endogenous class I antigen presentation pathway leading to the generation of HSP-peptide complexes. These complexes are ideal chaperones of antigenic peptides for the transfer of antigens to DCs for a number of reasons. Once generated within the cell, the HSP-peptide complex

might be released into the extracellular milieu during cell necrosis because of viral infection and intervention of cancer, resulting in taking-up by the immature DC and acting as a danger signal. At the same time, antigenic peptides chaperoned by HSPs are efficiently presented in the context of MHC class I and class II molecules and immediately activate the host's immune responses. As described earlier, Hsp90 binds precursor (pre-processed) peptides generated in the cells and thus, endosomal processing is a suitable mechanism for pre-processed peptides. HSP would also serve to protect the peptide antigen from degradation upon entry into the endocytic compartment of the DC. Finally, it has been suggested that some HSP family members may be capable of facilitating the loading of MHC class I molecules by an unknown mechanism. In addition, an as yet uncharacterized lysosomal enzyme may play a role in the processing of internalized antigens for generation of MHC I epitopes. Recently, Rock et al. have demonstrated that DC-restricted cathepsin S plays an important role in the processing of exogenous antigens for the generation of MHC I antigenic determinants in the early endosome (Shen et al., 2004).

SIGNIFICANCE OF CROSS-PRESENTATION IN VIVO: IMPACT ON EPITOPE GENERATION VIA ENDOSOMAL PROCESSING AND PROTEASOMAL PROCESSING

Although DCs are capable of using an endocytic exchange mechanism to create MHC class I-peptide complexes, typical somatic cells have only the classical pathway for the generation of MHC class I-presenting peptides. For $CD8^+$ T cells induced by cross-presentation to be functionally useful against pathogen-infected cells, it would seem that the epitope generation mechanism used should be the same as those in classical MHC class I processing. An endosomal exchange mechanism in which peptides are generated by different proteases in radically different conditions from those in the endogenous pathway, therefore seems unlikely to contribute substantially to the $CD8^+$ T cell repertoire. Cytoplasmic processing, including proteasomal proteolysis, and ER-based trimming would be expected to be involved to generate the same peptide sequences as those made by nonhematopoietic cells. In fact, although partial proteolysis may occur in the endocytic pathway, extensive experimental evidence suggests that exogenous antigens must reach the cytoplasm to be efficiently cross-presented. In DCs, the presentation of exogenous antigens is unaffected by both chloroquine and inhibitors of lysosomal proteolysis. Exogenous antigen presentation is, however, highly sensitive to specific inhibitors of the proteasome, indicating that cytoplasmic proteolysis is the main form of epitope generation in the cross-presentation pathway. In contrast, the HSP-mediated cross-presentation pathway has been shown to involve both a proteasomal pathway and an endocytic-recycling pathway. We have demonstrated that a tumor-derived Hsp70-peptide complex is efficiently cross-presented to peptide-specific CTLs by DCs and this presentation is dependent on TAP molecules. In addition, in vitro generated Hsp70- and gp96-antigen complexes have been shown to be cross-presented via

a TAP-dependent parhway (Castellino et al., 2000; Moroi et al., 2000; Suto and Srivastava, 1995). This fact suggests that processing and loading a peptide onto MHC class I requires translocation of the antigen from the endocytic compartment to the cytosolic pathway. Rodriguez et al. demonstrated that DCs have a unique membrane transport pathway linking the lumina of endocytic compartments and the cytosol (Rodriguez et al., 1999). Thus, in DCs, the exogenous HSP-chaperoned antigen in the endocytic compartment is released into the cytosol, where it follows the classical proteasome- and TAP-dependent class I pathway for presentation. Further studies to define the precise mechanisms for Hsp70- and Gp96-chaperoned peptide trafficking may reveal a new paradigm for cross-presentation.

WHO GETS TO 'EAT' … AND HOW TO 'EAT', THAT'S THE QUESTION!

Despite the extensive studies of cross-presentation, the precise identity of the APCs responsible has been hotly debated. Although the cross-presenting cell is cleary an APC of hematopoetic origin, cross-presentation activity has been detected in both macrophage and DC populations. Because of the lack of critical markers distinguishing these populations, the outcome may have been complicated by small contaminating fractions of the other cell type. More comprehensive studies extensively examining splenic and lymph node DC subsets in mice have identified $CD8^+$ DCs as the only population capable of cross-presenting ovalbumin and herpes simplex epitopes. Despite the capacity of macrophages and other DC subsets to aquire antigens, the ability to effectively stimulate cytotoxic T lymphocyte responses seems to be restricted this population (Pooley JI 2001, Smith JI 2003). However, for HSP-mediated cross-presentation, it has been reported that both macrophages and DCs are involved and the critical cell subsets in vivo must be defined. Tobian et al. reported that bacterial Hsp70 promotes alternate MHC class I processing through an endocytic mechanism in macrophages and a cytosolic mechanism in DCs (Tobian et al., 2004). This diversity of processing mechanisms may explain differences among studies regarding the relative roles of cytosolic and endocytic processing for MHC-I cross-presentation of exogenous Ags, including peptides chaperoned by HSPs. In addition, recent reports have demonstrated several types of HSP-receptors expressed by APCs, so it is necessary to clarify the method of endocytosis/phagocytosis for cross-presentation by the HSP-antigen complex and how cross-presentation is affected by the uptake mechanisms of the HSP-antigen complex. For example, different endocytic receptors may have different functional consequences.

Cross-Presentation by Exogenous Hsp90-Peptide Complex

Hsp90 is a one of the most abundant proteins within cells and is overexpressed in many cancer cells. Therefore, once cancer cells become necrotic, much Hsp90 would be released from cells and might be a danger signal, subsequently eliciting

cell-specific immune responses. It has been demonstrated that the tumor-derived Hsp90-peptide complex elicits tumor-specific immunity. At present, however, the processing pathway yielding the transfer of exogenous Hsp90-associated peptide antigens to MHC class I molecules is unknown.

We examined the roles of Hsp90 in MHC class I-restricted cross-presentation using bone marrow-derived dendritic cells (DCs) as APCs. First, we tested whether Hsp90-peptide complexes reconstituted in vitro were taken up and associated peptides presented in the context of MHC class I molecules by DCs. To monitor the MHC class I antigen-processing pathway, we used Hsp90 reconstituted *in vitro* with the C-terminal extended version of VSV8 (RGYVYQGL), VSV-C (RGYVYQGLKSGNVSC: 15mer) to moniter the processing of the precursor peptide. The Hsp90-VSV-C peptide complex was cocultured with DCs for 2 hours, followed by incubation with a VSV8-specific CTL clone. The culture supernatant was assayed for the production of IFN-γ. VSV-C-loaded Hsp90 was processed and presented by H-2K^b and recognized by the VSV8-specific CTL clone, but not Hsp90 or VSV-C alone. In the presence of an anti-H-2K^b mAb during the presentation assay, the presentation of VSV8 to the specific CTL clone was completely abolished. These data suggested that Hsp90-bound VSV-C peptides were processed to VSV8 within the cells with subsequently gained access to the MHC class I pathway. Intriguingly, this presentation occurred within 15 min, indicating that very rapid and efficient processing might be achieved within DC.

Next, we investigated whether the Hsp90-mediated MHC class I pathway required functional TAP molecules. To test this, we used DCs derived from the TAP1$^{-/-}$ mouse. Surprisingly, DCs from the TAP1$^{-/-}$mouse could also process and present Hsp90-bound VSV-C peptides as efficiently as DCs from the wild-type mouse.

We also tested another well-characterized H-2K^b-restricted OVA$_{258-265}$ antigen system, Hsp90 reconstituted *in vitro* with the C-terminal extended version of SL8 (OVA$_{258-265}$), SL8-C peptide (OVA$_{258-270}$: 13mer). The Hsp90-SL8-C peptide complex was cocultured with DCs for 2 hours, followed by incubation with SL8-specific B3Z T cell hybridoma. As shown in Figure 3B, the Hsp90-SL8C peptide complex was processed and presented by H-2K^b and recognized by B3Z T cell hybridoma, but not Hsp90 or SL8-C alone, in a TAP-independent manner. These experiments demonstrate that a TAP-independent pathway is used for Hsp90-mediated MHC class I presentation.

Intracellular Localization of Endocytosed Exogenous Hsp90-Peptide Complexes

Using laser confocal microscopy, we found that Hsp90-peptide complexes accumulated only in the endosome and did not reach the stage of the lysosome. We also examined whether Hsp90 accumulation in the endosome was due to temperature-dependent endocytosis. As expected, at 4°C, labeled Hsp90 remained on the cell surface, but internalization was evident after incubation at 37°C following a 10-min internalization period. According to a competition assay, DCs express Hsp90

receptor on the cell surfaces. Identification of receptor (s) will be necessary to elucidate the mechanism responsible for cross-presentation of exogenous Hsp90-antigen complexes.

The Pathway for Hsp90-Mediated Cross-Presentation

To investigate where the Hsp90-associated antigenic peptides bind to MHC class I molecules, we stained H-2K^b molecules and exogenous Hsp90-peptide complexes. After 20 min of endocytosis, Alexa-labeled Hsp90 colocalized with endocytosed H-2K^b molecules. The results showed the internalization and co-localization of H-2K^b and Hsp90 was evident in the early endosome. This finding suggested that HSP-bound peptides might be transferred to MHC class I molecules in the endosome, where recycled MHC class I molecules from the plasma membrane are available. The peptide-MHC class I complexes generated in the endosome are then transported to the cell surfaces of the DCs, where specific CTLs recognize them.

Recycling of endocytosed MHC class I molecules back to the cell surface has been observed. Some of the recycled MHC class I molecules can be loaded into endosomes with peptides derived from endocytosed molecules (Gromme et al., 1999). Therefore, to confirm whether this presentation really utilizes recycled MHC class I molecules, we treated DCs with primaquine, which blocks the membrane recycling pathway. DCs incubated in the presence of this drug could not present the Hsp90-chaperoned SL8C-derived SL8 peptide. This result indicated that precursor peptides chaperoned by Hsp90 or processed peptides entered into recycling endosomes and transferred onto recycling MHC class I molecules, which returned to the cell surface and stimulated B3Z T cell hybridoma. To analyze the involvement of vacuolar acidification of endosomal compartments, DCs were incubated with Hsp90-SL8C in the presence of chloroquine, a known inhibitor of acidification of endosomal compartments. Chloroquine strongly inhibited Hsp90-mediated presentation without affecting SL8 peptide presentation. Thus, acidification of endosomal compartments is necessary for processing of Hsp90-chaperoned precursor peptides (Figure 2).

Hsp90-Chaperoned Precursor Peptides are Processed by the Endosomal Protease

We used protease inhibitors to investigate the proteolytic processes involved in the Hsp90-mediated TAP-independent cross-presentation pathways. In wild-type DCs, a broadly active cysteine protease inhibitor, leupeptin, almost completely inhibited the cross-presentation of Hsp90-SL8C. In contrast, the aspartic protease inhibitor pepstatin did not affect the cross-presentation. Cathepsins S, D and L are known to be the major cysteine proteases in endocytic compartments. We therefore examined the roles of various cathepsins in this pathway. Cathepsin D- and cathepsin L-specific inhibitors did not affect the cross-presentation, whereas a cathepsin S inhibitor completely blocked cross-presentation. Cathepsin S is a cysteine protease

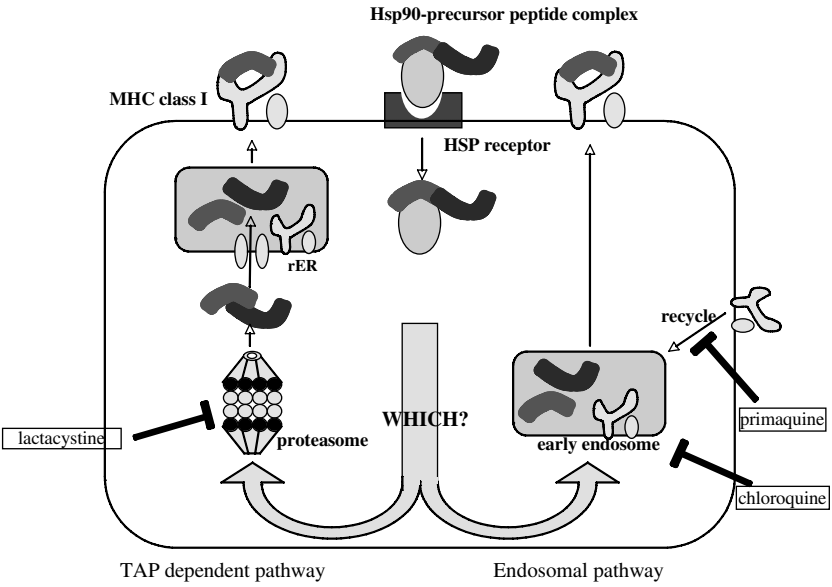

Figure 2. **Possible pathway of the Hsp90-antigen complex-mediated cross-presentation.** To elucidate how the Hsp90-antigen complex is processed for cross-presentation, we used DCs derived from TAP knockout mouse, and DCs were treated lactacystin as a proteasome inhibitor, chloroquine for blocking the acidification of endosomal compartment, and primaquine for blocking the recycling membrane transport

that is preferentially expressed in APCs, including DCs, macrophages, and B cells within endocytic compartments. Therefore, our data indicate that cathepsin S is a critical enzyme in TAP-independent Hsp90-mediated cross-presentation on MHC class I molecules and that the presented peptides are indeed generated in endosomal compartments.

Advantages of Hsp90-Antigenic Protein Complexes

As described above, we have shown that Hsp90-chaperoned precursor peptides are efficiently processed and presented by MHC class I molecules. To extend the range of HSP-based immunotherapy, we examined whether whole protein antigens chaperoned by Hsp90 were processed and presented by MHC class I molecules as well as class II molecules. The advantages of using protein antigens for cancer immunotherapy are that they can (1) provide an inherent polyvalent vaccine for $CD8^+$ T cells, and (2) they include $CD4^+$ helper epitopes, required for efficient CTL induction and proliferation. However, protein antigens themselves are not primarily immunogenic and therefore an immunostimulatory adjuvant is necessary for effective T cell responses. Given the well-known ability of HSP to forms complexes with naturally synthesized proteins, it is possible that Hsp90-protein antigen complexes could elicit antigen-specific CTL responses and Th responses

as well. Therefore, we investigated the impact of Hsp90 on the presentation of exogenous protein antigens using OVA as a model antigen. We observed that the Hsp90-OVA complex generated in vitro was very efficiently and selectively presented via the MHC class I pathway both in vitro and in vivo, and that cross-presentation involved both TAP-dependent and -independent pathways described below. These results would provide a rationale for the development of novel vaccination strategies for cancer immunotherapy.

HSP90-OVA COMPLEX IS EFFICIENTLY CROSS-PRESENTED BY DCS

We evaluated cross-presentation of the Hsp90-OVA protein complex. DCs were pulsed with Hsp90 alone, free OVA, a simple mixture of the two or the two in a complex generated in vitro for 2 hrs at 37°C, then fixed, washed and cultured with B3Z $CD8^+$ T cell hybridoma. The Hsp90-OVA complex elicited strong CTL responses, whereas Hsp90 or OVA alone did not lead to CTL responses. Notably, when we pulsed the simple mixture of Hsp90 and OVA, we did not detect significant CTL responses. These results show that binding to Hsp90 is essential for cross-presentation of OVA.

The Mechanism for Translocation of Hsp90-Chaperoned Antigens from Endosomes to Cytosol

The mechanism for escaping to the cytosol remains unknown, as is whether the Hsp90-antigen complex or its components separately escape to the cytosol. It is possible that the Hsp90-antigen complex first needs to be preprocessed in the endosomal compartments before being transferred to the cytosol to be further degraded by proteasomes. One possibility is that the mildly acidic pH in the endocytic compartments plays an important role for the transport of ingested antigens, and another is that delayed fusion with the late endosome/lysosome is important for the transport. Immature DCs maintained mildly acidic pH in the endocytic compartment even after antigen uptake and could transport these antigens into the cytosol (Hotta et al., 2006). Chloroquine treatment inhibits the acidification of the endocytic compartments. Our data indicated that chloroquine treatment inhibited Hsp90-OVA presentation by DCs in both TAP-dependent and -independent pathways. This suggested that antigen transport was dependent on mildly acidic pH-inducible machinery in the endocytic compartments of DCs. However, a recent report showed that treatment with chlorquine or NH_4Cl enhanced the efficiency of cross-presentation (Accapezzato et al., 2005). These treatments accelerated export of exogenous soluble antigens from endocytic compartments to cytosol, thereby enhancing cross-presentation. The difference between our results and theirs was the method of uptake by DCs. In our case, it occurred via receptor-mediated endocytosis, whereas they indicated that it occurred via phagocytosis or macropinocytosis. However, the regulators for the transport are still unclear. We

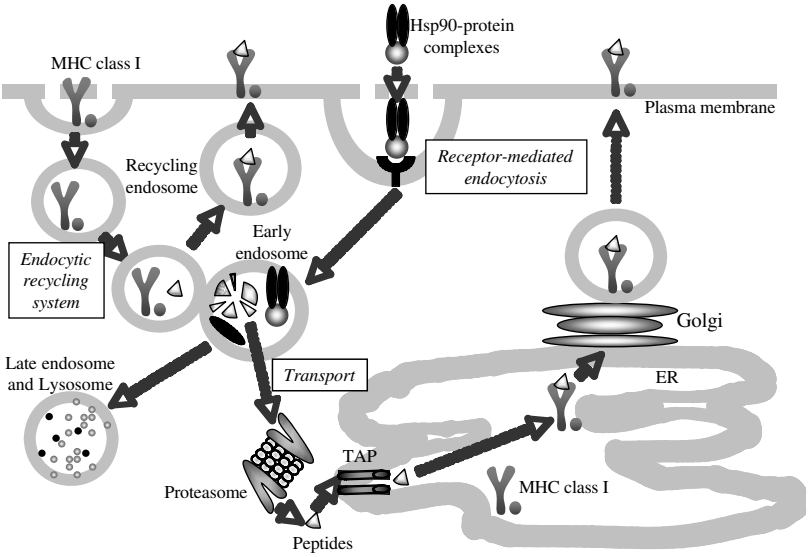

Figure 3. **The pathway for the Hsp90-antigen complex-mediated cross-presentation by DCs.** Internalized Hsp90-antigen complexes through receptor-mediated endocytosis follow 2 distinct MHC class I pathways. (1) Internalized Hsp90-antigen complexes are translocated to the cytosol and resultant peptide intermediates are imported into the ER in a TAP-dependent fashion. Hsp90-chaperoned antigens are degraded in the cytosol by proteasome and further trimmed by cytosolic peptidases. The resulting peptides are transported into the lumen of the ER for loading on newly synthesized MHC class I molecules. (2) Alternatively, internalized antigens chaperoned by Hsp90 are processed and loading in the endocytic pathway onto MHC class I molecules that are recycled from the plasma membrane, independently of TAP

are still on the road to complete understanding of HSP-mediated immune regulation, and further studies will be required to elucidate the precise mechanism (Figure 3).

CONCLUSION

Although HSPs are primarily cytosolic proteins, they play an important role as a "danger signal" in the extracellular milieu on behalf of immune surveillance. Above all, Hsp90 is one of the most abundant cytosolic proteins and elicits intriguingly efficient and rapid CTL responses. In this meaning, Hsp90 is a "smart and excellent guide" for the MHC class I cross-presentation pathway. A forthcoming issue is to elucidate the mechanism of the driving force toward the $CD8^+$ T cell response mediated by HSPs. In addition, the relationship between immune tolerance induced by HSP-mediated cross-presentation and the stimulation of DCs via toll-like receptors should be addressed. These findings will clarify the impact of HSP as a danger signal in the etiologies of autoimmune diseases and tumor immunity.

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