Hsp90 inhibitors: their effects on redox status and on the cytoarchitecture

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Table of Contents

Abbreviations

17AG	: 17-amino-17-demethoxy -geldanamycin
17AAG	: 17-allylamino-17-demethoxy-geldanamycin
Akt/PKB	: protein kinase B
Apaf1	: apoptosis protease activating factor 1
ARF	: alternate reading frame protein product
ARNT	: aryl hydrocarbon receptor nuclear translocator
BSA	: bovine serum albumin
cdc	: cell division control protein
CDDP	: cisplatin, cis -diamine-dichloro-platinum (II)
cdk	: cyclin dependent kinase
CMV	: cytomegalovirus
CsA	: cyclosporin A
DAPI	: 4',6-diamidino-2-phenylindole, dihydrochloride
DMSO	: dimethyl sulfoxide
dNTP	: deoxy nucleotide triphosphate
DPH	: 1,6-diphenyl-1,3-hexatriene
DPI	: diphenyleneiodonium chloride
DPPP	: diphenyl-1-pyrenylphosphine
DTT	: dithiothretol
eIF2	: elongation factor-2
EGF	: epidermal growth factor
ER	: endoplasmic reticulum
ErbB2/neu/HER2	: avian erythroblastic leukemia viral oncogene homolog 2
FITC	: fluorescein-5-isothiocyanate
GA	: geldanamycin
G-protein	: heterotrimeric G-protein
G2-phase	: generation-2 (meiotic) phase
Gab	: GRB-associated binder protein
GP	: geldampicin
GR	: glucocorticoid receptor
Grp94	: 94 kDa glucose-regulated protein
GSH	: reduced glutathione
HGF	: hepatocyte growth factor
HIF	: hypoxia induced factor
HRE	: hypoxia responsive element

Hsp	: heat shock protein
Hsp-s	: heat shock proteins
HSF	: heat shock transcription factor
HSE	: heat shock element
LB	: Lennex L broth
LB-agar	: Luria-Bertani-agar
LDH	: lactate dehydrogenase
LPO	: lipid peroxidation
MAPK	: mitogen activated protein kinase
MCS	: multiple cloning site
MDA	: malondialdehyde
MDM2	: murine double minute protein
MEK	: MAP kinase kinase
NADH	: nicotinamide adenine dehydrogenase
NQQ1	: NADH quinone oxidoreductase 1
NOS	: nitric oxide synthase
NP40	: nonidet P40
PAGE	: ployacrylamide gel electrophoresis
PBS	: phosphate buffer saline
PCR	: polymerase chain reaction
PEG	: polyethylene-glycol
PI	: propidium iodide
PI3K	: phosphatidylinositol triphosphate kinase
PMSF	: phenyl methyl sulfonyl fluoride
PR	: progesterone receptor
PU3	: purine based Hsp90 inhibitor
PU24FCl	: purine based Hsp90 inhibitor
PUFA	: polyunsaturated fatty acid
RA	: radicicol
Ras	: Rous sarcoma GTPase
Raf1	: Homo sapiens v-raf-1 murine leukemia viral oncogene homolog 1
RT-PCR	: reverse transcriptase-polymerase chain reaction
RTK	: receptor tyrosine kinase
SDS	: sodium dodecyl sulfate
STAT	: “signal transducers and activators of transcription” transcription factor
TBARS	: thiobarbituric acid reactive species
TBS	: Tris-buffered saline

TBST	: Tris-buffered saline containing 0.1% Tween 20
TCA	: trichloro-acetic acid
Tiron	: 1,2-dihydroxybenzene-3,5-disulfonate
p53/TP53	: tumor protein 53
TPR	: tetratricopeptide repeat
TRAP1	: tumor growth factor ? (TGF?) receptor associated protein
v-Src	: Rous sarcoma virus, p^{60}
v-Abl	: viral Abelson leukemia tyrosine kinase

Summary

The 90 kDa heat shock protein (Hsp90) is a highly abundant molecular chaperone. Hsp90 stabilizes the structure of several important signaling molecules suggesting a key role for this chaperone in cell proliferation and survival. In recent years Hsp90 inhibitors gained more and more attention as promising novel anticancer agents. Hsp90 inhibitors act at the N- and C-terminus of Hsp90. Although inhibition of Hsp90 may promote tumor cell death either by cytostasis or apoptosis, the mechanism behind this phenomenon needs to be further explored. Hence I measured the effect of Hsp90 inhibitors for the partial lysis of the Jurkat T lymphoid cell line. We were also interested, if the middle, charged linker region of Hsp90 is an active component in substrate binding or not. My major findings are the following:

1. Hsp90 inhibitors enhance the lysis of Jurkat cells by mild detergent treatment, by hypoxia or by the complement system.
2. Hsp90 inhibitors do not affect the lysis of bacteria, while they have a smaller effect on yeast cells, than on T lymphocytes.
3. The enhancement of the cell lysis has two components: it is partially derived from the increased amount of superoxides after the addition of Hsp90 inhibitors, while the rest of the effect comes from the inhibition of Hsp90.
4. Hsp90 inhibitors (especially geldanamycin) induce superoxide production, uneven lipid peroxidation of the plasma membrane and a change in membrane fluidity.
5. Hsp90 can be successfully inhibited by the specific hammerhead ribozyme we constructed.
6. Analyzing the binding of neuropeptide Y (NPY) to Hsp90 I showed that the binding of NPY to Hsp90 is grossly dependent on the ionic strength of the buffer, which gave a further evidence for the electrostatic nature of NPY/Hsp90 interactions, further supporting the role of the charged linker region in NPY binding.

My results uncovered an important role of Hsp90 in the maintenance of the cellular integrity, showed a novel element of the antitumor mechanism of Hsp90 drugs (which entered to phase II clinical trials recently) and helped to establish a functional role of the charged, linker region of Hsp90 by giving additional support for the identification of this

site as the third peptide binding site of Hsp90. Moreover, my studies gave the first example for the successful use of anti-Hsp90 ribozymes for the inhibition of Hsp90 function. Further experiments are on the way to explore the mechanism of the effects observed either by proving a role for Hsp90 in the maintenance of the cytoarchitecture or by establishing a role of Hsp90 in raft-dependent membrane stabilization.

1. Introduction

The Eukaryotic stress response is highly conserved, and involves the induction of heat shock proteins (Hsp-s). Hsp-s are detected in all cells, be it prokaryotic or eukaryotic. *In vivo* and *in vitro* studies have shown that various stressors transiently increase the production of Hsp-s as protection against harmful insults. Increased levels of Hsp-s occur after environmental stresses, infection, normal physiological processes, and gene transfer. They were first identified in *Drosophila melanogaster* salivary glands upon heat shock (*Ritossa, 1962*). The unique nature of Hsp synthesis was correlated with the acquisition of thermotolerance and cytoprotection, which suggested that Hsp-s are highly conserved proteins, and among many other cellular functions play an important role in cell physiology and metabolism. Later on the interest on Hsp-s been expanded tremendously as more and more functions of normal, resting cells were uncovered to involve Hsp-s besides their initially known protective role in stress. The multifunctional roles of Hsp-s in cells show that Hsp-s are one of the major regulatory proteins in the cell, and vital functions of cells are associated with Hsp-s. The role of Hsp-s in cell proliferation was reviewed (*Helmbrecht et al, 2000; Pechan, 1991*). Most of them are constitutively expressed, they are also induced under stress conditions and/or expressed in certain developmental stages.

In mammalian cells the stress response or the inducible response of heat shock proteins involves the induction of five major classes of Hsp families, namely, Hsp27, Hsp60, Hsp70, Hsp90 and Hsp110 (*Welch, 1990*). Hsp synthesis is tightly regulated at the transcription level by heat shock factors (HSF-s). Though there are various transcription factors reported, HSF1 was shown to be mainly involved in the induced transcription of Hsp-s. HSF1 is transcribed as a monomer, however, the active transcription factor exists as a trimer and binds to the heat shock elements located upstream of heat shock gene promoters thereby activating Hsp gene transcription (*Morimoto et al, 1992*). In addition, some members of HSF gene family exhibit both heat induction at the mRNA and protein level, and are constitutively expressed (*Morimoto, 1998*). Hsp-s function as molecular chaperones in regulating cellular homeostasis and promoting cell survival (*Bukau & Horwich, 1998; Hartl, 1996*).

1.1. Molecular chaperones

Though Hsp-s are inducible by a variety of stressors, their constitutive expression under non-stressful conditions show their important role in the maintenance of cellular homeostasis. Hsp-s are involved in maintaining appropriate folding and conformation of other proteins, hence they are called "molecular chaperones". They help in transport of proteins from one compartment to another inside the cell, and present old and damaged proteins to proteasomal degradation (*Bukau & Horwich, 1998; Hartl, 1996; Hartl & Hayer-Hartl, 2002*). Hsp-s are also believed to play a role in antigen-presentation and to serve as "danger signals" to help the immune system to recognize dead or damaged cells (*Nicchitta, 2003; Srivastava, 2000*).

The accumulation of Hsp-s is seen not only in stressful conditions but also in many pathophysiological conditions and tumors. Many types of tumors are associated with high expression of multiple Hsp-s compared to their normal parental cells (*Jaattela, 1999*) and Hsp90 in particular, is overexpressed in a wide variety of cancer cells (*Yufu et al, 1992*). In some instances, the differential expression of Hsp-s specifies the grade and type of tumor (*Conroy & Latchman, 1996; Ferrarini et al, 1992; Jameel et al, 1992; Kawanishi et al, 1999; Lebeau et al, 1991; Martin et al, 2000; Ostrerova et al, 1999*). Conditions, like Alzheimer's disease, prion disease, and Huntington disease, where the accumulation of misfolded proteins is the major cause of the neurodegenerative disorder *(Carmichael et al, 2000; Dou et al, 2003; Sittler et al, 2001; Warrick et al, 1999*), are associated with Hsp overexpression. Hsp-s help in cell recovery by refolding partially damaged functional proteins, and also by stabilizing association of cell survival factors. Misfolded proteins results in deregulated molecular and physiological functions within the cellular environment (*Scott & Frydman, 2003*).

Inhibition of Hsp-s either through molecular means (*using various antisense methods*) or pharmacological means (*using certain Hsp specific inhibitor drugs*) results in either cytostasis or apoptosis (*Nylandsted et al, 2000; Whitesell et al, 1994*). These observations suggest that tumor cells depend on Hsp-s for growth promotion, although the precise role behind this phenomenon needs to be explored. Tumor cells *in vivo* are often exposed to conditions such as glucose deprivation, hypoxia, low pH, and other

nutrient deprivation (*Brown & Giaccia, 1998; Tomida & Tsuruo, 1999*) thus need constantly elevated amounts of Hsp-s, which act as life-guards of the cell. Cancer cells undergo a series of mutations during the transformation process. Some of these affect the inhibitory signals, which are otherwise beneficial for the cell. These inhibitory signals alter several intracellular pathways resulting in loss of growth control, abnormal differentiation and other changes involved in carcinogenesis. Normal cells respond to a variety of overlapping signals, whereas cancer cells have limited signals for the same function. This phenomenon resulted in the hypothesis that selective inhibition of these intracellular pathways leads to anti-cancer treatment. The major role of Hsp90 in stabilizing these signaling molecules suggested that instead of using different small molecular targets that work at one or limited steps of signaling events, Hsp90 specific inhibitors are much more advantageous. In view of this targeting Hsp90 became central in recent tumor inhibition studies (*Blagosklonny, 2002; Dias et al, 2002; Gorre et al, 2002; Solit et al, 2002; Sreedhar et al, 2003b; Xu et al, 2002*). In addition, tumors are shown to be associated with activated Hsp90 complexes that are thought to facilitate tumor progression. Furthermore, tumor cells are much more sensitive for Hsp90 inhibitor drugs compared to normal cells (*Kamal et al, 2003*). In support of this the Akt survival pathway depends on Hsp90 will be affected by Hsp90 inhibition resulting in tumor cell death (*Basso et al, 2002*).

Among Hsp-s, Hsp90 has the ability to suppress and buffer mutations that accumulate during cellular transformation process, which would otherwise harmful to cells (*Rutherford, 2003*; *Rutherford & Lindquist, 1998; Sollars et al, 2003*). The association of Hsp-s with a wide variety of signaling molecules also suggests the importance of Hsp-s in induced and uncontrolled cell proliferation, which is characteristic of tumor cells (*Pratt & Toft, 2003*) and evoke therapies to control tumor growth by modulating Hsp levels.

Though the structure and function of Hsp-s vary between and within families, they all work in co-ordination at different stages of protein folding (*Hartl & Hayer-Hartl, 2002; Pratt & Toft, 2003*). Both Hsp90 and Hsp70 were shown to be associated with a number of signaling molecules, including v-Src, Raf1, Akt and steroid receptors suggesting an important role for these proteins in malignant cell transformation and

metastasis development (*Nollen & Morimoto, 2002; Pratt & Toft, 2003*). The discovery of some of the natural products like geldanamycin and radicicol with antitumor activity through inhibition of Hsp90 function opened a new era of therapeutic interventions (*Neckers et al, 1999; Neckers, 2002; Whitesell et al, 1994*).

1.2. Hsp90

Hsp90 is one of the most abundant proteins in the eukaryotic cell, comprising 1-2% of total proteins under non-stress conditions. Hsp90 is evolutionarily conserved, and is proven essential for cell survival. Its contribution to various cellular processes including signal transduction, protein folding and degradation, as well as morphological evolution was extensively studied. Hsp90 is primarily a cytosolic protein, however, it particularly translocates to cell nuclei upon stress (*Buchner, 1999; Csermely et al, 1998; Young et al, 1997*). There are two isoforms for this protein identified, namely, Hsp90-? (*inducible form*) and Hsp90-? (*constitutive form*). Its analogues include Grp94 in the endoplasmic reticulum and Hsp75/TRAP1 in the mitochondrial matrix (*Csermely et al, 1998*). The full functional activity of Hsp90 is gained in concert with other co-chaperones, playing an important role in the folding of newly synthesized proteins and stabilization and refolding of denatured proteins after stress.

Hsp90 is mainly a constitutive homodimer (*? ? or ? ?*), however, its monomers, heterodimers (*? ?*) and higher oligomers also exists. Its dimerization potential resides mainly at the carboxy-terminal 190 amino acids (*Garnier et al, 2001; Minami et al, 1994; Nemoto & Sato, 1998; Perdew et al, 1993; Radanyi et al, 1989*). Hsp90 is a phosphoprotein containing 2-3 covalently bound phosphate molecules per monomer (*Iannotti et al, 1988*), and the phosphorylation is thought to enhance its function. The monomer of Hsp90 consists of a conserved 25 kDa N-terminal and a 55 kDa C-terminal domains joined together by a charged linker region. Together with the C-terminal domain, this linker region helps in the dimerization of the protein (*Pearl & Prodromou, 2000*). Both termini are reported to bind to substrate polypeptides including client proteins and co-chaperones. The N-terminal domain contains an ATP binding site hence, Hsp90 is an ATP-dependent molecular chaperone (*Csermely & Kahn, 1991*). ATP binding helps dimerization because of a change in Hsp90 conformation (*Csermely et al,*

1993). Hsp90 also exhibits ATPase activity that is necessary for its chaperone function (*Maruya et al, 1999*). Recently it was shown that Hsp90 contains a second nucleotide binding site at the C-terminal domain (*Garnier et al, 2002; Marcu et al, 2000a; Soti et al, 2002*), however, the biological significance of this second nucleotide binding site needs to be further elucidated. Both the N- and C-terminal domains have been implicated in binding of substrate polypeptides *(Freeman & Morimoto, 1996; Scheibel et al, 1998; Young et al, 1997*). The tetratricopeptide repeat present in Hsp90 co-chaperones binds to the C-terminal MEEVD motif, and these interactions help in forming the chaperone complex.

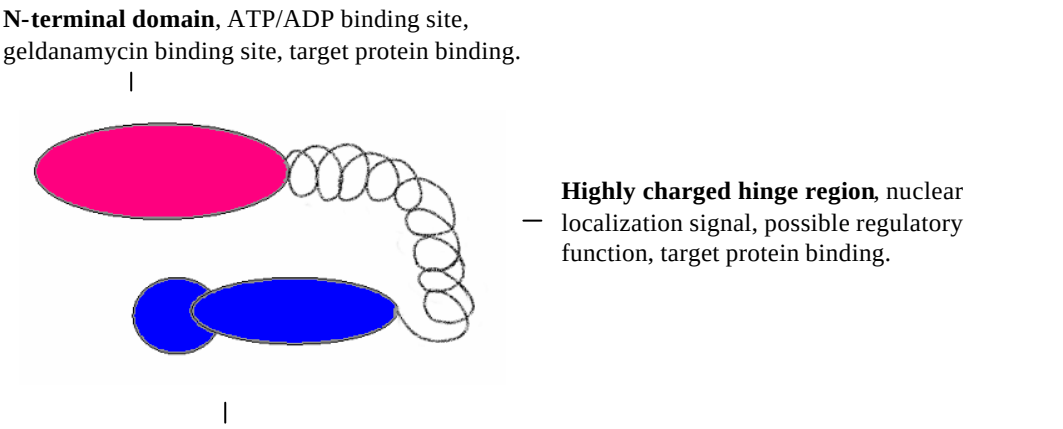

Figure 1. Domain structure of Hsp90. **The 25 kDa N-terminal and 55 kDa C-terminal domains are joined together by a charged domain.**

The middle domain *or* the hinge region was shown to increase the substrate specificity (*Scheibel et al, 1999*). Though there is no exact functional role attributed to this region, recently it was shown to contribute to the interaction of Hsp90 with its co-chaperone and to the binding to the client proteins (*Lotz et al, 2003; Mayer et al, 2003; Sato et al, 2000*). The substrate binding affinities of the middle domain needs to be explored further. Apart from its co-chaperones, Hsp90 binds to an array of client proteins, where the co-chaperone specificity varies and depends on the actual client. There is a growing list of Hsp90 client proteins and interestingly most of the clients include

molecules that are involved in signal transduction (*Pratt & Toft, 2003; Sreedhar et al, 2003b*).

Hsp90 forms several discrete sub-complexes; each containing different set of co-chaperones that function at different steps during the folding process of client protein (*Maruya et al, 1999; Pearl & Prodromou, 2000*). Unlike other chaperones, Hsp90 contains two independent chaperone sites that differ in their substrate specificity (*Pearl & Prodromou, 2000; Pratt, 1998; Young et al, 1997*) probably working in the form of a switch between Hsp90 and Hsp70 client protein interactions. The best understood molecular associations of Hsp90-multichaperone complexes are those with steroid receptors (*Georget et al, 2002*). The maturation of steroid receptors (*Pratt & Toft, 1997*) and their nuclear trafficking requires Hsp90. Steroid receptors also require molecular chaperones for their ligand dependent transcriptional activation *(Bose et al, 1996; Cheung & Smith, 2000*). Though there are reports that some Hsp90 co-chaperones can work independently of Hsp90 in preventing the aggregation of misfolded protein substrates, the complete competence in folding-assistance requires Hsp90 (*Buchner, 1999; Freeman et al, 1996; Johnson & Toft, 1994*). Co-chaperones also help Hsp90-client protein binding (*Chen & Smith, 1998; Dittmer et al, 1997*) through interactions between Hsp70 client proteins (*Czar et al, 1997*) and docking cytoskeletal proteins (*Pratt et al, 1999; Yarden & Sliwkowski, 2001*).

1.2.1. Hsp90-dependent signal transduction

Over the years, many different tyrosine and serine/threonine protein kinases have been selected as potential pharmacological targets in antitumor therapies, based either on their overexpression and/or dysfunction in a particular organ or tissue, or through their association in deregulated signal transduction/cell cycle pathways. Our current understanding is that a number of distinct tyrosine kinases play role in diverse but fundamentally important aspects of tumor progression such as growth, survival, metastasis and angiogenesis. Hsp90's role is implicated in many kinases from both tyrosine and serine/threonine family members. At cellular level, the ability of a cell to know whether to grow, divide, differentiate or die depends upon extracellular signals in an orchestrated manner. Several molecules like hormones, small peptides, surface

proteins from other cells etc. are involved in initiating these transduction mechanism through tyrosine and serine/threonine kinase cascades.

1.2.2. Tyrosine kinases

Hsp90 interacts with, and stabilizes a growing list of various kinases. Among them one of the major receptor tyrosine kinases (RTK-s) is ErbB2 (HER2 or *neu*). This kinase either works alone or in homo/hetero complexes with its homologues. ErbB2 was first identified as an oncogene, hence, the down regulation of ErbB2 signaling emerged as an anti-cancer strategy *(Nagy et al, 1999*). ErbB2 was shown as an *in vitro* substrate for Hsp90 chaperone complex, and inhibition of Hsp90 results in the dissociation of ErbB2 from the Hsp90 chaperone-complex (*Xu et al, 2002*). The Src family of tyrosine kinases was implicated in signal transduction following growth factor stimulation and integrin-mediated cell-substrate adhesion. Both v-Src and c-Src bind to Hsp90, where the chaperone maintains the kinase in an inactive form, and helps in its membrane recruitment as suggested by the modulation of Src activity by Hsp90 (*Xu & Lindquist, 1993*).

v-Abl and its cellular counterpart, c-Abl, shares sequence homology with Src members. Hsp90 affects the function and stability of this kinase (*An et al, 2000; Gorre et al, 2002*). At the nuclear level Wee1 tyrosine kinase catalyzes the inhibitory phosphorylation of the mitotic regulator cdk1 (cdc2), preventing mitosis during S-phase, and delaying it in response to DNA damage or developmental signals during G2-phase (*Munoz et al, 1999; Munoz & Jimenez, 1999*). Hsp90 is required for the assembly and/or disassembly of functional Wee1 protein complex (*Goes & Martin, 2001*). c-Met, a new member of RTK-s, stimulates the invasive growth of carcinoma cells, is tumorigenic and is overexpressed in many solid tumors (*To & Tsao, 1998*). c-Met, on activation by autophosphorylation, can associate with, and activate multiple signal transducing intermediates, such as Grb2, the p85 subunit of PI3K, STAT-3, and Gab1 (*To & Tsao, 1998*). Though there is no direct association of Hsp90 and the c-Met/HGF pathway, the co-ordinate regulation of c-Met and Hsp90 levels were shown (*Maulik et al, 2002; Stella & Comoglio, 1999*).

1.2.3. Serine/threonine kinases

The most important serine/threonine kinase members involved in malignant transformation and tumor progression include the major signaling cascades consisting of the Ras, Raf, MEK, and MAPK (*Kolch, 2000; Morrison & Cutler, 1997; Rosario et al, 1999*). Raf-1 is the most extensively studied member of the Raf family. Active Ras, in its GTP-bound state, binds to the amino-terminal regulatory domain of Raf-1 leading to the recruitment of Raf-1 into the cell membrane. Raf-1 is primarily located in the cytosol, and the cytosolic Raf-1 exists in a complex with Hsp90 *(Schulte et al, 1995; Schulte et al, 1996*), where Hsp90 binding to Raf is required for its activity (*Grammatikakis et al, 1999*). Both CK II (*Miyata & Yahara, 1992*) and the heme regulated eIF-2? kinase were identified in a complex with Hsp90 (*Shao et al, 2001*). Apart from its tight binding to Hsp90, CK II phosphorylates both isoforms of Hsp90 as well as the ER-resident Grp94 (*Cala & Jones, 1994*). Hsp90 also binds both co-translationally as well as post-translationally to eIF-2? kinase, and this binding is essential for maintaining the activity of the kinase (*Matts & Hurst, 1989; Xu et al, 1997*).

Akt/PKB is a downstream target for PI3K involved in the regulation of cell growth (*Nicholson & Anderson, 2002*). Several pieces of evidence show that Akt forms complexes with Hsp90, which enhance cell survival. However, this complex formation requires cdc37, a co-chaperone of Hsp90 (*Basso et al, 2002; Brazil et al, 2002*). At the nuclear level the major and initial cell cycle transition regulators, cdk4/cdk6 were also shown to form complexes with Hsp90, however, their binding involves their association with the co-chaperone, cdc37 (*Dai et al, 1996; Stepanova et al, 1996*). Recently it was shown that cdk9 acts preferentially by controlling processes such as transcription and the balance between differentiation and apoptosis, suggesting that this kinase can serve as a switch between many important cellular processes (*De Falco & Giordano, 2002*). Although Hsp70 is a general chaperone for cdk9, Hsp90 is also been shown to play a role in the regulation of cdk9 *via* cdc37 (*O'Keeffe et al, 2000*).

1.2.4. Transcription factors

The role of Hsp90 in glucocorticoid and progesterone receptor regulation was the best studied among the chaperone-dependent signaling events related to transcription

factors (*Pratt & Toft, 2003*). Another transcription factor, HIF-1? is associated with hypoxia-induced transcription of genes together with the nuclear protein, ARNT. The resulting HIF-1? /ARNT heterodimers interact specifically with the hypoxia responsive element (HRE), thereby increase the transcription, where Hsp90 maintains the conformation of HIF-1? /ARNT heterodimers (*Hur et al, 2002*).

The p53 tumor suppressor is an important regulator of cellular response to stress, abnormal cell proliferation, and DNA dama ge. In normal cells, p53 is maintained at very low levels because of rapid degradation through the ubiquitin-dependent proteasome pathway and is under the control of murine double minute protein (MDM2) (*Prives & Hall, 1999*). Hsp90 binding has been shown to contribute to the accumulation of mutant p53 (*Nagata et al, 1999*). Binding of Hsp90 inhibits the ability of MDM2 to promote p53 ubiquitinylation and degradation, resulting in the stabilization of both mutant p53 and MDM2 (*Peng et al, 2001a; Peng et al, 2001b*). Hsp90 appears to inactivate MDM2 by blocking the central domain of MDM2, which is normally involved in p53 regulation mimicking the effect of ARF to prevent mutant p53 degradation (*Wang & Chen, 2003*). HSF-1 tightly regulates the inducible transcription of heat shock genes (*Cotto & Morimoto, 1999*). Hsp90 negatively regulates the HSF-1 (*Zou et al, 1998*) and is involved in the termination of the stress response.

1.2.5. Non-signaling molecules that interact with Hsp90

The other major non-signaling molecules associated with Hsp90 are several cytoskeletal proteins such as, actin (*Kellermayer & Csermely, 1995; Koyasu et al, 1986*), tubulin (*Redmond et al, 1989; Sanchez et al, 1985*), intermediary filaments (*Czar et al, 1996; Fostinis et al, 1992*), dynein (*Galigniana et al, 2002*) or the Tau protein, which stabilizes the microtubules (*Dou et al, 2003*). Other Hsp90-interacting proteins include the G-protein family members G protein ? ? (*Inanobe et al, 1994*), G? $_o$ (*Busconi et al, 2000*), G? $_{12}$ (*Vaiskunaite et al, 2001*), nitric oxide synthase (NOS) (*Bender et al, 1999; Garcia-Cardena et al, 1998*), and the anti-apoptotic protein, Apaf-1 (*Pandey et al, 2000*). There is a growing list of Hsp90 associated proteins (*Pratt & Toft, 2003*) showing a large number of non-signaling molecules that interact with Hsp90.

1.3. *In vivo* roles of Hsp90: chaperones as possible organizers of the cytoarchitecture

The eukaryotic cytoskeleton contains three major components, microfilaments, intermediate filaments, and microtubules. The microtubule and actin cytoskeletal filament systems play a major role in mechanical cellular processes such as dynamic shape change, shape maintenance and intracellular organelle movement. Most of the cytoskeletal functions are attributed to the ability of polarized cytoskeletal polymers to assemble and disassemble rapidly, and to interact with binding proteins and molecular motors that mediate their regulated movement and/or assembly into higher order structures, such as radical arrays or bundles. Extensive research on this field showed the importance of Hsp-s in stabilizing the cytoskeleton by direct interaction with cytoskeletal proteins (*Liang & McRae, 1997; Sussman, 2002*). The major cytoprotective chaperone, Hsp70 was shown to interact with tubulin (*Decker et al, 2002*), and actin. Especially small heat shock proteins were extensively studied for their role in cytoskeletal stabilization (*Mounier & Arrigo, 2002*).

Hsp90 has been proposed to be involved in maintaining a fine cytoplasmic meshwork, called microtrabecular lattice apart from its known cytoskeletal role (*Csermely et al, 1998; Csermely, 2001*). The growing list of Hsp90-interacting cytoskeletal proteins suggests that Hsp90 plays a major role in preserving these structures, hence it is involved in maintaining the cell shape. In accordance with this, Hsp90 inhibition is associated with disturbances in the cytoskeleton and cytoskeletal signaling (*Pai et al, 2001*). Most of the theories related to cytoskeletal interactions are structural (*where microtubule and actin are physically linked*) versus regulatory (*actin and microtubules work independently through their effects on the signaling cascades*) interactions, however, the molecular basis for these interactions can be explained by the 'tensegrity model' in which actomyosin generates tension against stiff microtubule struts and adhesion to the substrate to stabilize or change cell shape (*Volokh, 2003*). *Kellermayer & Csermely* (*1995*) have demonstrated that during actomyosin complex formation and motility, Hsp90 competes for actin binding. Though Hsp90-actin binding was shown indirect, and is through a TPR containing protein, UNC-45 (*Barral et al, 2002*), several lines of indirect evidence, such as diffusion anomalies show that the

hypothesis on Hsp90 involvement in the maintenance of the cytoarchitecture may be still valid (*Csermely, 2001*).

1.3.1. *In vivo* roles of yeast and bacterial Hsp90

The budding yeast, *S. cerevisiae* and the fission yeast, *S. pombe* have two Hsp90 homologues, *Hsp82* and *Hsc82*. Yeast cells are viable when either of the two genes is disrupted, however, deletion of both genes is lethal (*Borkovich et al, 1989*). Compared to a wide array of signaling molecules that interact with Hsp90 in higher eukaryotes (*Pratt & Toft, 2003*) multiple substrates for yeast Hsp90 were not known except for the kinases, Wee1 in *S. pombe* (*Aligue et al, 1994*) and Ste1 in *S. cerevisiae* (*Louvion et al, 1998*). In contrast, the prokaryotic homologue of Hsp90, HtpG is dispensable for growth (*Bardwell & Craig, 1988*). The functional variance between pro-and eukaryotic Hsp90 proteins revealed that bacterial Hsp90 is unable to rescue Hsp90-deficient yeast strains (*Palmer et al, 1995*). It could be due to lack of both the highly charged hinge/middle domain and the C-terminal extension that is otherwise present in all eukaryotic Hsp90-s (*Scheibel & Buchner, 1997*). The functional significance of HtpG in efficient folding of nascent polypeptides similar to the DnaK, DnaJ/GrpE (Hsp70/Hsp40) complex and the GroEL/GroES (Hsp60/Hsp10) chaperone complex in *E. coli* (*Hartl, 1996; Johnson & Craig, 1997*) is not known. However, HtpG was shown to play a role in cold acclimatization and thermal management in cyanobacteria (*Hossain & Nakamoto, 2002; Tanaka & Nakamoto, 1999; Thomas & Baneyx, 2000*).

1.4. Hsp90 inhibitors and their clinical use

Over the years, Hsp90 gained much of attention because of its role in steroid receptor activation and in other pathways of signal transduction. However, its role in malignant transformation stabilizing the signaling intermediates leading to tumor development extends the importance of this molecular chaperone to be a new target for anticancer therapies (Table. 1). Tumors are known for their induced proliferative ability, where they are in high demand of signaling events and these events are associated with the deregulated cell cycle and escape from programmed cell death due to accumulation of certain mutations (*Pompetti et al, 2003*). Though drugs like geldanamycin shown to be

associated with the induction of reactive oxygen species (ROS) (*Billecke et al, 2002; Dikalov et al, 2002; Sreedhar et al, 2003a*), they are still known for their specific antitumor potential.

Table 1. Major cellular effects of Hsp90 inhibitors, which are associated with anti-tumor potential

Hsp90 inhibitor	Major cellular effect	Reference
Specific inhibitors		
Geldanamycin	Reversal of phenotype	*Uehara et al, 1986*
	Reversal of phenotype/differentiation	*Whitesell et al, 1994*
	Cytoskeletal rearrangements	*Davis & Carbott, 1999*
17AAG	Differentiation	*Munster et al, 2001b*
	Inhibition of metastatic phenotype	*Nguyen et al, 2000*
Purine scaffold analogues	Growth suppression/differentiation	*Chiosis et al, 2001; 2002*
Radicicol	Suppresses anchorage-dependent cell growth	*Kim et al, 2001*
Non-Specific inhibitors		
Cisplatin	Differentiation	*Mares et al, 2003*
Novobiocin	Inhibition of cell proliferation	
	Increases sensitivity to microtubule disrupting drugs	*Nordenberg et al, 1994*
	Differentiation	*Stocker et al, 1995*
Taxol	Growth suppression	*Fujishita et al, 2003*

Drugs like cisplatin induce apoptosis without involving ROS, which suggests a strong and direct antitumor potential (*Senturkar et al, 2002; Sreedhar et al, 2003a*). Recent reports show that the inhibition of Hsp90 is associated with degradation of Hsp90 client, HER-2 resulting in the induction of apoptosis. Further, this inhibition was shown to be successful with tumors of poor prognosis and chemoresistance (*Munster et al, 2002*).

In several tumor models the selective inhibition of Hsp90 function causes degradation of important signaling proteins that are involved in cell proliferation, cell cycle regulation, and apoptosis (*Maloney & Workman, 2002*). These findings can be explained by the proposition that many growth-regulating proteins in tumor cells depend

on either stable or transient interactions with Hsp90 for their function. Hence, inhibition of Hsp90 function through pharmacological interference makes these tumor cells more vulnerable for anti-Hsp90 drugs. The advantage of using Hsp90 inhibitors over other pharmacological targets is their selectivity on multiple signaling pathways, whereas other drugs may target one or limited signaling pathways, which do not count for an extensive signaling network. In addition, tumor cells may find alternative pathways to circumvent specific drug effects. In such cases, alternative pathways help them in the development of drug resistance as well as contribute for the malignant as well as metastatic phenotype. Towards this direction there are several Hsp90-specific drugs developed and some of them are already in phase I and phase II clinical trials (Table. 2).

Table 2. Hsp90 clients influenced by various Hsp90 inhibitors

Hsp90 client protein	**Hsp90 Inhibi tor**	**Reference**
Tyrosine kinases		
$p60^{v\text{-}Src}$, $p60^{c\text{-}Src}$	Geldanamycin	*Whitesell et al, 1994*
		An et al, 2000
	Novobiocin	*Marcu et al, 2000b*
	Radicicol	*Soga et al, 1999*
$p56^{lck}$	Geldanamycin	*Hartson et al, 1996*
Hck	Geldanamycin	*Scholz et al, 2001*
$p^{185\ erb2}$	Geldanamycin	*Xu et al, 2001*
		Tikhomirov & Carpenter, 2000
	17AAG	*Munster et al, 2002*
	PU3	*Chiosis et al, 2001*
	PU3 derivatives	*Chiosis et al, 2002*
	Geldanamycin dimers	*Zheng et al, 2000*
	Radicicol	*Soga et al, 1999*
	Cisplatin	*Smith et al, 2002*
$p120^{\ bcr\text{-}abl}$	Geldanamycin/17AAG	*Nimmanapalli et al, 2001*
		An et al, 2000
	Radicicol	*Shiotsu et al, 2000*
Serine/ threonine kinases		
v-Raf, c-Raf, B-Raf,	Geldanamycin	*Schulte et al, 1995*
Gag-Mil, Ste11		*Stancato et al, 1997*
		Grammatikakis et al, 1999
	17AAG	*Nimmanapalli et al, 2003*
	Novobiocin	*Marcu et al, 2000b*
	Radicicol	*Soga et al, 1999*
Heme-regulated	Geldanamycin	*Uma et al, 1997*
eIF-2? kinase		*Shao et al, 2002*
cdk4	Geldanamycin	*Stepanova et al, 1996*

	Radicicol	*Soga et al, 1999*
	Cisplatin analogues	*Kuang et al, 2001*
cdk9	Geldanamycin	*O'Keeffe et al, 2000*
Akt	17AAG	*Solit et al, 2003*
PI3 Kinase	Geldanamycin	*Fujita et al, 2002*
	Ligand-geldanamycin	*Chiosis et al, 2001*
Transcription factors		
Glucocorticoid receptor	Geldanamycin	*Whitesell & Cook, 1996*
Progesterone receptor	Geldanamycin	*Smith et al, 1995*
Estrogen receptor	Radicicol	*Lee et al, 2002*
Aryl hydrocarbon (Ah) receptor	Geldanamycin	*Kazlauska et al, 2000*
Heat shock factor	Geldanamycin	*Ali et al, 1998* *Zou et al, 1998*
HIF-1?	Geldanamycin	*Minet et al, 1999*
	Radicicol	*Kurebayashi et al, 2001*
p53	Geldanamycin	*Blagosklonny & el-Diery, 1996*
	Novobiocin	*Marcu et al, 2000b*
	Radicicol	*Soga et al, 1999*
Polymerases		
Hepatitis-B reverse transcriptase	Geldanamycin	*Hu et al, 1996*
Telomerase	Geldanamycin	*Holt et al, 1999*
	17AAG/Geldanamycin	*Villa et al, 2003*
	Novobiocin	*Haendeler et al, 2003*
	Cisplatin	*Zhang et al, 2002*

Most of the initial studies with Hsp90 inhibitors showed reversal of phenotype. However, later studies confirmed that this phenomenon causes either the association with induced cellular differentiation or growth inhibition/apoptosis. Alternations in the extensive cellular network of signaling pathways are thought to be the major cause of the phenotype change either through differentiation or cytostasis. Recent developments in Hsp90 interaction with various signaling intermediates revealed that Hsp90 interacts with thousand-and-one signaling proteins, which are linked to one to another, hence, inhibition of one molecular interaction leads to the impairment of multiple pathways.

1.4.1. Geldanamycin

The first Hsp90 inhibitor drug with antitumor potential identified was geldanamycin, a natural product isolated from *Streptomyces hygroscopicus* (*Be Boer &*

Dietz, 1976). Though the antitumor potential of geldanamycin was initially thought to be due to specific tyrosine kinase inhibition (*Whitesell et al, 1992*), later studies revealed that the antitumor potential relies on the depletion of oncogenic protein kinases *via* the ubiquitin proteasome pathway (*Mimnaugh et al, 1996; Whitesell et al, 1994*). The major regulatory signaling proteins which are affected by geldanamycin include the oncogenes, *erbB2, EGF, v-Src, Raf-1* and *cdk4* as well as the nuclear hormone receptors including both the estrogen and androgen hormone receptors (*Pratt & Toft, 2003*). Subsequent immunoprecipitation (*Schulte et al, 1997; Whitesell et al, 1994*) and X-ray crystallographic (*Jez et al, 2003; Stebbins et al, 1997*) studies revealed that geldanamycin directly binds to Hsp90, and inhibits the formation of Hsp90-multichaperone complex required for client protein maturation, resulting in the ubiquitin-mediated degradation of Hsp90-client proteins. Geldanamycin binds to the N-terminal domain of Hsp90 and competes for ATP binding. The geldanamycin-Hsp90 crystal structure also shows that this binding inhibits the substrate protein binding (*Prodromou et al, 1997; Stebbins et al, 1997*). Geldanamycin also binds to Grp94, the Hsp90 analogue that is abundant in ER and coordinately regulated by other ER chaperones (*Chavany et al, 1996*).

Though geldanamycin shows both anti-tumor and selective Hsp90 binding potential, it had difficulties to enter clinical trials due to its high hepatotoxicity in human tumor models (*Supko et al, 1995*). Thus, a search for new classes of Hsp90 inhibitors with less toxicity began, and was successful in developing its analogue, 17AAG. 17AAG possesses all the Hsp90-related characteristics of geldanamycin (*Kelland et al, 1999; Schulte & Neckers, 1998*) but with lesser toxicity (*Page et al, 1997*), hence, 17AAG could enter phase I and recently phase II clinical trials. Though 17AAG is metabolized by cytochrome P450-CYP3A4, this metabolite was found be a stable form, and retains both Hsp90 inhibitory and antitumor activities (*Kelland et al, 1999*). Both geldanamycin and 17AAG can be metabolized by NQO1, which is known to potentiate antitumor activity by stabilizing the tumor suppressor p53 (*Brunton et al, 1998; Kelland et al, 1999*). Similarly to geldanamycin, 17AAG also binds to Grp94 (*Egorin et al, 1998*). Several geldanamycin-testosterone compounds (*Kuduk et al, 1999; Kuduk et al, 2000*) and ligand-geldanamycin compounds (*Chiosis et al, 2001; Zheng et al, 2000*) were also developed, and showed selective effects in certain tumors.

Interestingly, the comparison of various tumor and experimental models suggests that the mode of action of Hsp90 inhibitors vary. In some cases, they induce apoptosis (*van den Berghe et al, 2003*) and in other cases they induce either differentiation or cytostasis (*Hostein et al, 2001*).

1.4.2. Radicicol

Radicicol is a macrocyclic antibiotic isolated from *Monosporium bonorden*. (*Delmotte & Delmotte-Plaqee, 1953*) Because of its potential to reverse the malignant phenotype similar to geldanamycin (*Kwon et al, 1992; Zhao et al, 1995*), it was also initially thought to be a tyrosine kinase inhibitor. However, later studies showed its role in Hsp90 client protein degradation (*Schulte et al, 1998*).

Radicicol is involved in the degradation of NQQ1 followed by the degradation of mutant p53, which is involved in the malignant transformation (*Asher et al, 2002*). The antitumor potential for NQQ1 is because of its involvement in stabilizing the tumor suppressor protein p53 (*Asher et al, 2002*) and failure of these results in p53 mediated apoptosis.

Radicicol inhibits the peptide binding to Grp94 though the peptide binding, and radicicol binding sites are different in Grp94 (*Vogen et al, 2002*). However, radicicol lacks antitumor activity *in vivo* in experimental models because of its instability. The oxime derivatives of radicicol (*Agastuma et al, 2002*) exhibit antitumor activity *in vivo* as well as *in vitro* hence serve as good anticancer drug candidates. Radicicol binds to the N-terminal domain of Hsp90 with much higher affinity than the structurally different drugs, geldanamycin and 17AAG (*Roe et al, 1999*).

Table 3. Hsp90 clients influenced by geldanamycin

Hsp90 client protein	**Hsp90 Inhibitor**	**Reference**
G?$_0$	Geldanamycin	*Busconi et al, 2000*
G?$_{12}$	Geldanamycin	*Waheed & Jones, 2002*
NOS	Geldanamycin	*Garcia-Cardena et al, 1998* *Bender et al, 1999* *Joly et al, 1997*
Centrin/centrosome	Geldanamycin	*Lange et al, 2000*
Apoprotein B	Geldanamycin	*Gusarova et al, 2001*

Retrovirus cell attachment protein	Geldanamycin	*Gilmore et al, 1998*
Protease activated receptor	Geldanamycin	*Pai et al, 2001*
Catalytic unit of calcineurin (cna2)	Geldanamycin	*Imai & Yahara, 2000*
Mammalian aminoacyl-tRNA synthetase	Geldanamycin	*Kang et al, 2000*
Atrial natriuretic peptide receptor	Geldanamycin	*Kumar et al, 2001*

1.4.3. Cisplatin

Cisplatin (CDDP) was first identified 125 years ago and its clinical development started in the 1970's for its effective antitumor activity in a wide variety of tumors (*Fuertes et al, 2002; Wong & Giandomenico, 1999*). Its biochemical ability to form DNA-adducts was thought to be the major cause of its antitumor activity (*Jamieson & Lippard, 1999*). Its non-DNA targets include phospholipids, especially phosphatidyl serine and RNA (*Judson & Kelland, 2000; Speelmans et al, 1997*). However, it also interacts with thiol-containing proteins and peptides, such as cytoskeletal proteins (*Louie & Meade, 1999*). The specific binding of cisplatin to Hsp90 was also demonstrated (*Itoh et al, 1999*). Later studies showed that it binds to the C-terminal domain of Hsp90 and specifically interferes with the nucleotide binding at this site (*Soti et al, 2002*).

1.4.4. Novobiocin

Novobiocin (NB) belongs to the family of coumarin antibiotics, and is known to inhibit bacterial DNA synthesis by direct binding to DNA gyrase. The novobiocin-binding site of DNA gyrase is similar to the Hsp90 ATP-binding site (*Gormley et al, 1996*). Novobiocin binds to a previously unrecognized ATP-binding domain in the carboxy terminus of Hsp90, and inhibits its function. However, binding of novobiocin to Hsp90 inhibits geldanamycin binding suggesting an extensive interaction between the N- and C-terminal domains in regulating Hsp90 chaperone function (*Marcu et al, 2000a, Soti et al, 2002*).

1.4.5. Purine scaffold inhibitors

Hsp90 contains a conserved N-terminal ATP/ADP binding pocket and nucleotide binding regulates the chaperone function of the protein (*Richter et al, 2002*). Earlier studies with various Hsp90 inhibitors showed that most of Hsp90 inhibitors developed so far directly bind to the N-terminal ATP/ADP site resulting in a change of Hsp90 conformation and a consequent interference with its chaperone function (*Neckers, 2003*). In a recent development, PU3, a purine-based Hsp90 inhibitor was designed using X-ray crystallographic data (*Chiosis et al, 2001*). PU3 has similar effects like geldanamycin in inhibiting Hsp90 client protein degradation and in possessing a robust antitumor potential. However, the trials to modify and improve PU3 led to the development of PU24F-Cl, which binds to the N-terminus of Hsp90 with 30-folds higher affinity than the parent compound PU3, approximating the binding affinity of 17AAG. PU24F-Cl was found more selective over Hsp90 inhibitors. Its water-solubility is also an advantage over geldanamycin and 17AAG (*Chiosis et al, 2002*). However, PU24F-Cl might not show the specific intracellular accumulation typical to more hydrophobic geldanamycin analogues.

1.4.6. Taxol

Taxol is a plant-derived antitumor agent. Its antitumor action is ascribed to its ability to block mitosis by binding and stabilizing microtubules (*Schiff & Horwitz, 1980; Wani et al, 1971*). Members of the Hsp90 and Hsp70 families were recently identified as targets of taxol (*Byrd et al, 1999*). However, taxol-induced cell death is found independent of the Raf kinase, which is one of the usual targets of Hsp90 (*Torres & Horwitz, 1998*). Taxol needs to be explored as to see its role in among a large variety of Hsp90 client protein degradation, whether it is directly/inversely related to its binding to Hsp90 to influence/interfere with its chaperone function.

1.4.7. Miscellaneous inhibitors

Growing interest of compromising Hsp90 chaperone function in antitumor treatments raised the interest to explore novel antitumor drugs/compounds. Nucleotide analogues such as diadenine oligo phosphates-*APnA* (*Soti et al, 2003a*) were shown specifically bind and interfere with Hsp90 chaperone function. Pharmacological

companies like, Ribotargets (www.ribotargets.com) or Telik (www.telik.com) advanced further to find out molecular means of Hsp90 inhibition. Our own study reveals the advantage of use of antisense hammerhead ribozyme over chemical compounds (*Sreedhar et al, 2003a*).

1.4.8. Overlapping functions of Hsp90 inhibitors

The benzoquinone ansamycins, geldanamycin and 17AAG both inhibit Hsp90 ATPase activity through their direct binding to the N-terminal ATP-binding site required for Hsp90 chaperone activity (*Maloney & Workman, 2002*). This results in the failure of Hsp90 binding to its client proteins and their recruitment for proteasome-mediated degradation. Similarly, radicicol binds to the N-terminal domain of Hsp90 and inhibits its intrinsic ATPase activity (*Roe et al, 1999*). The small molecular inhibitors PU3 and PU24F-Cl also show specific binding to the N-terminal domain of Hsp90 (*Chiosis et al, 2002*), and are potent inhibitors of Hsp90 function. In contrast, cisplatin binds to the second nucleotide-binding site at the C-terminal domain of Hsp90 (*Rosenhagen et al, 2003; Soti et al, 2002*).

1.4.9. Advantages of Hsp90 inhibitors

In most cases Hsp90 inhibition was shown to induce either cytostasis or apoptosis (*Hostein et al, 2001*). However, there are some reports showing that at low doses Hsp90 inhibitors induce cell differentiation (*Munster et al, 2001b*). Though the differences in the downstream effects of Hsp90 inhibition leading to these various final outcomes in the fate of the tumor cells are not known, several prominent features of Hsp90 inhibition are associated with all of these effects (*Lopez-Maderuelo et al, 2001; Nimmanapalli et al, 2001; Nimmanapalli et al, 2003*). There is a selectivity of drug-induced effects among the type and grade of tumor. *Clarke et al* (*2000*) showed from human colon carcinoma cells that 17AAG depletes Raf and Akt through Hsp90 inhibition without affecting other client protein expression. In certain cancers, single administration of an Hsp90 inhibitor, such as 17AAG was shown more effective for a variety of client protein degradation (*An et al, 2000*).

In a recent report, Hsp90 inhibition results in either differentiation or apoptosis depending on the cell type (*Lopez-Maderuelo et al, 2001*). Cell cycle arrest is the crossroad for this decision. Geldanamycin and 17AAG (*Munster et al, 2001a; Srethapakdi et al, 2000*) were shown to induce G1-phase arrest. Small molecular purine inhibitors were also exhibit a similar phenomenon (*Chiosis et al, 2002*). These pre-clinical studies emphasize the important role of Hsp90 inhibitors in the clinical implications, of course with certain exceptions. However, the combinatorial treatment with low doses of these drugs will be much more effective. For example, in case of Bcr-Abl positive leukemias, a low dose of geldanamycin was shown to be sufficient to sensitize cells to apoptosis in presence of ineffective concentrations of doxorubicin through caspase activation (*Blagosklonny et al, 2001*). In another example, taxol resistant erbB2 overexpressing breast cancer cells, 17AAG in combination with taxol showed more cytotoxic effects (*Munster et al, 2001a; Sauville, 2001*).

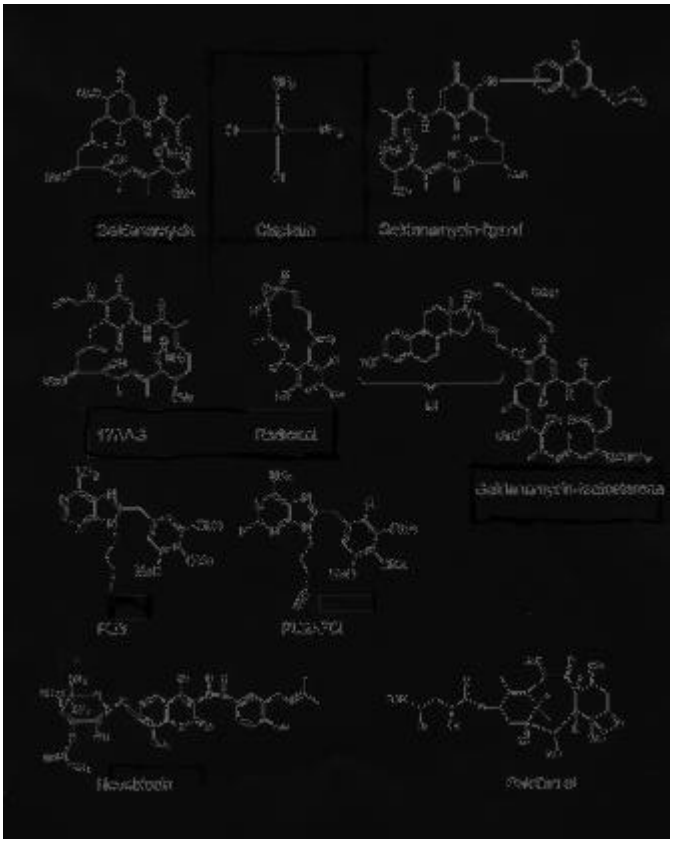

Fig 2. **Structures of Hsp90 inhibitors. N-terminal inhibitors:** geldanamycin, 17AAG, radicicol, PU3, PU24FCl, novobiocin, geldanamycin-ligands and geldanamycin-testosterone complexes; **C-terminal Hsp90 inhibitors:** cisplatin, novobiocin; **non-specific inhibitors:** paclitaxel.

Table 4. Hsp90 inhibitors in clinical trials

Drug candidate	Major effect	Company and web site
Specific inhibitors		
Geldanamycin analogues	N-terminal Hsp90 binding	Conforma Inc. (www.conforma.com) Kosan Bioscience (www.kosan.com)
Geldanamycin-testosterone	N-terminal Hsp90 binding (targeted specificity)	-
Radicicol	Hsp90 inhibition	Kyowa Hakko Kogyo Ltd. (www.kyowa.co.jp)
Purine - scaffolds	Hsp90 inhibition	
?	Hsp90 inhibition	Ribotargets (www.ribotargets.com)
?	Hsp90 inhibition	Telik Co (www.telik.co)
Non-specific inhibitors		
Cisplatin	C-terminal Hsp90 binding	-
Novobiocin	N & C-terminal Hsp90 binding	-
Taxol	N-terminal binding (?)	-
Diadenosine oligophosphates (Ap_nA)	N-terminal Hsp90 binding	-

1.5. Hsp90, Hsp90 inhibitors and cellular redox status

Oxidative stress is an inbalance between oxidant exposure and anti-oxidative protection within the cellular environment resulting in a range of responses that differ greatly from the type of stress and are associated with the sensitivity of the cell function and viability. There is a cross talk between the amount of Hsp-s and the intracellular redox homeostasis. Hsp-s, like ?-crystalline, Hsp27 and Hsp70 were extensively studied for their antioxidant properties. These proteins also contribute for maintaining the intracellular redox homeostasis (*Ahn & Thiele, 2003; Graf & Jakob, 2002*). Though the exact involvement of Hsp90 in redox homeostasis regulation is not known, Hsp90 was

shown to possess reactive cysteines and was able to reduce cytochrome *c* suggesting a role for this chaperone in modulating the redox status in resting and apoptotic cells (*Nardai et al, 2000*).

Although Hsp90 inhibitors exhibit selective effects inducing the degradation of Hsp90 client proteins, they are also associated with other effects unrelated to their binding to Hsp90. Geldanamycin, which contains a quinone group is known to induce reactive oxygen species, and in general, the cytotoxicity of the ansamycin antibiotics has been attributed to free radical generation (*Benchekroun et al, 1994; Dikalov et al, 2002; Shi et al, 2002; Sreedhar et al, 2003a; Suttinamongkol et al, 2000*). Radicicol is also involved in the free-radical formation from non-peroxide compounds (*Tanaka et al, 1999*), while cisplatin and novobiocin have multiple targets, which are independent of Hsp90.

2. Objectives

In the present study, we wanted to understand the physiological significance of Hsp90 inhibitors as well as the consequences of Hsp90 inhibition with respect to cellular integrity. The rationale behind these experiments was to test, whether Hsp90, a cytoprotective chaperone, binding to "thousand-and-one" substrates and other proteins is involved in the maintenance of cellular integrity (*Csermely, 2001*) and whether its inhibition makes cells more "lysis-prone". The first experiments were very promising: geldanamycin, a well-established Hsp90-inhibitor (*Stebbins et al, 1997; Whitesell et al, 1994*) induced a significant increase in lysis rate of various cells (*Csermely et al, 2003; Pato et al, 2001; Sreedhar et al, 2003a*). However, later experiments demonstrated that the extent of geldanamycin-induced enhancement of cell lysis was dependent on the experimental conditions, namely, if cells were shaken during the experiment or not. At this time the first results of geldanamycin-induced superoxide generation appeared (*Dikalov et al, 2002; Billecke et al, 2002*). These results turned our attention to examine the contribution of superoxide-related *versus* Hsp-related events to diminished cellular integrity after Hsp90 inhibition. Using various Hsp90 inhibitors (*Whitesell et al, 1994; Stebbins et al, 1997; Schnaider et al, 2000*) as well as anti-Hsp90 hammerhead ribozymes we demonstrated that besides a putative increase in membrane fragility by geldanamycin-induced superoxides, inhibition or lack of Hsp90 *alone* also results in a compromised cellular integrity. Moreover, cell lysis after hypoxia and complement attack was also enhanced by Hsp90 inhibition suggesting the maintenance of cellular integrity by Hsp90 in tumor cells. Our results show the first successful use of an anti-Hsp90 ribozyme in manipulating Hsp90 levels, and demonstrate a novel element of Hsp90-related cytoprotection, its role in the maintenance of cellular integrity. Attempts were also made to understand the role of Hsp90 with increasing cellular complexity from bacteria and yeast in comparison with mammalian cells with cytolysis experiments and found that bacterial response to Hsp90 inhibition is almost negligible whereas yeast showed an intermediate effect between bacteria and mammalian cells (*Sreedhar et al, 2003a*).

Since Hsp90 inhibitors bind both to the N- and C-termini of Hsp90, we were interested if the middle charged linker region also plays an active role in Hsp90 function.

A study to examine the substrate binding characteristics of Hsp90, especially the middle charged region, was carried out using various peptides and we found that neuropeptide-NPY, a positively charged amphipathic ?-helical peptide binds to Hsp90 by ionic interactions to a previously unrecognized site immediate next to the amino terminus of the protein (*Ishiwatari et al, 2003*).

Specific Objectives

1. To measure the cellular integrity when Hsp90 chaperone function is compromised

The rationale behind these experiments was to test, whether Hsp90, a cytoprotective chaperone, binding to "thousand-and-one" substrates and other proteins is involved in the maintenance of cellular integrity (*Csermely, 2001*) and whether its inhibition renders cells more "lysis-prone". Using various Hsp90 inhibitors (*Schnaider et al, 2000; Stebbins et al, 1997; Whitesell et al, 1994*) as well as anti-Hsp90 hammerhead ribozymes (*Sreedhar et al, 2003a*) we wanted to demonstrate that lack of Hsp90 *alone* also results in a compromised cellular integrity.

2. To measure the role of Hsp90 in yeast and bacteria in maintaining the cellular integrity

Cellular integrity is associated with the increasing complexity of the cytoarchitecture. Observing a role for Hsp90 to maintain cellular integrity we wanted to study, whether cells with a less important contribution of Hsp90 to their viability and with a smaller degree of cellular organization than the human Jurkat cell line, namely bacterial or yeast cells are sensitive to geldanamycin-induced additional lysis or not.

3. To see whether Hsp90 inhibitors alters the cytoskeleton

Besides being a partner of a large number of co-chaperones and substrates, Hsp90 binds to filamentous actin and tubulin (*Czar et al, 1996; Kellermayer & Csermely, 1995; Koyasu et al, 1986*) and the involvement of the cytoskeleton in the traffic of Hsp90-substrates has also been demonstrated (*Galigniana et al, 2002; Pratt & Toft, 2003*). In the present study an attempt was made to extend these observations as to see the effect of Hsp90 inhibition on cytoskeletal alterations.

4. To find out the peptide binding characteristics of middle charged region of Hsp90

Though there is an extensive analysis of both C and N-terminal domains of Hsp90, the importance and the functional significance of the middle domain is lacking. Since the understanding of the functional significance of the middle/charged region of Hsp90 is necessary, binding of various peptides were studied and the electrostatic binding of neuropeptide Y to the charged domain of Hsp90 was demonstrated.

3. Materials and methods

3.1. Cell cultures

The human T-lymphocyte cell line (*Jurkat, J32*) was provided by *M. Kamoun* (*Department of Pathology and Laboratory Medicine, Philadelphia PA, USA*) and was cultured in RPMI-1640 medium (*GIBCO-BRL*) supplemented with 10 % heat inactivated bovine foetal calf serum, 100 U/ml penicillin, 100 ?g/ml streptomycin, 2 mM glutamine and 0.2 % commercial antimycotic solution (*GIBCO-BRL*).

3.2. Yeast cultures

Wild type strains of bakers yeast, *Saccharomyces cerevisiae* (*S-288*) and fission yeast, *Schizosaccharomyces pombe* (*L-972*) were provided by Prof. Anna Maraz (*Department of Microbiology and Biotechnology, St. Istvan University, Budapest, Hungary*) and were grown in liquid YEPD medium. Yeast cells from the midlog phase of growth (at 30 ?C) were used for making yeast spheroplasts and protoplasts.

3.3. Bacterial cultures

The bacteria used in the present study were as follows, XL1-Blue [*Genotype: sup*44 *hsd*R17 *rel*A1 end A1 *gyr*A46 *thi rel*A*lac*$^-$ F' [*pro*AB$^+$ *lac*I$_q$ *lac*Z?M15Tn*10* (*tet*^{r})], DH5? [*Genotype: sup*44? *lac*U69 (? 80*lac*Z? M15) *hsd*R17 *rec*A1 *gyr*A96 *thi*-1 *rel*A1], and BL21-(DE3) [*Genotype*: *hsd*S *gal* (?*cIts*857 *ind*1 Sam7 *nin*5 *lac* UV5-T7 gene-1]. Bacteria were inoculated in liquid LB (*Sigma*) and were incubated at 37 ?C, with constant shaking (200 rpm).

3.4. Preparation of yeast spheroplasts and protoplasts

Yeast cells from the midlog phase of growth (at 30 ?C) were used for making yeast spheroplasts and protoplasts. First, yeast cells were treated with 0.1 mg/ml of lyticase in case of *S. cerevisiae* (*Jazwinski, 1990*) and 1 mg/ml lysing enzyme in case of *S. pombe* (*De Sampaio et al, 1999*) at 30 ?C for 30 or 45 min to make spheroplasts or protoplasts, respectively. Spheroplasts and protoplasts were collected in isotonic 1 M sorbitol and used for treatments.

3.5. Preparation of bacterial protoplasts

Bacteria from the mid-log phase of growth (at 37 ?C) were collected, centrifuged at 3000 g for 10 min at 4 ?C, suspended in 1 ml of 20 % w/v sucrose in 50 mM Tris.HCl pH 8.0. The cell wall was digested with freshly made lysozyme (*Sigma*, 0.5 mg/ml in 25 mM Tris.HCl, pH 8.0) for 15 min in between scrolling tubes at 37 ?C. Cells were spun at 2000 rpm for 2 min, the pellet containing the protoplasts was used for immediate experimental purpose. Protoplasts were stored in 0.9 % NaCl isotonic solution and used for experimental analysis.

3.6. Purification of rat liver Hsp90

Rat liver Hsp90 protein was a kind gift from *Dr. C. Soti* (*Soti et al, 1998*). Its isolation was performed follows. Fresh rat livers were collected from 2 months old Wistar rats after sacrificing the animals according to the Animal Ethics Regulations. An isolation buffer (basic buffer: 10 mM Hepes, pH 7.4, 1 mM ATP, 1 mM DTT, 0.5 ?g/ml PMSF, 5 ?g/ml aprotinin, 2 ?g/ml leupeptine) and were homogenized in a Dounce homogenizer followed by a 20 min centrifugation (14,000 rpm; *Beckman JA20 rotor*). After passing through 6 layers of gauze cloth the supernatant was subjected to 30 % ammonium sulfate precipitation for 1 h at 4 ?C and was centrifuged (14,000 rpm; *Beckman JA20 rotor*). The supernatant was passed through different chromatography columns, Butyl-Sepharose (basic buffer), DEAE-Bioscale-20 Cellulose (basic buffer + 0.15 M NaCl) Hydroxyapatite (basic buffer + 0.4 M phosphate, 5 mM NaCl) and Q-Chromatography (basic buffer + 0.5 M NaCl) with dialysis (one exchange of 2 liters) after every flow through using buffers, buffer B (10 mM Hepes, pH 7.4, 1 mM ATP, 1 mM DTT, 1 mM EDTA), buffer D (10 mM phosphate, pH 6.8, 5 mM NaCl, 0.1 mM DTT), buffer H (20 mM Tris.HCl, pH 7.4, 0.1 mM EDTA, 0.1 mM DTT), and buffer Q (10 mM Hepes, pH 7.4, 0.1 mM EDTA, 0.1 mM DTT), respectively.

3.7. Lactate dehydrogenase measurements

The activity of lactate dehydrogenase (LDH) was measured using the direct spectrophotometric assay of *Wroblewski & La Due* (*1955*) in the presence of pyruvate and NADH. In 2 ml-s of a 50 mM Hepes buffer (pH 7.4) containing 30 ?M pyruvate and

30 ?M NADH 10 ?l-s of Jurkat cell supernatant were added after the indicated treatments with drugs and detergents, and changes in optical density were measured at 340 nm for 5 min. Special care was taken to avoid the absorption of LDH to the cuvette wall during the activity measurement. The percentage of LDH release was calculated by dividing the activity of LDH in the supernatant by the LDH activity measured after complete cell lysis achieved by sonication. None of the detergents and drugs affected LDH activity, when added directly to the reaction mixture at the concentrations used in whole cell experiments.

3.8. Superoxide production measurements

Superoxide production in geldanamycin-treated Jurkat cells was assayed by the lucigenin-enhanced chemiluminescence method (*Xie & Wolin, 1996*). 2 x 10^6 Jurkat cells (2 x 10^6 cells/ml) after a treatment with various drugs were washed free of the used inhibitors, re-suspended in 10 mM Hepes, pH 7.4, and added to the scintillation vial containing 5 ?M lucigenin in the same Hepes buffer. Lucigenin chemiluminescence with and without cells was recorded as cpm (counts per minute) for about 10 min in 0.1 min intervals in a *Beckman LS7800 liquid scintillation counter* using a single photon mode. Chemiluminescence values were corrected to background chemiluminescence without added cells.

3.9. Membrane fluidity measurements

The procedure was adapted from *Revathi et al* (*1994*). Jurkat cells after treatment with the drug indicated, were incubated with 1 ?M 1,6-diphenyl-1,3,5-hexatriene (DPH) for 15 min in the dark in triplicates. Cells were washed free of DPH, re-suspended in 2 ml of PBS, and the fluorescence was immediately measured using a steady state spectrofluorimeter (*M300-Edinburgh Instruments*) in 1 cm path length quartz cuvettes with an excitation and emission wavelengths of 357 and 430 nm, respectively. Fluorescence polarization was measured after adapting cells in dark with constant stirring.

3.10. Construction, cloning of anti-Hsp90 hammerhead ribozymes

The design of anti-Hsp90 hammerhead ribozyme was adapted from the studies of *Little & Lee* (*1995*). The ribozyme was designed to cleave in a highly conserved segment of the coding region of Hsp90 at its N- or C-terminus, respectively. Partially overlapping nucleotides for both Hsp90? and Hsp90? N-terminal (sense strand: AGC-TTC-TGC-CTC-TGA-TGA-GGC-CGA-AAG-GCC-GAA-AAA-GGC-AAA-CTC-GAG-ATT-AAT-AAA-AGC, antisense strand: GGC-CGC-TTT-TAT-TAA-TCT-CGA-GTT-TGC-CTT-TTT-CGG-CCT-TTC-GGC-CTC-ATC-AGT-GGC-AGA) and C-terminal (sense strand: AGC-TTC-ATC-CTG-ATG-AGG-CCG-AAA-GGC-CGA-AAT-ACC-TAG-ACT-CGA-GAT-TAA-TAA-AAG-C, antisense strand: GGC-CGC-TTT-TAT-TAA-TCT-CGA-GTC-TAG-GTA-TTT-CGG-CCT-TTC-GGC-CTC-ATC-AGG-ATG-A were synthesized. These sense and antisense oligonucleotides were annealed at room temperature for 15 min in a 50 mM Tris.HCl (pH 7.6) buffer. Prior to cloning, these oligonucleotides were further extended with 5 Units of Klenow polymerase in a standard PCR buffer (50 mM Tris.HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl and 10 ?M dNTP mix) for 60 min at 37 ?C. The end-filled products were precipitated using ethanol, re-suspended in sterile double distilled water, and double digested with HindIII and NotI. For cloning the cloning vector, pcDNA3 with an hCMV promoter and a polylinker containing the bovine growth hormone poly-A sequence was selected. In the vector the upstream ATG (at nucleotide position 995) after the XbaI and ApaI sites was disrupted by introducing a stop codon followed by XhoI, NheI, NotI and SacII restriction sites. The vector was digested with HindIII and NotI restriction enzymes, the digested vector was dephosphorylated using calf intestinal phosphatase at 37 ?C for 15 min and the enzyme was inactivated by phenol:chloroform extraction. The anti-Hsp90 hammerhead ribozyme oligonucleotides were then inserted as described in *Sambrook et al* (*1989*) at the HindIII and NotI sites within the multiple cloning site of pcDNA3. Ligation reactions were carried out in a total volume of a 10 ?l buffer containing 50 mM Tris.HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5 % PEG-8000. The double digested insert and the vector were ligated in a molar ratio of 3:1 with 1U of T4 DNA ligase at 16 ?C for 12 h. XLN-blue competent cells were transformed with the ligation mix, and plated on Luria-Bertani (LB) agar plates containing 60 ? g/ml ampicillin. The colonies were selected from

the plate, and were subjected to plasmid miniprep. The positive clones were selected by Southern blot analysis using the respective end-labelled, annealed antisense oligo.

3.11. Plasmid isolation

Plasmid DNA was isolated using a *QIAGEN* plasmid-midiprep isolation kit according to the manufacturer's instructions provided with the kit. The purity was always checked both spectrophotometrically and by running on 1 % agarose gel.

3.12. Southern blot analysis

To confirm the ligation of antisense ribozymes, isolated plasmid DNA was run on a 1 % agarose gel and the DNA was capillary transferred to a nylon membrane *(*N+, *Amersham)* using 2 X SSC buffer (0.3 M NaCl and 30 mM sodium citrate, pH 7.0). The ribozyme was radiolabelled with 10 ?Ci of ^{32}P as described by the supplier (Promega) using the Klenow enzyme as follows. 1 ?g of DNA was mixed with 1 ?l of 10 x Klenow buffer, 2 mM dNTP-s without dATP, 10 ?Ci of ^{32}P-phosphate , and 1 U Klenow enzyme incubated for 10 min at 37 ?C, the reaction was stopped by adding 0.1 M EDTA. The probe was purified by a Sephadex-G 25 spun column and the per cent ^{32}P incorporation was calculated using scintillation counting (Beckman LS7800). The probe with high specific activity (2 x 10^8 cpm/?g DNA) was used for Southern blotting in 1 M sodium phosphate buffer, pH 7.4) containing 7 % SDS for 4 h in a hybridization chamber (*Amersham*) at 65 ?C (*Southern, 1975*). The unbound probe from the blot was washed off with serial washes of SSC and the blot was exposed to an X-ray film.

3.13. RNA isolation

Total RNA from Jurkat cells was isolated using Trisol (*Sigma*) solution. First, cells (2 x 10^6 /ml) were suspended in 1 ml of Trisol and left at room temperature for 5 min, followed by the addition of 0.2 ml chloroform:isopropanol (49:1) solution. Samples were mixed vigorously by hand for 15 sec and were incubated for 3 min at room temperature. Tubes were centrifuged at 10,000 rpm for 20 min (*Sorval 5B, SS34 rotor*), the aqueous phase was collected and the RNA was precipitated with 0.5 ml isopropanol at room temperature for 10 min. The RNA pellet was collected by further centrifugation

at 12,000 rpm for 10 min (*Sorval 5B, SS34 rotor*), the pellet was washed twice with 70 % ethanol, air dried and used for further experiments. RNA integrity was checked on a 1 % agarose gel containing 40 mM 3-(N-morpholino)-propanesulfonic acid, 10 mM sodium acetate, 1 mM EDTA, 0.1 % deionised formamide and 0.1 % formaldehyde.

3.14. RT-PCR analysis

The first strand cDNA was made from 5 ?g of total RNA (10 ?g/?l) in a reaction mixture containing 2.5 U of RNase inhibitor, 2 ?l of reverse transcriptase (RT) buffer (5x, stock), 10 pmoles of oligo dT primer, 1 ?l dNTP mix (dATP, dCTP, dGTP, dTTP 2 mM each), 2 ?l of $MgCl_2$ (25 mM, stock), 1 U reverse transcriptase enzyme (*MBP Pharmaceuticals*) in a total volume of 10 ?l, and was incubated at 43 ?C for 1 h. From the reaction mixture 1.5 ?l was taken for the first round of PCR (30 cycles, each cycle: 94 ?C -one min, 55 ?C -one min, 72 ?C –two min with a final extension for 10 min at 72 ?C) with full length *Hsp90* specific primers: sense 5'- CCT-GAG-GAA-GTG-CAC-CAT-GGA-GAG-3' and antisense 5'-CTA-ATC-GAC-TTC-TTC-CAT-GCG-AGA-CG-3'; sense: 5'-AGC-TCG-AGA-CCT-GAG-GAA-ACC-CAG-ACC-CAA-GAC-3' and antisense: 5'-CAT-GGT-ACC-TTA-GTC-TAC-TTC-CTT-CCA-TGC-GTG-ATG-TGT-C-3' for *Hsp90?* and *Hsp90?* , respectively.

3.15. Transfection and screening of Jurkat cells

Jurkat cells were transfected with either pcDNA3 (*mock*), or the respective anti-Hsp90 hammerhead ribozyme using the poly-cationic reagent, Lipofectamine. The entire transfection protocol was performed according to the manufacturer's instructions in a 96 well plate (*NUNC^{TM}, Nalgene Nunc International, NY*) in multiples. Control and ribozyme-transfected cells were screened for cell viability using trypan blue dye exclusion. Neomycin selection was not feasible, since the rate of cell death with ribozyme transfections was constantly increasing showing the importance of high Hsp90 levels for the survival of eukaryotic cells. Hsp90 content was checked by immunoblot with anti-Hsp90 antibody. Cells on the second day of transfection, where cell death was not yet much prevalent were used for further experiments.

3.16. SDS polyacrylamide gel electrophoresis and Western blot analysis

Cell lysates were mixed with *Laemmli* buffer (*Laemmli*, 1970) containing 100 ?M DTT for 5 min and the samples were subjected to 10% SDS-PAGE. Proteins were transferred from the gel to nitrocellulose membrane using a semidry protein gel transfer apparatus. Transfer of proteins was confirmed by Ponceau-S staining, and the blot was processed for Western blot analysis using a primary antibody followed by a horseradish peroxidase-conjugated secondary antibody. Labelled bands were visualized using an enhanced chemiluminescence kit (*Amersham*).

3.17. Immunofluorescence

Jurkat cells after respective treatments were washed twice with phosphate buffered saline, pH 7.6 (PBS), fixed with 80 % ethanol and left in the refrigerator for 6 h. Then the fixative was removed with a brief spin, cells were washed once with PBS, permeabilized with 0.1 % Triton X-100 and blocked with 1 % gelatin in Tris.HCl buffered saline, pH 7.6, containing 0.1 % Tween-20 (TBST) for 1 h, followed by first antibody (1:50 dilution) in TBST buffer containing 1 % BSA for 1 h. Cells were washed with TBST (3 x 5 min), the fluorescently tagged secondary antibody was added (1:200 dilution) and samples were incubated for an additional 45 min. The secondary antibody was removed with a TBST wash (3 x 5 min), anti-fade solution was added and permanent slides were made by sealing them with wax/paraffin. Slides were viewed under fluorescence microscope (*Nikon Eclipse, E400, Japan*).

3.18. Propidium iodide staining

Jurkat cells were subjected to various drug treatments and were fixed with 80 % ethanol as described earlier, the nuclei were stained with 50 ?g/ml propidium iodide (*Sigma*) in distilled water containing 100 ?g/ml RNase (*Fluka*) and assessed for apoptotic cells under fluorescent microscope (*Nikon Eclipse, E400, Japan*).

3.19. Trypan blue exclusion

Cultured cells were tested for cell viability by incubating cells with 0.4 % trypan blue (*Sigma*) for 90 seconds at room temperature and by visualizing cells under microscope.

3.20. Phalloidin and DAPI staining

Cell after respective treatments were washed twice with PBS and fixed with 8 % paraformaldehyde solution (*Sigma*) for 10 min followed by one PBS wash, were permeabilized with 0.1 % Triton X-100 for 3 min, washed with PBS and were stained with 1:10,000 dilution of phallodin (*Molecular Probes*) for 45 min. Then 300 ?M DAPI (*Molecular Probes*) was added to the samples and samples were incubated for an additional 15 min at room temperature. After a 1 h incubation the staining solution was removed, a mounting medium (*Vectashield mounting medium, Vector laboratories, Inc. Burlingane, CA*) was added to the cells and cells were visualized under fluorescent microscope (*Nikon Eclipse, E400, Japan*).

3.21. Xanthine oxidoreductase assay

The fluorimetric assay for measuring xanthine oxidase and dehydrogenase was adapted from *Beckman et al* (*1989*). Into 1 ml-s of 50 mM potassium phosphate buffer (pH 7.8) the following solutions were added in the order described: first the sample (Jurkat cell extract), then 10 ?M pterin, then 10 ?M methylene blue and finally 50 ?M allopurinol. The fluorescence was measured for 1 min after addition of each solution at 345 nm. The reaction was finally calibrated by adding 10 ?l of 0.1 ?M isoxanthopterin (IXPT). The enzyme activities, xanthine oxidase and xanthine dehydrogenase were obtained from the same assay and the whole assay was carried out in a 1 ml quartz cuvette in 1ml total volume. The blank was always maintained without sample. Units of enzyme activity were expressed as 1 ?mol/min/mg of product formed at 25 ?C.

3.22. Measuring thiobarbituric acid reactive substances (TBARS)

TBARS were assayed by the method developed by *Buege & Aust* (*1976*) with modification. The assay mixture contained 0.1 ml of Jurkat cell extract, 0.4 ml of 0.9 % NaCl, 0.5 ml of 3 % SDS, 2 ml of thiobarbituric acid reagent (15 % w/v TCA, 0.375 % w/v thiobarbituric acid, 0.25 N HCl). The solution was heated at 95 ?C for 30 min, cooled under running tap water, extracted by adding 3 ml of *n*-butanol. After centrifugation at 3000 rpm for 15 min the butanol phase was assayed spectrophotometrically at 532 nm. Various concentrations of tertramethoxypropane (TMP) served as external standards and Jurkat cells treated with H_2O_2 were used as a positive control. The malondialdehyde (MDA) formed from the breakdown of polyunsaturated fatty acids (PUFAs) during lipid peroxidation was expressed in nmol/mg protein.

3.23. Visualization of lipid peroxidation using DPPP (diphenyl-1-pyrenylphosphine)

The procedure was adapted from *Takahashi et al* (*2001*). To visualize the extent of lipid peroxidation induced by Hsp90 inhibitors (especially by geldanamycin), Jurkat cells (2 x 10^6 cells/ml) were washed three times with PBS, pre-incubated in PBS at 37 ?C containing 150 ?M DPPP for 5 min, washed by three times by PBS, re-suspended in RPIM-1600 medium, transferred to cell incubator, incubated with/without geldanamycin for 2 h and visualized under fluorescent microscope. To visualize the membrane-associated lipid peroxidation, Jurkat cells were pre-incubated with DPPP (50 ?M) as described earlier, and were treated with geldanamycin for 30 min. The whole procedure was done in dark.

3.24. Real-time detection of lipid peroxidation

The procedure was adapted from *Manevich et al* (*2002*). DPPP-labeled cells were made as described above, and were suspended in PBS (2 x 10^6 cells/ml). Real-time kinetics of oxidized DPPP fluorescence in Jurkat cells after addition of 2 ?M geldanamycin or 50 nmols of H_2O_2 was continuously measured at 380 nm (*excitation, 350 nm*) in a *Perkin Elmer* spectrofluorimeter equipped with a single photon-counting system with excitation/emission slits of 1 and 3 nm-s, respectively.

3.25. Effect of geldanamycin on mitochondrial permeability transition

Isolation of mitochondria: Mitochondria were isolated from rat liver as follows: one month female Wistar rat was sacrificed, the liver was removed and washed twice with PBS, once with ice cold washing buffer (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$), homogenized in a glass potter homogenizer in isolation buffer (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 250 mM sucrose, 1 mM EGTA, 1 mM EDTA, 1 mM PMSF, 1 ? g/ml leupeptine, 1 ? g/ml aprotinin, 1 ? g/ml benzamidine) by 40 strokes. The sample was centrifuged at 3500 rpm for 10 min (*Sorval 5B centrifuge*), the supernatant was collected and re-centrifuged at 12,000 rpm for 10 min (*Sorval 5B, SS34 rotor*). The pellet containing mitochondria was suspended in a buffer containing 400 mM mannitol, 50 mM Tris.HCl, pH 7.2, 5 mg/ml BSA, 10 mM KH_2PO_4 and used for further experiments.

MPT assay: To assess the induction of permeability transition the *in vitro* swelling of mitochondria by was assayed in CFS buffer (220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 5 mM KH_2PO_4, 2 mM ATP, 50 ? g/ml creatine phosphokinase, 10 mM phosphocreatine) with 2 ? M/ml rotenone. Mitochondria were treated with geldanamycin or geldanamycin + purified Hsp90 in the presence or absence of 30 ? M cyclosporin A (CsA) and the absorbance variation caused by swelling was measured using a double beam spectrophotometer at 520 nm as described earlier (*Kondoh et al, 2001*).

3.26. Preparation of competent bacteria

Competent cells for bacterial transformation were prepared as follows. From 10 ml of overnight cultures of bacteria 500 ? l was inoculated to 250 ml LB and the optical density was monitored during the growth at 37 ? C to have cells in midlog phase (OD_{600}=0.2 to 0.3). Cells were spun at 4 ? C in GSA rotor (*Sorval 5B*) for 15 min at 5000 rpm. The pellet was re-suspended in ice cold 12 ml of sterile filtered TSB buffer (7.5 ml 2 x LB, 3.8 ml 40 % PEG 6000, 1.5 ? l 1 M $MgSO_4$, 1.5 ? l 1 M $MgCl_2$ and 750 ? l of DMSO). Cells in small aliquots were quick frozen in liquid nitrogen and stored at –80 ? C for further use.

3.27. Bacterial cell transformation

1 to 80 ?l of DNA, 20 ?l of 5 x KCM solution (0.5 M KCl, 0.15 M $CaCl_2$, 0.25 M $MgCl_2$) and 100 ?l of competent cells (which were thawed on ice) were mixed together, first incubated on ice for 20 min, at room temperature for 10 min and after the addition of 600 ?l fresh LB the incubation was continued for an additional 45 min at 37 ?C with shaking. Cells were spun, were re-suspended in 50 ?l fresh LB and were spread on LB plates with respective antibiotics. After 5 h of incubation at 37 ºC cell colonies were monitored and used for further experiments.

3.28. Apoptotic DNA ladder detection

Jurkat cells after various treatments were fixed in ethanol for 6 h at 4 ?C, washed with PBS and suspended in citrate-phosphate buffer, pH 7.4, having concentrations of 0.2 M and 0.1 M, respectively in a ratio of 192:8. DNA was extracted after different treatments with 0.2 % NP40, 0.1 mg/ml RNase, 100 ?g/ml proteinase K, and was run on 0.7 % agarose gel for 8 h. DNA fragmentation was visualized by staining the gel with ethidium bromide (50 ?g/ml) under UV.

3.29. Surface plasmon resonance (SPR)

Binding of peptides to purified Hsp90 was determined by surface plasmon resonance analysis using a carboxy dextran coated Biacore 1000 biosensor system (*Biacore AB, Uppsala, Sweden*). All the solutions for the SPR measurements were passed through a 0.22-micron Millipore filter and degassed using vacuum at room temperature. Fisrt the sensor chips were incubated in a buffer containing 10 mM Hepes, pH 7.4, 0.15 M KCl and 0.001 % Tween-20, for 10 min (*at 25 ?C*) at a flow rate of 5 ?l/min. The sensor chip was activated with 200 mM of 1-ethyl-3-[3-dimethylaminopropyl]-carbodiamide and 50 mM N-hydroxy-succinamide in MilliQ deionized water for 5 min at 25 ?C. Rat liver Hsp90 (5 ?g/?l) was coupled to the chip in a buffer containing 10 mM Na-formiate, pH 4.0, for 5 min at 25 ?C. The remaining active sites of the resin were inactivated by incubation with a 1 M ethanolamine-HCl solution (pH 8.5) for 5 min at 25 ?C. After coupling, non-covalently bound Hsp90 was removed by 1 (2 min) flux of 10 mM HCl. Binding of peptides to Hsp90 was analyzed in a running buffer and after each

binding experiment the sensor chip was washed with a short flux of 10 mM HCl. The contribution of ionic interactions to the binding of Hsp90 to NPY was assessed by using high ionic strength potassium chloride and sodium chloride solutions. All the binding experiments were repeated with mock coupled sensor chips, where in the coupling procedure Hsp90 was omitted. Binding curves (sensorgrams) were corrected by subtracting the electrostatic binding of the peptide.

3.30. Statistical analysis

Data are presented as means ? SEM of minimum three independent experiments unless otherwise indicated, and were analyzed with unpaired Student's t-test. p ? 0.05 was accepted as indicating a statistically significant difference compared with controls. At the figures ? stands for p ? 0.05, ?? stands for p ? 0.01 and ??? stands for p ? 0.001.

4. Results

4.1. Geldanamycin induces an accelerated lysis of Jurkat cells

In order to test, whether Hsp90, a cytoprotective chaperone forming a complex with a lot of cytoplasmic proteins is involved in the maintenance of cellular integrity

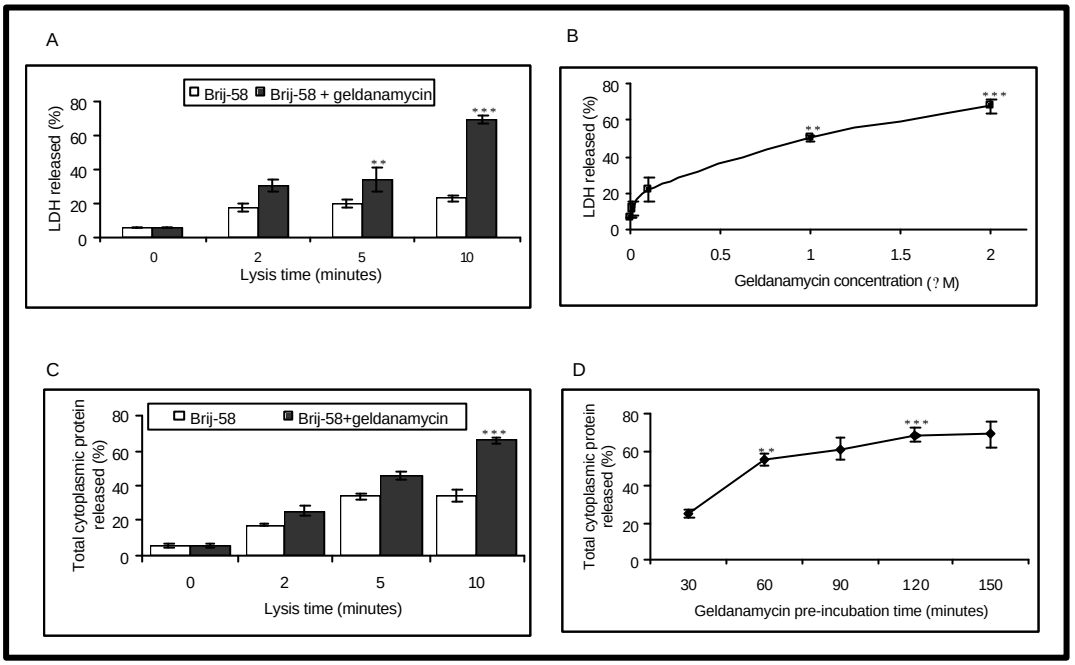

Fig 1. Geldanamycin increases detergent-induced lysis of Jurkat cells. 2 x 10⁵ cells (2 x 10⁵ cells/ml) were incubated in the absence (open symbols) or presence (filled symbols) of GA at final concentrations and times indicated. Cells were lysed with 0.005% Brij-58, and the activity of released LDH or the amount of total proteins were measured as described in Materials and Methods. 100% lysis was achieved by sonication and results were normalized to this value. Panel A: Effect of GA treatment on LDH-release. Cells were incubated without or with 2 ? M GA for 2 h and lysed with Brij-58 for the times indicated. Panel B: Concentration dependence of geldanamycin-induced increase of LDH-release. Cells were incubated without or with GA for 2 h at final concentrations indicated and lysed with Brij-58 for 10 min. Panel C: Effect of GA treatment on total protein-release. Cells were incubated without or with 2 ? M GA for 2 h and lysed with Brij-58 for the times indicated. Panel D: Time dependence of geldanamycin-induced increase of total cytoplasmic protein release. Cells were incubated with 2 ? M GA for times indicated and lysed with Brij-58 for 10 min.

(*Csermely, 2001*) Jurkat cells were subjected to mild detergent lysis with Brij-58. Lysis conditions were optimized to achieve an approximately 20 % lysis of lactate dehydrogenase (LDH) in a 10 min detergent treatment (Fig. 1A, *open bars*). Preincubation of Jurkat cells with geldanamycin, a specific inhibitor of Hsp90 (*Stebbins*

et al, 1997; Whitesell et al, 1994) induced a significant increase in the extent of released LDH (Fig. 1A, *filled bars*). In agreement with previous data (*Schnaider et al, 2000; Stebbins et al, 1997; Whitesell et al, 1994*) geldanamycin treatment alone, without additional Brij-58 lysis did not induce a significant lysis of Jurkat cells (*data not shown*). Geldanamycin-induced lysis was not specific to Brij-58, but could be observed if primary cell lysis was induced by other detergents, like the plasma membrane-specific digitonin or by hypotonic shock (*data not shown*). The effect was concentration dependent, showing saturation above 2 ?M geldanamycin (Fig. 1B and *data not shown*).

The geldanamycin-induced enhancement of the release of LDH, a well-known cytoplasmic marker protein might be the consequence of a heretofore-unknown interaction between LDH and Hsp90, where geldanamycin would dissociate LDH from Hsp90, and cause its accelerated release. However, the geldanamycin-induced enhancement of cell lysis was a general feature of all cytoplasmic proteins, including Hsp90 itself, Hsp90 substrates, like the Raf and Lck kinases and total cytoplasmic proteins (Fig. 1C and *data not shown*). The extent of maximal release was similar for LDH and total proteins showing that the inhibition of Hsp90 induced a fairly general destabilization of cellular structures. The geldanamycin-induced enhancement of cell lysis was not instant but required at least one hour pre-incubation with the drug to be effective (Fig. 1D). Our data are in good agreement with the concentration- and time-dependence of geldanamycin-induced changes in Hsp90 substrate proteins (*Schnaider et al, 2000; Stebbins et al, 1997; Whitesell et al, 1994*).

The geldanamycin-induced enhancement of cell lysis was similar to the increase in cell lysis after disruption of cytoskeletal elements with colchicine or cytochalasin (*data not shown*). However, the effect of geldanamycin was not changed (*data not shown*) if lysis was performed in the presence of actin or tubulin stabilizing buffers (*Carraway et al, 1982; Osborn & Weber, 1997*), which suggests that the geldanamycin-induced effect is not a direct consequence of cytoskeletal disorganization. A highly similar pattern of geldanamycin-induced increase in Brij- or hypoosmotic shock-induced hemolysis was observed in mouse red blood cells. Time- and concentration-dependence of geldanamycin-induced additional hemolysis was very similar to the lysis rates obtained

with Jurkat cells (*Pato et al, 2001*; *Csermely et al, 2003; data not shown*) showing the generality of the effects observed.

4.2. Geldanamycin-induced additional cell lysis is partially dependent on oxidative stress

Interestingly, there was a marked difference in the extent of geldanamycin-induced additional lysis, if cells were shaken during the experiment. When geldanamycin treatment was combined with additional shaking, a larger increase in cell lysis was observed (Fig. 2A). Shaking the cells after geldanamycin treatment, but before detergent treatment might help to disrupt cellular structures otherwise preventing the faster release of LDH and cytoplasmic proteins in the presence of Brij-58. However, shaking-induced differences were larger at larger geldanamycin concentrations (Fig. 2B), which suggested that the two effects are not independent from each other.

Reaching this point in our experiments the first results of geldanamycin-induced superoxide generation appeared (*Billecke et al, 2002; Dikalov et al, 2002*). What if shaking provided additional oxygen inducing a larger amount of superoxides? The addition of reduced glutathione as an antioxidant to the incubation medium reduced the geldanamycin effect (Fig. 2C). On the contrary, the effect of other Hsp90 inhibitors, like the much more effective N-terminal inhibitor, radicicol (*Soga et al, 1998*), the C-terminal inhibitor, cisplatin (*Itoh et al, 1999; Soti et al, 2002*) and novobiocin, which inhibits nucleotide binding to Hsp90 at both termini (*Marcu et al, 2000a; Soti et al, 2002*) was not changed by glutathione addition (Fig. 2C).

Geldampicin, an ineffective geldanamycin analogue (*Whitesell et al, 1994*) did not induce a large increase in cell lysis, and the effect of all other Hsp90 inhibitors was in between the control and geldanamycin-induced level being roughly equal with the lysis after the simultaneous addition of geldanamycin *and* reduced glutathione (Fig. 2C). These data raised the possibility that the geldanamycin-induced additional cell lysis was a result of *both* geldanamycin-induced superoxide production and Hsp90 inhibition.

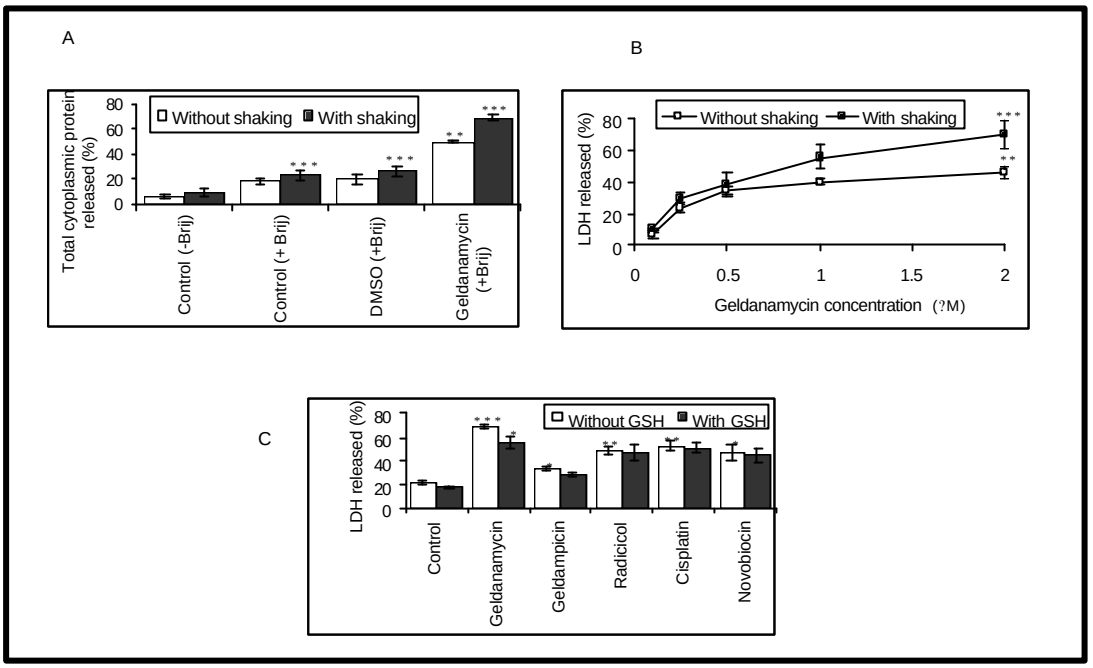

Fig 2. Geldanamycin- (but not other Hsp90 inhibitor-) induced increase of Jurkat cell lysis is dependent on oxidative conditions. 2 x 10^5 cells (2 x 10^5 cells/ml) were incubated in the absence or presence of various Hsp90-inhibitors for 2 h at a final concentration of 2 ?M unless otherwise indicated. 100% lysis was achieved by sonication and results were normalized to this value. Panel A: Effect of shaking to geldanamycin-induced additional release of total cytoplasmic proteins. Cells were incubated with GA for 2 h at a final concentration of 2 ?M without (open bars) or with (filled bars) additional shaking and lysed with Brij-58 for 10 minutes. Panel B: Effect of shaking on the concentration dependence of geldanamycin-induced increase in LDH-release. After GA incubation at final concentrations indicated, cells were incubated with Brij-58 without (open diamonds) or with (filled squares) shaking. Panel C: Effect of GSH on the increase of LDH-release induced by various Hsp90 inhibitors. Cells were incubated without or with 2 ?M geldanamycin (GA), 2 ?M geldampicin (GP), 2 ?M radicicol (RA), 2 ?M cisplatin (CDDP) or 0.1 mM novobiocin (NB) for 2 h in the absence (open bars) or presence (filled bars) of 2 mM reduced glutathione, and lysed with Brij-58 for 10 min.

4.3. Geldanamycin induces superoxide production in Jurkat cells and increased membrane fluidity

Observing an oxidative stress-related component of geldanamycin-induced additional cell lysis, we wanted to obtain a direct evidence for geldanamycin-induced superoxide production in Jurkat cells. Indeed, geldanamycin, but not the inactive analogue, geldampicin (*Whitesell et al, 1994*) induced a significant increase in lucigenin chemiluminescence (Fig. 3A), which is a clear indication of geldanamycin-induced

superoxide production in Jurkat cells. To assess, if geldanamycin affects the status of Jurkat cell membranes, we opted to measure membrane fluidity by measuring the fluorescence polarization of the commonly used probe, 1,6-diphenyl-1,3,5-hexatriene (DPH, *Revathi et al, 1994*). Geldanamycin induced a significant decrease in DPH fluorescence polarization (Fig. 3B). On the contrary to our results with geldanamycin, another N-terminal inhibitor of Hsp90, radicicol (*Soga et al, 1998*) did not induce a significant change in DPH fluorescence polarization (*data not shown*). Our data suggest that geldanamycin-induced superoxide production may lead to an increased membrane fluidity, membrane disorganization in Jurkat cells, which may contribute to their increased sensitivity to lysis conditions.

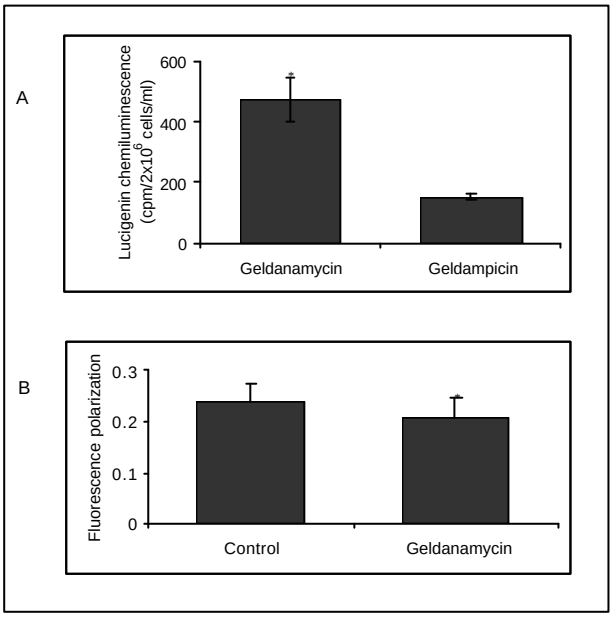

Fig 3. Effect of geldanamycin on superoxide production and membrane fluidity. 2 x 10^6 cells (2 x 10^5 cells/ml) were incubated in the absence or presence of geldanamycin (GA) or geldampicin (GP) for 2 h at final concentrations of 2 ?M, respectively. Superoxide production or fluorescence polarization of the membrane probe, DPH was measured as described in Materials and Methods. Panel A: Effect of GA and GP on superoxide production. Cells were incubated without or with GA, and their superoxide production was measured. Data were normalized to background superoxide level of control cells. Panel B: Effect of GA on membrane fluidity. Cells were incubated without or with GA and their membrane fluidity, was measured.

4.4. Incorporation of DPPP into the cell membranes

Observing a change in membrane fluidity and membrane polarization, which are in way related to superoxide production, we made an attempt to visualize the extent of lipid peroxidation upon GA treatment. Because of its specificity for lipid oxidation, DPPP (*Takahashi et al, 2001*) was examined for its incorporation into plasma membrane. In these experiments cells (2 x 10^6 /ml) were labelled with high concentrations of DPPP followed by geldanamycin treatment and were viewed under fluorescent microscope. Due to the intracellular oxidation upon geldanamycin treatment cells were fluorescently labelled with DPPP suggesting geldanamycin-induced lipid peroxidation (Fig. 4B and 4C). H_2O_2 was used as a positive control for oxidative effects (Fig. 4D, 4E and 4F).

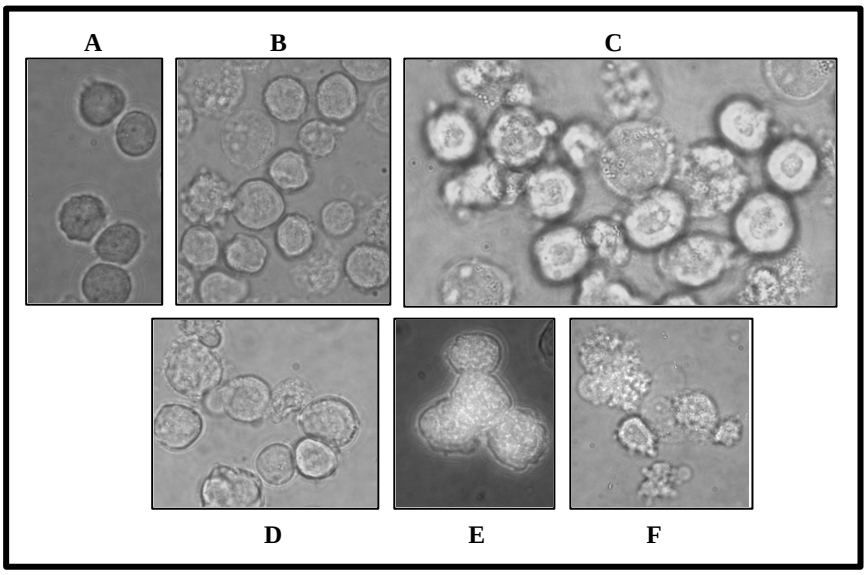

Fig 4. Incorporation of DPPP into Jurkat cells. Cells were pre-incubated with 200 ?M DPPP for 15 min at 37 ?C followed by 2 ?M geldanamycin treatment. Fluorescence microscopic images were taken with (B & C) and without geldanamycin (A) incubation for 2 (B) and 8 h (C), respectively. Hydrogen peroxide was used as a positive control. 50 ?M H_2O_2 was used for the current experiment for 15 (D), 45 (E) and 120 (F) min, respectively.

4.5. Visualization of lipid peroxides upon geldanamycin treatment

The effect of geldanamycin on intracellular oxidation raises two possibilities for the geldanamycin effects, 1) the oxidation is associated with the redox cycling of the compound, 2) it is associated with Hsp90 chaperone function inhibition. Recently Hsp90 has been shown to be associated with membrane lipid rafts (*Shah et al, 2002; Waheed & Jones, 2002*). Our data of superoxide production, lipid peroxide production together with

a change in membrane polarization suggested the localized action of oxidative effects on the plasma membrane. To assess the localization of lipid peroxides Jurkat cells were processed as described earlier for DPPP treatment in PBS, however, DPPP was used at a 4000-fold lesser concentration to see the plasma membrane-associated effects better. Under these circumstances geldanamycin induced a localized membrane oxidation that appeared as patches on the surface of plasma membrane (Fig. 5D). Though there was certain amount of cytosolic oxidation in absence of Hsp90, ribozyme transfected cells failed to produce similar effects (Fig. 5C) presumably due to the pre-translational inhibition of Hsp90.

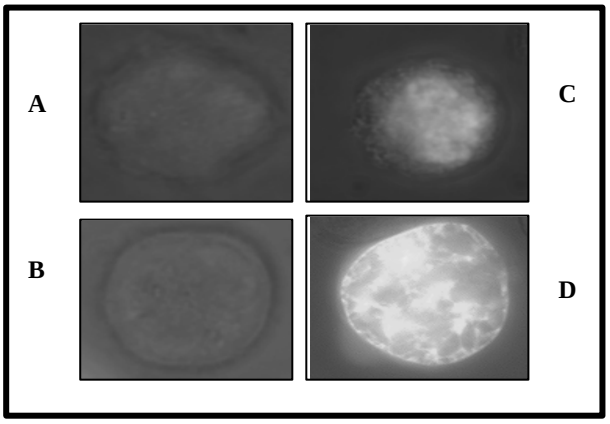

Fig 5. Incorporation of DPPP into Jurkat cell membrane. Cells were pre-incubated with 50 nmols of DPPP for 15 min with (C and D) and without (A and B) 2 ?M geldanamycin for 30 min and then fluorescence microscopic images were taken. Normal (B and D) and ribozyme transfected (A and C) cells were compared for membrane lipid peroxidation.

4.6. Fluorimetric measurements of membrane lipid peroxidation

DPPP if added before geldanamycin treatment may report mixed cellular redox changes such as redox cycling of the compound together with the consequences associated with Hsp90 inhibition. To rule out this possibility, Jurkat cells were preincubated with geldanamycin for 30 min or hydrogen peroxide for 5 min followed by the addition of DPPP. The cells were measured immediately for DPPP fluorescence (Fig. 6). The geldanamycin-induced effect is saturable. In contrast hydrogen peroxide treated cells exhibit a constant elevation of oxidation. The initial shift of oxidation upon geldanamycin treatment suggested that geldanamycin treatment indeed induces an oxidative step, which can not be a mere result of non-specific redox cycling of DPPP.

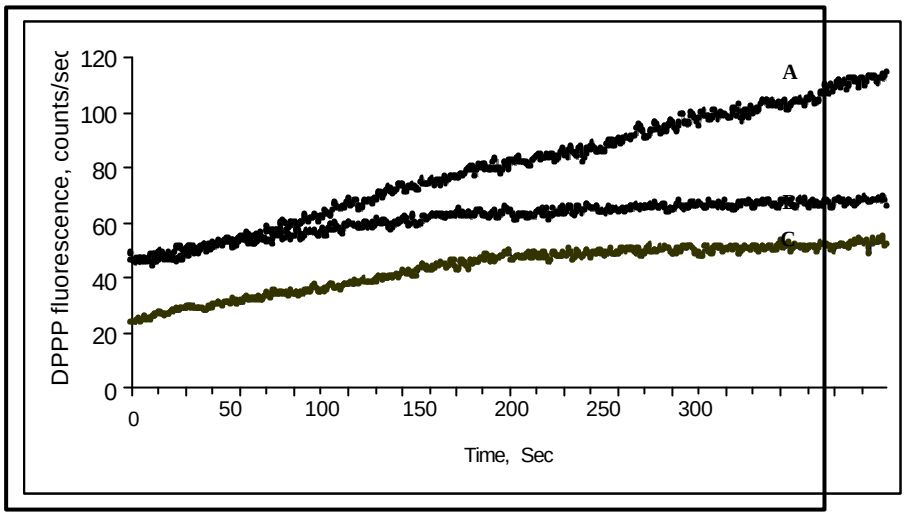

Fig 6. DPPP fluorescence detection of ?OH-mediated lipid peroxidation in plasma membrane. DPPP fluorescence was measured after addition of hydrogen peroxide (50 ?M, A), geldanamycin (2 ?M, B) and without any pre- treatment (C).

4.7. Measuring intracellular lipid peroxidation

As an additional independent evidence for lipid peroxidation besides DPPP fluorescence we measured the amount of malondialdehyde (MDA) as thiobarbituric acid reactive species (TBARS) (*Chen et al, 1995*). Jurkat cells were treated with different concentrations of geldanamycin for 2 h and processed for TBARS measurements as described in Materials and Methods. Geldanamycin increased TBARS in a concentration-dependent manner (Fig. 7). Geldampicin at 2 ?M concentration showed a lesser extent of lipid oxidation. Both geldanamycin and geldampicin show a change in TBARS production compared to control. However, the extent of contribution of these lipid peroxides in changing the cytoarchitecture needs to be further elucidated.

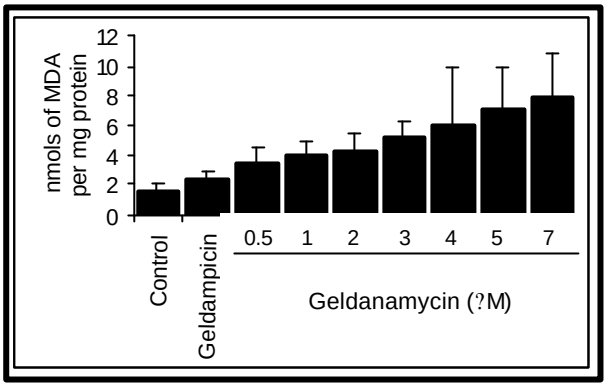

Fig 7. Estimation of TBARS upon geldanamycin treatment. The extent of lipid peroxidation was estimated by measuring the MDA content per mg protein upon geldanamycin treatment. Geldanamycin was used at different concentrations (0.5, 1, 2, 3, 4, 5 and 7 ? M) and geldampicin (2 ? M) was also used as a second control apart from the control without drug treatment.

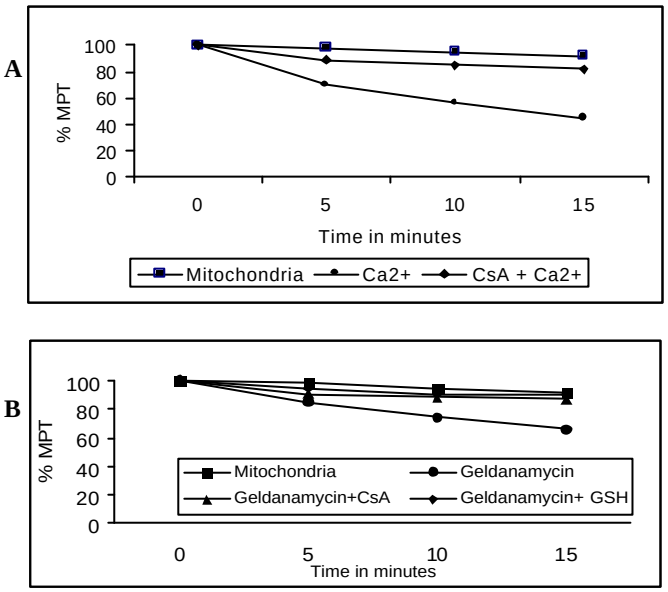

Fig 8. Effect of geldanamycin on mitochondrial membrane permeability transition (MPT). Mitochondria (1 mg protein/ml) were placed into a cuvette, and their absorbance was measured using a double beam spectrophotometer at 520 nm. Mitochondria were pre-treated with and without cyclosporin A (CsA) as indicated on the figure followed by geldanamycin, with and without glutathione (GSH) and the optical density of the sample was monitored for the ensuing 15 minutes.

4.8. Measuring mitochondrial membrane permeability upon geldanamycin treatment

A redox change in intracellular environment results in mitochondrial swelling which results in rupturing of inner mitochondrial membrane, and a consequetive release of cytochrome c (*Kanno et al, 2002*). To see whether geldanamycin-induced superoxides were associated with mitochondrial membrane permeability transition (MPT), fresh rat

liver mitochondria were treated with geldanamycin, and the extent of MPT was measured spectrophotometrically. Geldanamycin at 2 ?M concentrations was associated with a change in MPT (Fig. 8B). Calcium was used as a positive control to see the MPT and cyclosporin A was used to inhibit MPT (Fig. 8A) Though the geldanamycin-induced MPT is less than half of that induced by calcium, it can be inhibited by reduced glutathione and cyclosporin A, substantiating a role for geldanamycin in changing the MPT. The effect was also compared in the presence and absence of extrinsic Hsp90, where Hsp90 addition completely nullified the effect of geldanamycin (*data not shown*).

4.9. Anti-Hsp90 hammerhead ribozymes reveal a truly Hsp90-dependent component of geldanamycin-induced additional lysis of Jurkat cells

Since none of the Hsp90-inhibitors have truly specific effects to Hsp90, [geldanamycin induces superoxide production independent of Hsp90, (*Bellecke et al, 2002; Dikalov et al, 2002*); radicicol is an inhibitor of citrate lyase, (*Ki et al, 2000*); cisplatin and novobiocin both have a wide spectra of effects at the concentration they inhibit Hsp90] we wanted to use a tool, which really specifically inhibits Hsp90 to assess the ratio of superoxide-dependent and Hsp90-dependent components of geldanamycin effects. Utilizing the idea of *Little and Lee* (*1995*) to diminish Grp94, we constructed two anti-Hsp90 hammerhead ribozymes specific to the N- and C-termini of Hsp90 mRNA (Fig. 9B). Both ribozymes cleave a sequence, which is the same in Hsp90-? and Hsp90-? and conserved in a wide range of species (Fig. 9B and 10A). Transfection of Jurkat cells with the anti-Hsp90 hammerhead ribozymes resulted in an efficient reduction of Hsp90 levels, while keeping the pattern of total cellular proteins intact (Fig. 11A). According to our expectations, both the N-terminal and the C-terminal anti-Hsp90 ribozymes, either alone or in combination induced a significant acceleration of Brij-induced Jurkat cell lysis (Fig. 11D; *the difference between DMSO- and GA-treated cells was statistically significant at a level of p? 0.001*). Interestingly, the C-terminal ribozyme was somewhat less efficient than the N-terminal which might be related to its smaller degree of homology (Fig. 10A). On the contrary, lipofectamine-treatment or vector-transfection had only marginal effects (Fig. 11D). The ribozyme-induced additional lysis

could be further increased by geldanamycin treatment. This increase was normalized by the addition of glutathione in all cases (Fig. 11D). Substitution of glutathione with the superoxide scavenger, Tiron (*Ledenev et al, 1986*) or the NO-synthase, flavin containing enzymes and NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI; *Dikalove et al, 2002; Stuehr et al; 1991*) gave smaller, but similar effects (Table. 1).

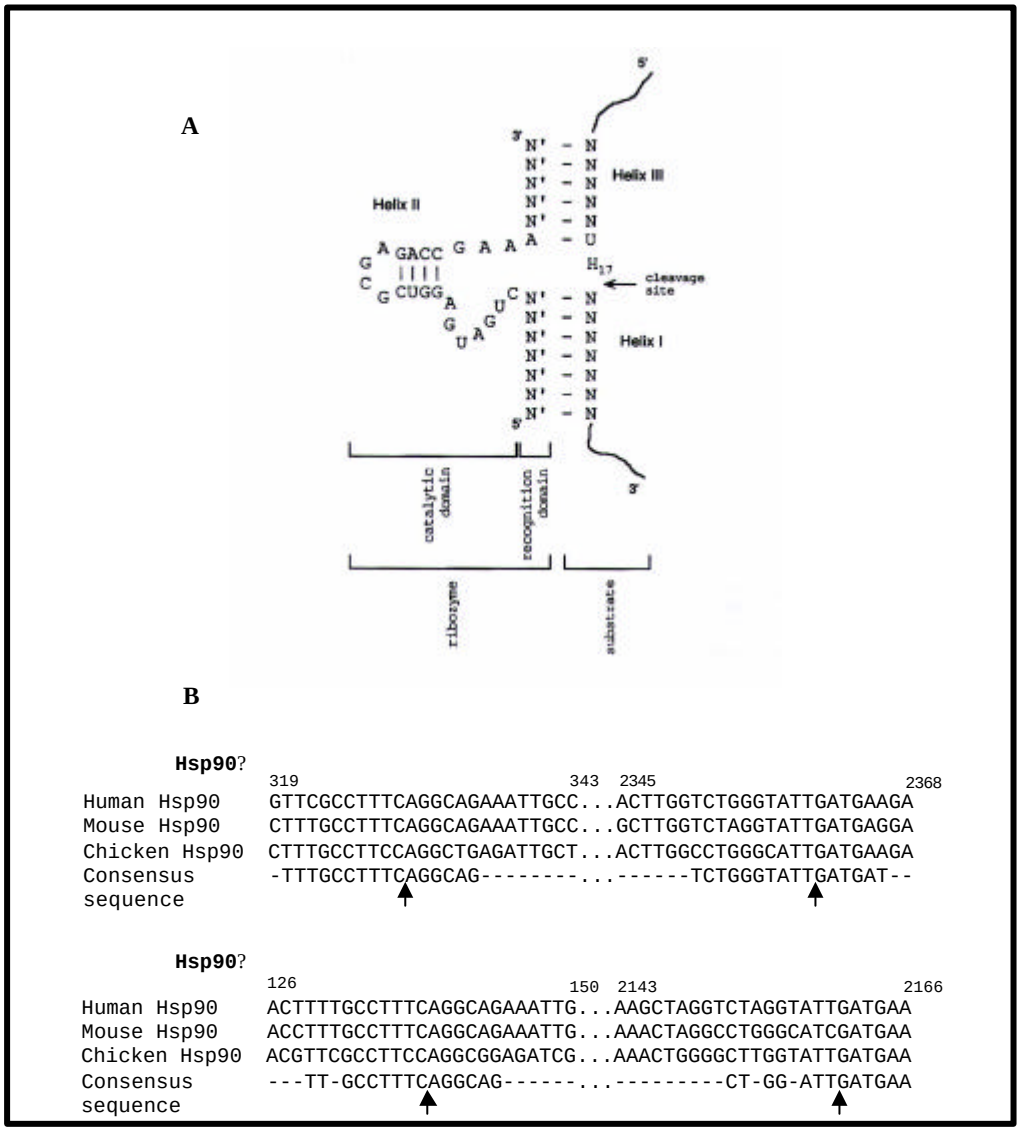

Fig 9. Panel A: Typical structure of a hammerhead ribozyme. Panel B: Conservation of ribozyme target sequences in Hsp90. Sequences of human, mouse and chicken Hsp90-? and Hsp90-?, as well as their consensus sequences in the vicinity of the ribozyme cleavage site (marked with an arrow) are shown for both the N- and C-terminal anti-Hsp90 hammerhead ribozymes. Nucleotide numbers refer to human Hsp90.

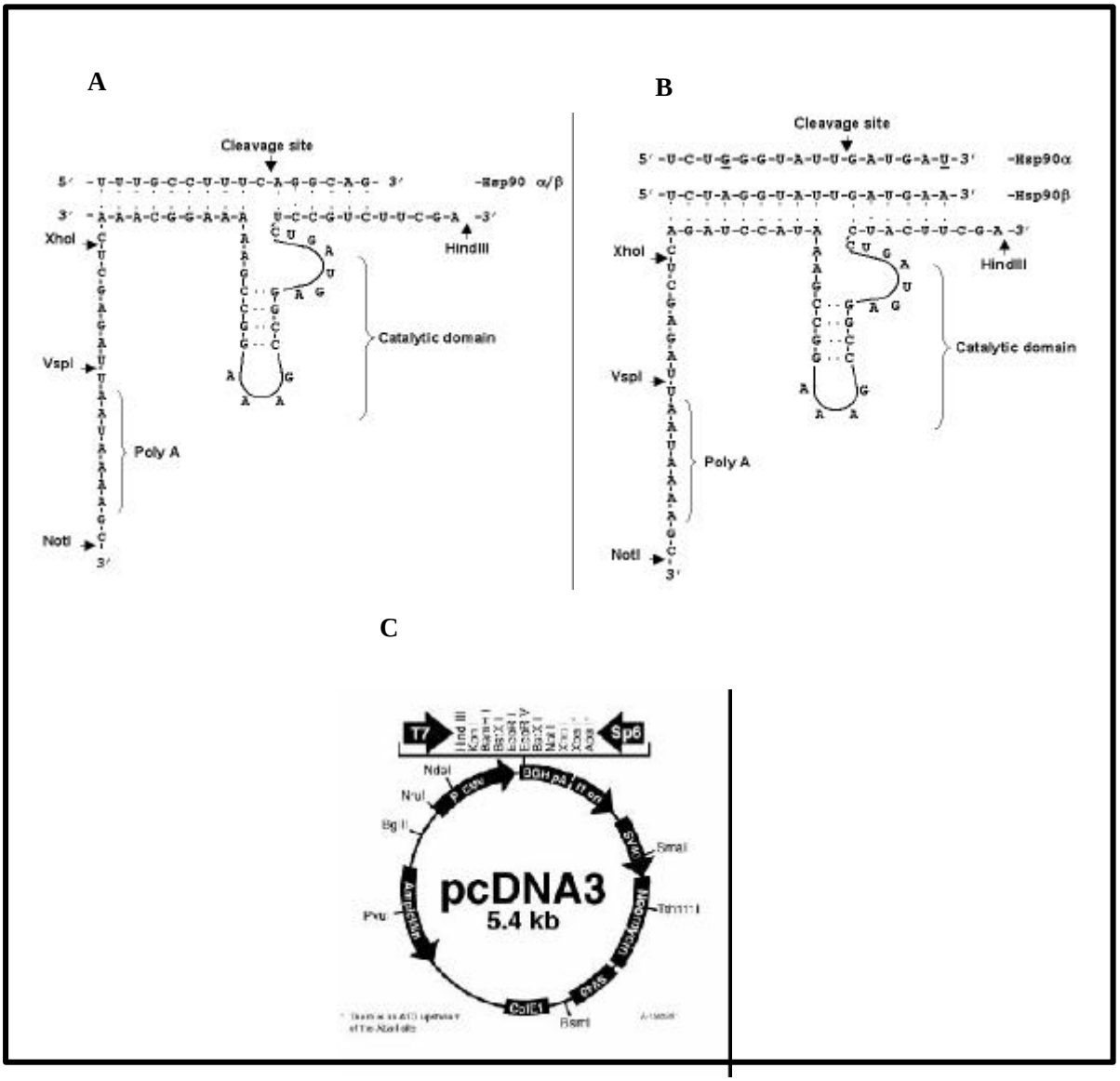

Fig 10. Anti-Hsp90 hammerhead ribozymes. A & B: The structure of the mRNA segments containing a conserved region of the N-terminus (Hsp90-N) or the C-terminus (Hsp90-C) of human Hsp90-? and Hsp90-? hammerhead ribozymes are shown. The cleavage sites of various restriction enzymes, the mRNA cleavage site and the "poly-A" sequence are indicated. The compatibility between Hsp90 isoforms was shown next to each ribozyme. C: The mammalian expression vector used in the present study showing the multiple cloning site (MCS).

The effect of DPI was the smallest of all antioxidants studied, showing a rather small contribution of specific redox systems to Jurkat cell lysis. The ribozyme experiments gave a strong support to our conclusion that geldanamycin induces the destabilization of Jurkat cells by both superoxide- and truly Hsp90-dependent mechanisms. Hsp90 seems to be important in the maintenance of cellular integrity.

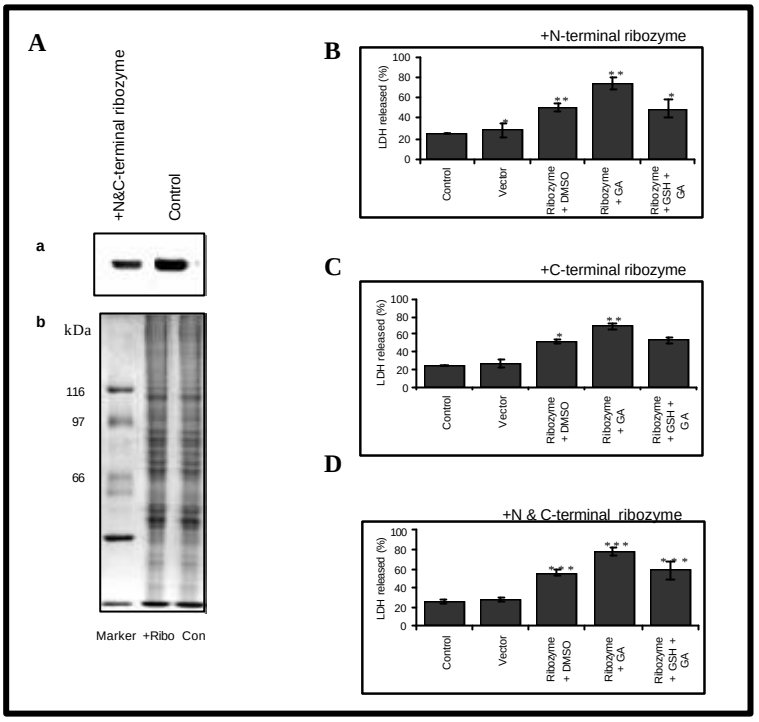

Fig 11. Effect of anti-Hsp90 hammerhead ribozymes on detergent-induced lysis of Jurkat cells. A: Hsp90 content of ribozyme-transfected Jurkat cells. "a": Immunoblot analysis of control and ribozyme transfected Jurkat cell lysates with polyclonal anti-Hsp90 antibody. "b": Coomassie blue-stained SDS-PAGE of control or ribozyme transfected Jurkat cell lysates. Panels B, C and D show the effect of anti-Hsp90 ribozymes on Jurkat cell lysis; ribozymes against the N-terminus, C-terminus and N & C-termini of Hsp90, respectively, were incubated in the presence or absence of geldanamycin and reduced glutathione for 2 h at final concentrations of 2 ?M and 2 mM, respectively. Cells were lysed with 0.005% Brij-58 for 10 min and the activity of released LDH was measured as described in Materials and Methods. 100% lysis was achieved by sonication and results were normalized to this value.

LDH released (%)	**+ Hsp90N ribozyme**	**+ Hsp90C ribozyme**	**+Hsp90C& N ribozyme**
51?4 (Control)	69?4	74?6	78?5
GSH (2 mM)	49?9	53?4	59?9
Tiron (5 mM)	56?6	67?8	68?10
DPI (5 ?M)	60?6	66?10	64?7

Table 1. Effect of antioxidants on ribozyme-transfected Jurkat cell lysis. The extent of Jurkat cell lysis in presence of the various antioxidants, GSH, Tiron and DPI was measured by measuring the released LDH activity as described in Materials and Methods after geldanamycin treatment for two hours. Control, represents cells after geldanamycin treatment. Ribozyme transfected cells for Hsp90 N-terminus ribozyme, Hsp90 C-terminus ribozyme, both Hsp90 N & C terminus ribozymes together are marked.

4.10. Geldanamycin-induced lysis acceleration in bacteria and yeast cells

Observing a role for Hsp90 to maintain cellular integrity we wanted to study, whether cells with a less important contribution of Hsp90 to their viability and with a smaller degree of cellular organization than the human Jurkat cell line, namely bacteria or yeast cells are sensitive to geldanamycin-induced additional lysis or not. Lysis conditions were optimized to achieve a 20 to 30 % lysis of total cytoplasmic proteins. On the contrary to our results with Jurkat cells, geldanamycin induced no significant additional lysis in *E. coli* (Fig. 12).

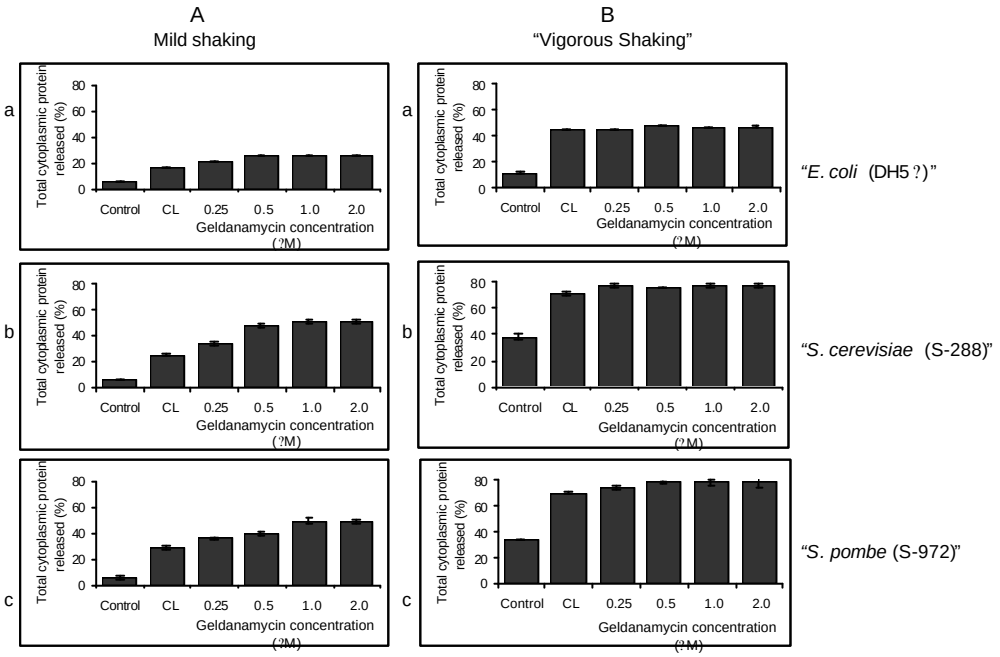

Fig 12. Effect of geldanamycin on bacteria and yeast cell lysis. Bacterial protoplasts from E. coli cells (DH5? strain) were made from mid-log phase as described in Materials and Methods. Similarly, S. cerevisiae (S-288 strain) and S. pombe (S-972 strain) cells from 5 ml overnight cultures in YEPD medium were inoculated to 100 ml cultures in 1:100 dilution and cells were further incubated at 30?C with constant shaking for 12 h. Yeast protoplasts were made as described in Materials and Methods. 2 x 10^5 protoplasts per ml were treated with geldanamycin at final concentration indicated for 2 h and were subjected to mild hypotonic lysis using 0.45 M NaCl or 0.65 M sorbitol in case of bacteria or yeast, respectively. Lysis was measured with mild shaking (2000 rpm; panels A) and with vigorous shaking (10,000 rpm; panels B). Lysed protoplasts were centrifuged and the released total protein was measured as described in Materials and Methods. 100 % lysis was achieved by distilled water treatment and results were normalized to this value. Geldanamycin-induced changes are statistically significant at a level of p > 0.001.

In *S. cerevisiae* and *S. pombe* yeast cells the additional lysis after geldanamycin treatment was significant, but smaller than that observed in Jurkat cells (cf. Figs. 1B and

12A). As in case of Jurkat cells, lysis rate was strongly dependent on the extent of shaking in all cell types studied. No shaking induced no appreciable lysis, however, vigorous shaking induced a close-to-maximal cell lysis suggesting that bacterial and yeast protoplasts lacking a sophisticated cellular architecture are more sensitive to cell lysis than eukaryotic cells (*data not shown*, Fig. 12B). Since we could not be sure that geldanamycin inhibits the bacterial Hsp90 homologue, HtpG similarly than eukaryotic Hsp90, we prepared heat preconditioned bacterial cells at 44 ?C and measured their lysis rate. Elevation of molecular chaperones in bacterial cells did not induce any change in cell lysis rate (*data not shown*) indicating that in bacterial cells chaperones may not playing a prominent role in the maintenance of cellular integrity.

4.11. Hsp90 inhibition enhances hypoxia-induced cell lysis

To address the physiobgical significance of our cell lysis experiments using mild detergent treatment, we wanted to study, if Hsp90 inhibition induces an enhanced cell lysis in hypoxia, a usual phenomenon in tumors. Hypoxia was induced using cobalt chloride (*Wang et al, 2000*). Geldanamycin and radicicol both caused a significant increase of hypoxia-induced cell lysis in Jurkat cells. The effect was reduced in presence of reduced glutathione (Fig. 13A). Other Hsp90 inhibitors, such as cisplatin and novobiocin showed approximately 50 % less enhancement of hypoxia-induced Jurkat cell lysis, and, on the contrary to geldanamycin and radicicol, addition of glutathione had no significant effect on the extent of cisplatin- and Novobiocin- induced lysis (Fig. 13A).

To see and compare the differences between Hsp90 inhibitor-induced cellular effects and those after ribozyme-targeted Hsp90 inhibition, ribozyme transfected Jurkat cells were subjected to hypoxia. Hypoxia-induced cell lysis was amplified to more than two fold in ribozyme-transfected cells (Fig. 13B). Vector (mock) transfected cells did not show any significant change. Addition of geldanamycin to ribozyme-transfected cells showed no further change in hypoxia-induced cell lysis. Similarly, addition of glutathione did not reduce the combined effects of anti-Hsp90 ribozyme and geldanamycin (Fig. 13B).

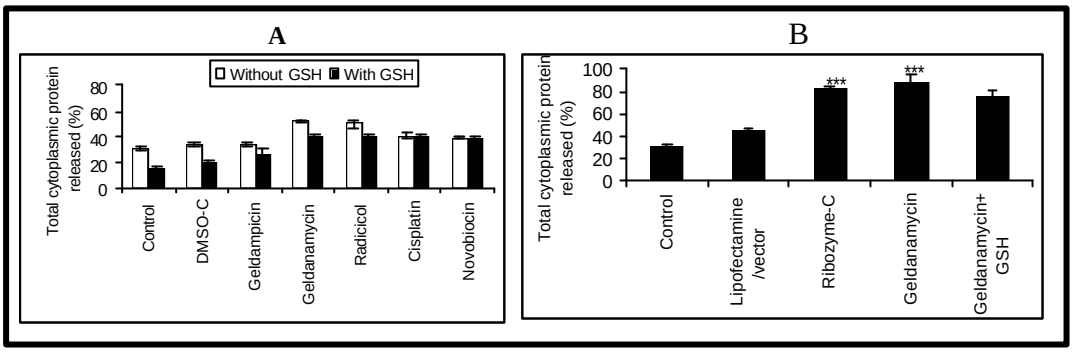

Fig 13. Effect of geldanamycin on hypoxia induced cytolysis. Panel A: 2 x 10^6 Jurkat cells were pre-incubated with 0.2 mM $CoCl_2$ for 2 h together with geldanamycin both in the absence (open bars) and presence (filled bars) of glutathione and their lysis was measured as described in Materials and Methods. Panel B: Effect of anti-Hsp90 hammerhead ribozymes on hypoxia induced cell lysis. Jurkat cells were transfected with vector or anti-Hsp90 ribozyme and cell lysis was measured after $CoCl_2$ incubation as described in panel A and in Materials and Methods. To distinguish the oxidation induced effects from Hsp90 effects antioxidant glutathione was added during geldanamycin treatment. Data represent mean ± SD of three independent experiments.

4.12. Hsp90 inhibition enhances complement-mediated cell lysis

To demonstrate the physiological significance of the enhanced cell lysis associated with Hsp90 inhibition further, we have chosen the complement-mediated immune lysis of the malignant Jurkat cell line, as a model of immune-attack on human tumor cells. Though all effective Hsp90 inhibitors showed some increase of cytolysis when Jurkat cells were preincubated with them before the addition of human serum, again geldanamycin and radicicol showed on significant differences (Fig. 14A). Addition of glutathione induced a slight decrease in cell lysis (Fig. 14A).

The Hsp90 antisense ribozyme induced a massive increase in complement-mediated cell lysis, which was only marginally affected with the addition of geldanamycin without or with glutathione suggesting that Hsp90 loss is the major cause of increased cytolysis after complement attack (Fig. 14B). Addition of an exogenous source such as mouse sera or rabbit sera completely lysed tumor cells (*data not shown*).

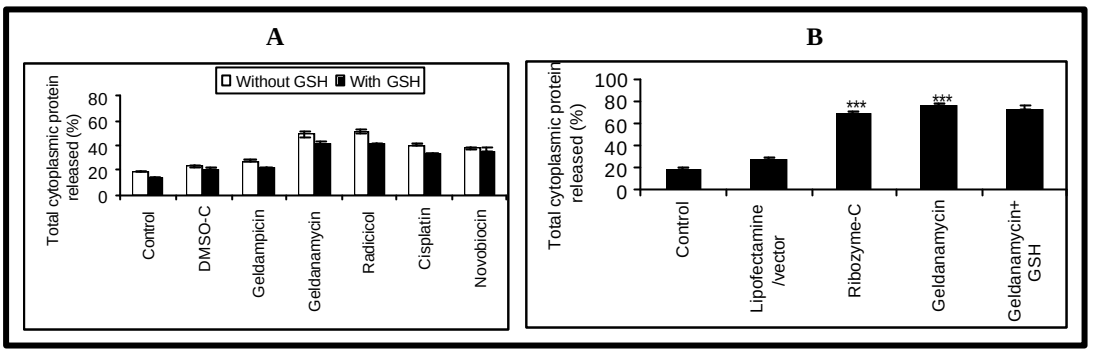

Fig 14. Effect of Geldanamycin on complement induced cytolysis. Panel A: 2 x 10^6 cells were pre-incubated with various anti-Hsp90 inhibitors in the absence (open bars) or presence (filled bars) of glutathione. Cells were lysed with human serum for 10 min at 30?C and cell lysis was measured by estimating the per cent total cytoplasmic protein release as described in Materials and Methods. Panel B: Effect of anti-Hsp90 hammerhead ribozymes on complement-induced lysis. Jurkat cells were transfected with vector or anti-Hsp90 ribozyme and complement-induced cell lysis was measured both in presence and absence of antioxidant glutathione together with geldanamycin as described in panel C and in Materials and Methods. Data represent mean ± SD of three independent experiments.

4.13. Geldanamycin induces actin-reorganization in vivo

Steroid receptors require intact cytoskeleton for their translocation and signaling and geldanamycin inhibits their movement (*Galigniana et al, 1998*). Therefore we wanted to study if geldanamycin affects the actin cytoskeleton. To visualize geldanamycin-induced effects, Jurkat cells were treated with geldanamycin and were stained with actin-phalloidin. Results showed an actin-rearrangement with a filopodia like morphology (Fig. 15). This phenomenon could be either a result of geldanamycin-induced ROS or due to a direct inhibition of Hsp90.

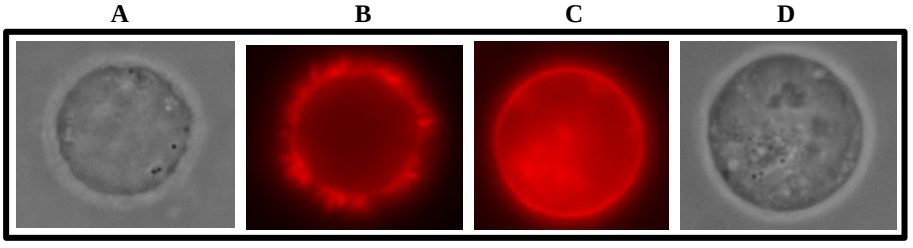

Fig 15. Geldanamycin induces actin reorganization. Jurkat cells were treated with 2 ?M of geldanamycin for 2 h and fixed with 4 % formaldehyde for 5 min, after washes with PBS as described in Materials and Methods. Samples were stained with actin-phallodin for 20 min. The stained cells were visualized under fluorescent microscope. Panels A and D: geldanamycin treated and control cells, respectively, Panels B and C: actin staining of geldanamycin treated and control cells, respectively.

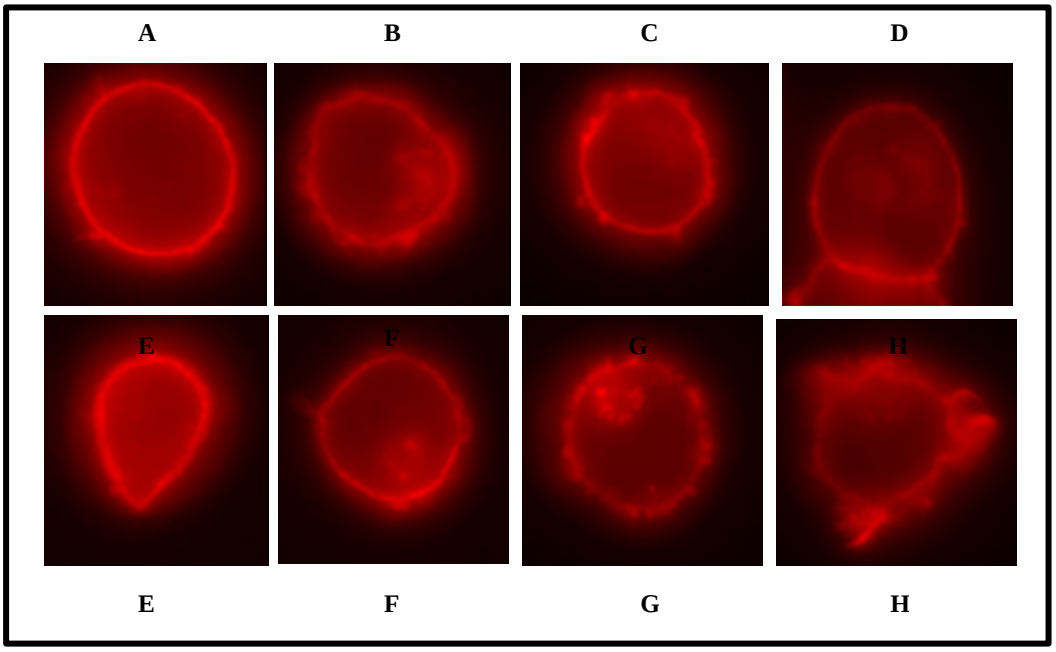

Fig 16. Effect of various Hsp90 inhibitors on the actin cytoskeleton. Jurkat cells were treated with various concentrations of Hsp90 inhibitors and analogues for 2 h, fixed with 4% formaldehyde for 5 min, stained with actin-phallodin for 20 min and visualized under fluorescent microscope as described in Materials and Methods. Panels, A: control; B: novobiocin; C: cisplatin, D: transplatin; E: DMSO; F: geldampicin; G: 5', 5'''-P1, P4-adenosine tetraphosphate (Ap_4A); H: taxol.

4.14. Effect of various Hsp90 inhibitors on actin reorganization

Though geldanamycin-treated Jurkat cells show distinct morphology with respect to actin cytoskeleton, the role of geldanamycin-induced ROS in this phenomenon cannot be ruled out. Hence, Jurkat cells were treated with various Hsp90 inhibitors and analogues and were stained for F-actin. All Hsp90 inhibitors showed similar changes in actin cytoskeleton compared to controls (Fig. 16). These results confirmed the role of Hsp90 in stabilizing the actin-cytoskeleton. The effect of geldanamycin-induced actin reorganization seems to be a result of Hsp90 inhibition. The inactive analogues, geldampicin and transplatin failed to induce significant changes (Fig. 22F and 22D, *respectively*). Treatment with vehicle, DMSO was also without any major effect (Fig. 22E and 22A, r*espectively*).

4.15. Screening of peptide binding to Hsp90

Commercially available, biologically active peptides were screened for binding to Hsp90 using surface plasmon resonance (SPR) biosensor chips containing immobilized Hsp90. In this method, a complex of Hsp90 and interacting peptides is detected by measuring the change in refractive index caused by the absorption of various peptides to the Hsp90-covered chip surface. SPR has been proved and efficient method for the characterization of the interaction of Hsp90 and other proteins and peptides (*Csermely et al, 1997; Miyata & Yahara, 1995*).

Peptide	Total amino acids	Basic amino acids	Acidic amino acids	Hydrophobic amino acids	Req(RU)/ng Hsp90mm^2
NPY 13-36	24	5	2	9	1,253±86
MCD-peptide	22	9	0	6	202±24
VIP	28	6	2	10	98±6
Histatin 5	24	12	2	2	66±8
Dynorphin A	13	5	0	5	43±5
Glucagon	29	4	3	8	40±4
[Tyr^1]-somatostatin	14	2	0	5	29±2
PTH 1-34	34	7	4	13	25±6

Table 2. Binding of various peptides to immobilized Hsp90. Thirty-eight peptides were examined for binding to immobilized Hsp90 by SPR analysis as described in Materials and Methods. The eight peptides indicated in the Table showed a significant binding. Negligible binding was detected using the following 30 peptides: Angiotensin I, Angiotensin II, Bradykinin, Bradykinin potentiator B, Bradykinin potentiator C, Calcitonin, Kallidin, ??casmorphin-7, DSIP, ?-endorphin, Guanylin, Uroguanylin, Insulin, Laminin penta peptide, Magainin, ??mating factor, Neurokinin A, Neuromedin B, Neurotensin, Nociceptin, Oxytocin, Chomogranin A, Peptide T, Platelet factor 4, Serum thymic factor, Somatostatin, Substance P, Substrate for tyrosine protein kinase, Substrate for renin, and Vasopressin. Data represent mean + S.D. of three separate experiments.

From an extensive screen of various biologically active peptides the results of our collaborator (*Ishiwatari et al, 2003*), demonstrated that neuropeptide Y binds with high affinity to Hsp90 (Table. 2 and Fig. 17).

4.16. Specificity of NPY13-36 binding to Hsp90

To analyze the location of binding sites for NPY13-36 on Hsp90 further, deletion mutants of Hsp90 were fused to glutathione S-transferase (GST) (Fig. 17), and were analyzed by SPR. Binding experiments revealed that some deletion mutants of Hsp90

showed lower binding (Req values) for NPY13-36 compared to full-length Hsp90. Full length Hsp90 showed a binding corresponding to approximately 1,500 resonance units (RU), whereas the mutant 1-47/290-732 and 1-312 showed half of this binding level (approximately 750 RU, Fig. 17 B). Almost all the rest of binding was abrogated in the mutant 1-47/459-732. These lines of evidence suggest that there are at least two NPY13-36 interaction sites on Hsp90, the first is located between the 47th and 290th residues (Fig. 17A, Region 1) and the other is located between the 312 and 459th residues (Region 2). The NPY peptide showed far lower binding to the mutant 1-47/459-732 and 535-732, showing that NPY13-36 does not bind to either the N-terminal 47 amino acids or the C-terminus of Hsp90.

Consistent with these results, binding of NPY13-36 was insensitive to geldanamycin and was not augmented by ATP (*Ishiwatari & Yahara, unpublished observation*). Region 1 contains the highly charged domain (amino acids 206-287), which is also referred as the acidic region, because in this region acidic amino acids are dominant. Further substantiating that this domain particularly contributes to the interaction, a mutant NPY13-36 with arginine to alanine substitutions (NPY13-36-RA, *data not shown*) hardly interacted with Hsp90. In addition, the full-length NPY peptide binds to the polypeptide comprising amino acids 213-275 of chicken Hsp90 by SPR analysis (*Soti & Csermely, unpublished observation*).

4.17. Effect of potassium and sodium chloride on peptide binding to Hsp90

From the experimental data it is evident that there is a strong binding between the peptide NPY and Hsp90. However, further examination of binding of Hsp90 to immobilized NPY13-36 with various charge modifications suggested that the positively charged amino acids on NPY are important structural elements reorganized by Hsp90. To demonstrate this further, we repeated the binding experiments with increasing salt concentrations, especially with potassium and sodium chloride solutions (Fig. 18). The results showed that both salts inhibited this binding at high concentrations (Fig. 18 A and B).

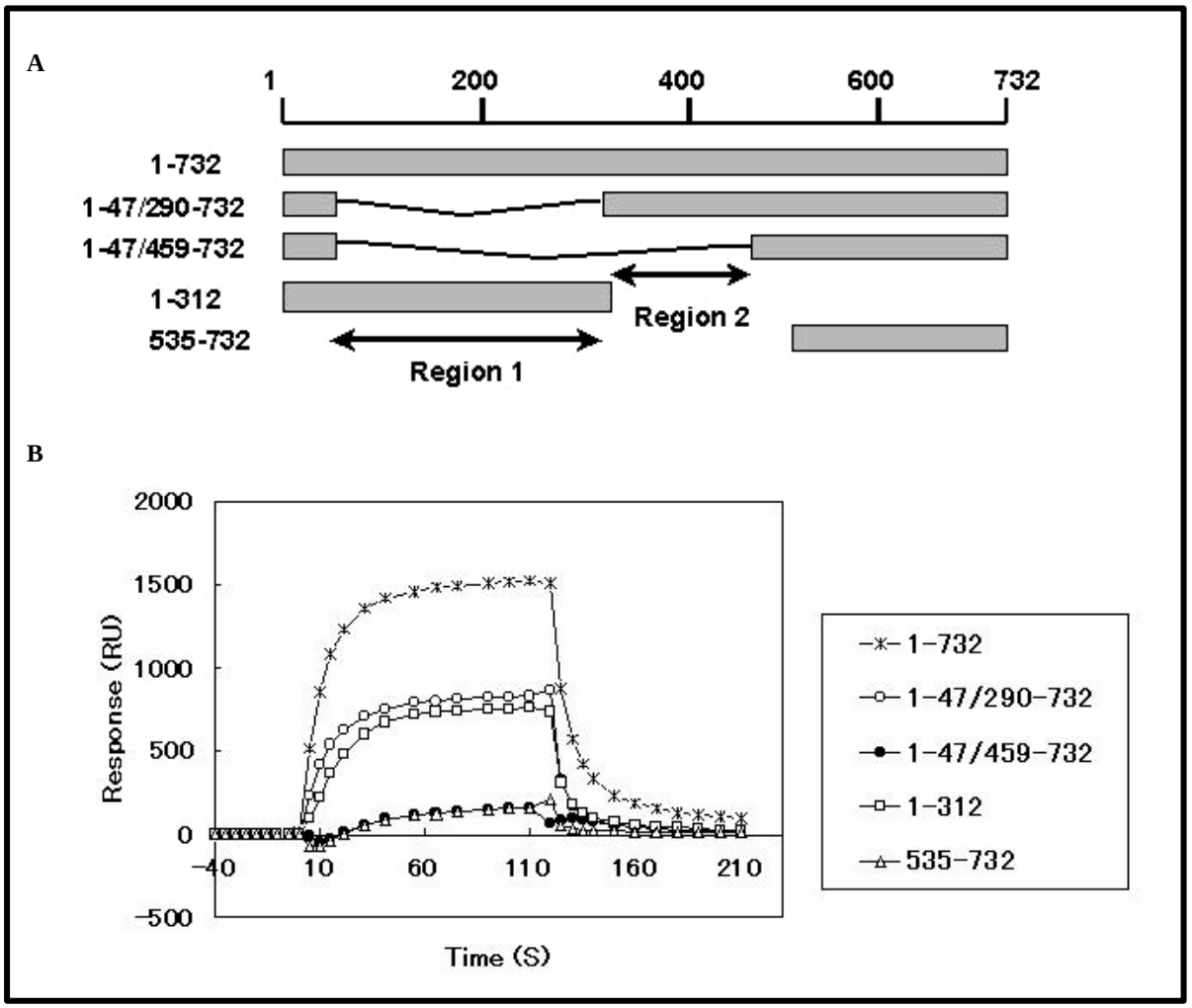

Figure 17. Binding of NPY13-36 to deletion mutants of Hsp90. Panel A: Hsp90 mutants with various deletions. Panel B: Sensorgrams of NPY13-36 binding to the deletion mutants. Recombinant proteins were purified from E. coli lysates and immobilized on surface plasmon resonance sensor chips at amounts proportional to their molecular weight (full length 1-732; 85K, 4,100 RU; 1-47/290-732; 57K, 2,800 RU; 1-47/459-732; 42K, 2,500 RU; 1-312; 50K, 2,700 RU; 535-732; 27K, 2,100RU). Data are representatives of three independent experiments.

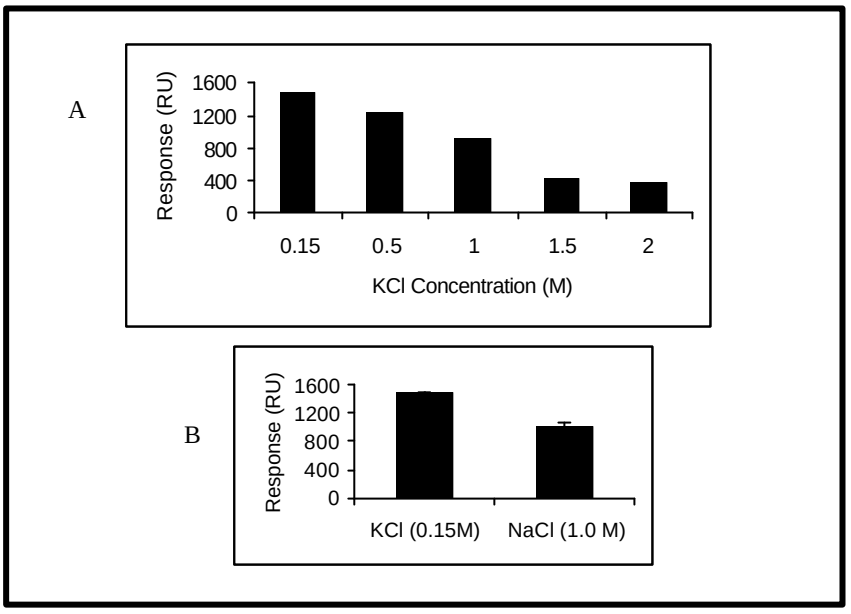

Fig 19. Neuropeptide Y (NPY13-36) binding to immobilized Hsp90 was inhibited at higher ionic strength. Peptide (20 ?M) binding to Hsp90 was analyzed by surface plasmon resonance. Panel A: Effect of KCl (0.1, 0.5, 1.0, and 2.0 M) on NPY binding to Hsp90. Panel B: Effect of sodium chloride (1.0 M) on NPY binding to Hsp90. Sensorgrams were corrected to non-specific binding to the reference sensor chip.

5. Discussion

Heat shock proteins (Hsp-s) interact with multiple key components of signalling pathways that regulate growth and development (*Pratt & Toft, 2003*). Growing interest on these proteins revealed the biochemical mechanisms behind these interactions unravelling the molecular relationship between signaling molecules and Hsp-s that are crucial for normal cellular development. Cells with deregulated cellular functions often are associated with high expression of Hsp-s (*Jaattela, 1999)* and depletion of Hsp-s in transformed cells was shown to be an effective antitumor intervention (*Nylandsted et al, 2000; Whitesell et al, 1994*). Hsp90-inhibitor based chemotherapy accelerated the development of new types of Hsp90-related antitumor drugs (*Csermely et al, 1998; Neckers, 2002; Neckers, 2002; Sreedhar et al, 2003a; Whitesell et al, 1994*). Though the major impact of Hsp90 inhibitors are attributed to their interference at the signaling cascades through Hsp90 inhibition, their pleotropic cellular effects needs to be explored further. Towards addressing these effects we have chosen to measure the cell lysis after treating tumor cells with various Hsp90 inhibitor-drugs because cell lysis is one of the most commonly used methods to test cellular integrity.

Lysis rate-anomalies (*Clegg & Jackson, 1988; Miseta et al, 1991*) together with diffusion anomalies (*Luby-Phelps, 2000; Verkman, 2002*) were used as important arguments for the organization of the cytoplasm. Since cellular integrity is preserved after a partial cell lysis to a large extent (*Clegg & Jackson, 1988; Miseta et al, 1991*) partial lysis provides a highly sensitized, but still somewhat organized cellular system, where the contribution of various components to both the cytoplasmic organization and cellular stability can be studied. The first set of experiments were suggesting that Hsp90 inhibition enhanced detergent-induced cytolysis both in Jurkat T-lymphocytes and in mouse red blood cells (Fig. 1, *Pato et al, 2001; Csermely et al, 2003, data not shown*).

When we started to study the effect of various Hsp90 inhibitors on cytolysis using mouse red blood cells with hypotonic shock (*Pato et al, 2001; Csermely et al, 2003*), the first reports of superoxide production from the Hsp90 inhibitor, geldanamycin were appeared (*Dikalov et al, 2002; Billecke et al, 2002*). Also our cytolysis experiments varied with the experimental conditions, such as shaking of cells after the drug treatment (Fig. 2B). Moreover the lysis rate could be reduced, with the addition of GSH and other

antioxidants such as Tiron and DPI suggesting the role of superoxides in the cytolytic process (Fig. 2C, *data not shown*). To assess the Hsp90 inhibitor-induced effects on the redox status further, we studied the changes associated with the membrane fluidity and membrane polarization together with superoxide production (Fig. 3B and 3A). We found that in our system geldanamycin induced a significant amount of superoxides, which made further experiments necessary to see whether the effects of geldanamycin on cytolysis were a result of Hsp90 inhibition or were due to redox cycling of the compound.

5.1. Dual role of Hsp90 inhibitors as modifiers of the redox status and Hsp90 function

Accelerated superoxide production is a recently observed feature of geldanamycin, being the first Hsp90-independent function of this highly specific Hsp90 inhibitor (*Bi1lecke et al, 2002; Dikalov, et al 2002*). Superoxide production has been first implicated in geldanamycin action as an alternative route of endothelial NO-synthase function leading to an uncoupled superoxide production parallel with a decreased NO synthesis (*Tsuruo et al, 2003*). The role of Hsp90 as a "switch" from superoxide to NO production was also demonstrated in neural NO synthase (*Song et al, 2002*). However, later studies extended these findings and showed that geldanamycin is able to increase superoxide production independently of endothelial NO synthase activation both in *in vivo* and *in vitro* systems, possibly by its quinone group, which may participate in redox cycling (*Dikalov et al, 2002*). Similar data were reported on neural NO synthase (*Billecke et al, 2022*). Importantly, the non-quinone Hsp90 inhibitor, radicicol had no direct superoxide producing effect (*Billecke et al, 2002*). This is in agreement with our findings, that in contrast to the effects of geldanamycin, radicicol action cannot be attenuated by reduced glutathione (Fig. 2C). An earlier study proposed that radicicol might exert its antimalarial action *via* heme-dependent free radical generation but this assumption has not been tested directly (*Stuehr et al, 1991*). However, a recent study indicated that radicicol converts endothelial NO synthase from an NO-producer to a superoxide-generator independently of a direct superoxide production (*Pritchard et al, 2001*). Interestingly, the geldanamycin structural analogue, geldampicin was much less potent superoxide activator than the parent compound (Fig. 3A). Geldampicin contains a piperidine derivative directly attached to the quinoidal segment of the drug (*Whitesell et*

al, 1994), which possibly hinders its participation in redox cycling by stabilization redox-independent oxygen binding to geldampicin (*personal communication of Drs. Zsuzsanna Riedl and Judit Jakus*).

Geldanamycin-induced superoxides may induce lipid-peroxidation, which would lead to a significant membrane damage partially explaining the increased cell lysis observed in our study. In support of this our membrane fluidity data suggest that geldanamycin induces a gross change in the membrane organization, which is parellel with the increasing amounts of lipid peroxides after geldanamycin treatment. Lipid peroxidation induces a decrease in membrane fluidity (*Minamide et al, 1992; Patel & Block, 1986*). In contrast, we have found an increased membrane fluidity after geldanamycin action (Fig. 3B) suggesting that there are different reasons besides lipid peroxidation behind the observed change in membrane organization. Increased membrane fluidity may be concomitant with increase in membrane fragility (*Shertzer et al, 1992*). Thus geldanamycin seems to contribute by a partially oxidation-dependent, but heretofore-unknown mechanism to the observed increase in membrane sensitivity. It cannot be excluded, however, that Hsp90 inhibition alone, independently of any geldanamycin-induced oxidative changes also induces an increase in membrane disorganization.

Hypoxic conditions diminish synthesis of nitric oxide from the nitric oxide synthase (NOS) enzyme. NOS enzyme activities are mainly influenced by the intracellular redox state and specifically by oxygen tension. Elevated oxygen conditions (hyperoxia) increase the steady-state level concentration of nitric oxide by stimulating NOS activity, which depends on Hsp90 chaperone function. Geldanamycin was shown to prevent the extracellular oxygen induced NOS activity by inhibiting the Hsp90-chaperone complex with NOS, which is otherwise involved in the hyperproduction of nitric oxide (*Thom et al, 2003*). In addition, hypoxic conditions are associated with an increased production of the Hsp90 protein suggesting that Hsp90 protects cells from for hypoxia (*Almgren & Olson, 1999*). Similarly, radicicol also plays a role in modulating the eNOS activity through Hsp90 inhibition (*Ou et al, 2003*). Our data showing that Hsp90 inhibition enhances hypoxia induced cell lysis add a novel and interesting element of the protecting role of Hsp90 in hypoxia.

Earlier studies showed that radicicol does not redox cycle (*Billecke et al, 2002*). In agreement with this we observed negligible superoxide producing effects of radicicol. Similarly, radicicol did not induce a change in membrane order and decrease in membrane polarization (*data not shown*). Hence, from our results radicicol-induced additional cell lysis were mainly attributed to Hsp90 inhibition. In addition, geldanamycin induced a concentration dependent TBARS suggesting the predominance of drug-induced oxidative effects rather than the Hsp90-associated effects at higher geldanamycin concentrations (Fig. 7). These results are in support of experiments with hydrogen peroxide induced oxidative stress in renal carcinoma cells. Here enhanced lipid peroxidation was associated with reduced Hsp90 levels. However, if Hsp90 remained intact, no lipid peroxidation was observed (*Nowzari et al, 2000*). Though there is no direct experimental evidence, Hsp90 may protect cells against oxidative damage. However, the mechanism behind this process needs to be explored. In support of our assumption, *Nardai et al* (*2001*) have demonstrated *in vitro* that the reactive cysteines present in Hsp90 are sensitive to oxidative damage and probably regulate the chaperone function of Hsp90.

Though there is an assumption that reduced glutathione may contribute for the reactivation of reduced or oxidized thiol groups of Hsp90 for resistance towards cytolysis in our experiments, similar effects with other antioxidants prove that the antioxidative property is not limited to glutathione, therefore a direct and exclusive effect on Hsp90 thiol groups can not be ascertained in all the experiments.

Though at higher concentrations Hsp90 inhibitors, such as geldanamycin and 17AAG exhibit renal and hepatic toxicity, at nanomolar concentrations their anti-metastasis effects are predominant (*Page et al, 1997*). There are several assumptions that the toxicity in part is due to the production of superoxides by the ansamycin drugs by an unknown metabolic pathway. From our results it is clear that the amount of superoxides produced by these drugs does not correlate with enhanced cell lysis under physiological conditions as seen with hypoxia-induced and immune-mediated cytolysis (Fig. 13A and 14A), which argues against superoxide-induced cell lysis as a major contributor for Hsp90-induced toxicity. Results with isolated rat mitochondria upon geldanamycin treatment showed a change in membrane permeability trasition, which could be reduced

by GSH, or Cyclosporin, further confirming a role of geldanamycin in membrane permeability (Fig. 8B,) through an unknown mechanism. These observations need a thorough *in vivo* screening and analysis to confirm.

Although cisplatin was shown to be associated with the production of superoxides *in vivo* at higher concentrations, which are associated with the decreased antioxidant potential (*Baliga et al, 1999; El-Sayed & El-Masry, 2003*), in the current experimental set-up we used very low concentrations of the drug, which showed no significant reduction in the antioxidant potential, or increase in superoxide production (*seen by supplementing with GSH;* Fig. 2C). There were no reports that novobiocin alters the redox-homeostasis (*Stocker et al, 1995*). Altogether our results revealed that there is a varying level of the contribution of superoxides in the action of from various Hsp90 inhibitors. Our results with geldanamycin on membrane lipid peroxidation also suggests that inhibition of Hsp90 chaperone function results in localized lipid oxidation in the plasma membrane, which probably associated with the lipid rafts (Fig. 5D). This observation, however, requires further experiments to get confirmed.

As a possible example of oxidation-independent membrane action of geldanamycin, *Suttitanamongkol et al.* (*2000*) published an interesting study, which showed that geldanamycin-treatment *alone* induced the lysis of platelets by inducing extensive membrane blebbing and disrupting the plasma membrane structure. These data raised the possibility that the geldanamycin-enhanced lysis of Jurkat cells is due to a detergent effect of the drug. However, there are several pieces of evidence, which refute this assumption:

In our studies geldanamycin *alone* did not induce a significant lysis of Jurkat cells (*Schnaider et al, 2000*, and *data not shown*).

Suttitanamongkol et al (*2000*) used geldanamycin at a high concentration (18 ?M), which most probably produced aspecific effects. A recent study (*Chiosis et al, 2003*) showed that geldanamycin is accumulated in various cells. However, in our experiments detergent treatment was performed after the excess of geldanamycin has already been washed away, and the geldanamycin-induced sensitization for lysis also occurred in experiments (*i.e. when applied together with hypoxia or complement-induced lysis*)

when detergent treatment was not performed. This excludes the possibility that geldanamycin was especially enriched in detergent treated cells.

The effect of geldanamycin was gradually developed over a long preincubation period extending for several hours in our experiments (Fig. 1C). This slowly developing action is a typical feature of geldanamycin effects (*Schnaider et al, 2000; Stebbins et al, 1997; Whitesell et al, 1994*). On the contrary, detergent effects are prompt, requiring seconds to minutes to develop.

The structural analogue of geldanamycin, geldampicin did not induce the acceleration of Jurkat cell lysis (Fig. 2C). Detergent effects are not so sensitive to minor changes in detergent structure.

The structurally unrelated Hsp90 inhibitors, radicicol, cisplatin or novobiocin gave a smaller, but significant increase in Jurkat cell lysis compared to that observed after geldanamycin treatment (Fig. 2C).

Jurkat cells structure was analyzed after geldanamycin treatment with electron microscopy and no signs of membrane blebbing or disintegration were seen (*Janos Kovacs & Peter Csermely, data not shown*).

Recent studies indicated a role of Hsp90 in maintaining the membrane raft structure. Geldanamycin treatment efficiently dissociated several Hsp90-related protein complexes in lipid rafts (*Waheed & Jones, 2002; Shah et al, 2002*). Since lipid rafts are usually isolated after a nonionic detergent extraction, the similarity of experimental conditions argues for a role of Hsp90-inhibition induced raft disorganization in the decrease of cellular integrity observed in our studies. In support of this Jurkat cells after treatment with geldanamycin showed oxidative patches on cell membrane (Fig. 5D) leaving us with an assumption that there is a localized lipid peroxidation upon geldanamycin treatment.

5.2. Hsp90: a chaperone potentially affecting the cytoarchitecture

Hsp90 associates with filamentous actin, tubulin and other cytoskeletal proteins *in vitro* (*Csermely et al, 1998; Csermely, 2001*). The Hsp90-chaperone system is shown to facilitate nuclear translocation of steroid receptors, which requires an intact cytoskeleton (*Galigniana et al, 1998*). In addition, most of the signalling molecules depend on Hsp90

for their folding and transport and most of their traffick was shown to involve Hsp90 (*Pratt & Toft, 2003*). *Kellermayer & Csermely* (*1995*) have demonstrated the first direct visualization of binding of Hsp90 to actin filaments. Interestingly, both actin and Hsp90 are ATP-binding proteins (*Csermely & Kahn, 1991; Martonosi et al, 1960; Pollard et al, 1992*) and there is a dissociation of Hsp90-F-actin complex in the presence of ATP in *in vitro* motility assay (*Kellermayer & Csermely, 1995*). Our data also showed that there is a high degree of actin-cytoskeleton re-organization as seen by actin-phalloidin staining with Hsp90 inhibitors (Fig. 15). This effect is not limited to one Hsp90 inhibitor, and all anti-Hsp90 drugs revealed similar cytoskeletal reorganization (Fig. 16).

Further experiments with anti-Hsp90 hammerhead ribozymes demonstrated that a diminished Hsp90 function *alone* compromises cellular integrity (Fig. 11D). The role of Hsp90 can be a *direct* disorganization of cellular structures after the disruption of various Hsp90-related complexes or an *indirect* action of Hsp90 inhibition *via* the incorrect folding of an Hsp90 client protein, which is important in the maintenance of cellular integrity. Our data showing a prolonged action after Hsp90 inhibition (Fig. 1C) suggest the contribution of an Hsp90-client protein to the effects observed.

The observation, that Hsp90 inhibition does not affect the lysis of bacterial protoplasts, and has a smaller enhancement of yeast protoplast lysis than that of higher eukaryotic cells (Fig. 12) is in good agreement with the increasing role of this chaperone in the maintenance of cellular life from prokaryotes to yeast and higher eukaryotes (*Buchner, 1999; Csermely, 2001; Sreedhar et al, 2003a*) as well as with the development of more and more sophisticated cytoskeleton in these organisms. Hsp90 is known to interact with filamentous actin and tubulin (*Czar et al, 1997; Kellermayer & Csermely, 1995; Koyasu et al, 1986*) and the involvement of the cytoskeleton in the traffic of Hsp90-substrates has been repeatedly demonstrated (*Galigniana et al, 1998; Pratt, 1998*). These findings raise the possibility that Hsp90 might contribute to an increased cellular integrity by the maintenance of cytoskeleton-related structural elements in eukaryotic cells.

5.3. Applications and significance of the results in the clinical practice

Hsp90 inhibitors (*geldanamycin analogues, radicicol and purine scaffold inhibitors*) were recently introduced to the clinical practice as anticancer agents (*Chiosis et al, 2002; Neckers, 2002*). Our findings may help to establish a novel element of the mechanism of action of these drugs by showing the role of Hsp90 inhibition to sensitize cells for various lytic events. To assess the decreased cell stability after the inhibition of Hsp90 function in experiments, which are more relevant to physiological conditions than mild detergent treatment, or hypotonic shock, we have examined the effect of Hsp90 inhibitors and the disruption of Hsp90 by anti-Hsp90 ribozyme on hypoxia-induced and complement-mediated cytolysis of Jurkat cells. Both conditions mimic quite well the lytic conditions usual for tumor cells. Our results demonstrated a clear enhancement of cell lysis under both conditions after any type of Hsp90 inhibition used. Our findings suggest the probability of a general contribution of Hsp90 to maintain cellular integrity. The role of heat shock proteins in natural cell reactivity is well demonstrated (*Manzo, 1998*). Similarly, immune cell-mediated lysis is also associated with the production of superoxides (*Krishnaswamy et al, 2001*). Our results show that both effects take place when Hsp90 inhibitors enhance cell lysis, but our anti-Hsp90 ribozyme experiments show that the inhibition of Hsp90 itself seems to be predominant. Sodium arsenite was shown to sensitize Jurkat cells for immune mediated cytolysis (*Scott & Dawson, 1995*). However, in this case the complexity of the stress response both in tumor and by-stander cells as well as the relative toxicity makes the treatment non-suitable for selective tumor therapy. On the contrary, selective depletion of Hsp90 seems to be an effective mode of cell sensitization to both hypoxia- and immune-mediated cell lysis, which adds a novel element to the mechanism of action of Hsp90 inhibitor drug candidates. This phenomenon may help the immune system to attack tumor cells. Similarly, a lysis sensitization may cause a shift from tumor cell apoptosis to necrosis, which gives a further help for the activation of the immune system (*Soti et al, 2003b*).

5.4. Identification of a novel peptide-binding region in Hsp90

Although the functional significance of Hsp90 is known to a large extent in assisted protein folding/refolding of non-native and/or labile polypeptides, the structural

details how Hsp90 recognizes substrates with respect to its domains have not been well characterized. Hsp90 inhibitors bind to both the N- and C-terminal domains of the protein. Both domains also contain a substrate binding site. The role of the charged linker region connecting these domains in substrate recognition is largely unexplored.

By surface plasmon resonance experiments, a physiologically active peptide, neuropeptide Y (NPY13-36) was identified as an Hsp90 binding substrate from a large number of peptide screening (Table. 2 & *Ishiwatari et al, 2003*). Earlier reports suggested that the middle domain together with the N-terminal domain are required for the Hsp90 peptide binding (*Scheibel et al, 1998; Young et al, 1997*). In the present study, we proposed another peptide binding domain (residues 181-372), which is not located in the N-terminal geldanamycin/ATP-binding domain of Hsp90 (1-210 residues) (Fig. 17, *Ishiwatari et al, 2003*). Since mutant NPY13-36 and mutant Hsp90 failed to bind to each other (Fig. 17, *Ishiwatari et al, 2003; Soti & Csermely; unpublished observations*) and the binding was drastically disrupted in buffers with high ionic strength (Fig. 18) it seems that electrostatic interactions particularly contribute to the binding of NPY13-36 to Hsp90.

Hsp90 is known to preferably interact with polypeptides in the late folding stage where considerable secondary structure is present. Although there are many substrate proteins reported, the structural features of substrates recognized by Hsp90 and the actual binding sites on Hsp90 have not been characterized. Based on data of *Ishiwatari et al* (*2003*) we propose that one of the primary structure preferences governing high affinity binding to Hsp90 is positive charges aligned on a face of the ?-helix, and substrates having this structure may effectively bind to the region of amino acids 181-372 in Hsp90. Regarding the interaction between glucocorticoid receptor and Hsp90, it was shown that a predicted ?-helix region in the hormone-binding domain is necessary for efficient Hsp90 binding (*Xu et al, 1998*). In addition, studies with antibodies against Hsp90 (*Kang et al, 1994; Tbarka et al, 1993*) and deletion mutants of Hsp90 suggest that a possible glucocorticoid receptor-binding site is encompassed by the NPY binding region (*Jibard et al, 1999*). Moreover, a binding site for serine/threonine kinase Akt/PKB was mapped in this region (amino acid residues 327-340 of Hsp90?) (*Sato et al, 2000*). Thus, this

structural basis might be involved in binding of some native substrates to Hsp90, although further experiments are required to confirm this possibility.

5.5. Future experiments

Our lipid peroxidation experiments with Jurkat cells upon geldanamycin treatment suggest that lipid peroxidation is associated with the raft structure as seen by DPPP fluorescence (Fig. 5D). In support of this, recent studies indicated a role for Hsp90 in maintaining the membrane raft structure. Geldanamycin treatment efficiently dissociated several Hsp90-related protein complexes in lipid rafts (*Shah et al, 2002; Waheed & Jones, 2002*). Since lipid rafts are usually isolated after a nonionic detergent extraction, the similar experimental conditions in our studies argue for a role of Hsp90-inhibition induced raft disorganization in the decrease of cellular integrity. It will be interesting to address how Hsp90 is involved in raft structures and also whether it serves as an antioxidant protein in specific rafts?

The hypothesis of Hsp90 interaction with filamentous structures, the microtrabecular lattice (*Csermely et al, 1998; Csermely, 2001*) is quite challenging. It will be interesting to know, whether Hsp90 is involved either directly or through its clients to maintain these structures. The loss of cellular integrity as seen by both detergent, hypoxia and complement lysis experiments (*Sreedhar et al, 2003a*) as leakage of proteins from the cytosol to the extracellular environment suggests that Hsp90 inhibitors alter the cellular integrity. Further experiments are needed to show, if Hsp90 distribution becomes more disorganized after Hsp90 inhibition in cells, which would correspond to the disorganization of a meshwork-like structure.

Studies with peptide binding to Hsp90, which led to the discovery of a novel peptide binding region on Hsp90 showed us that there is still a lot to understand about Hsp90 and its molecular interactions.

5.6. Conclusions

In conclusion, the major finding of the present study is that Hsp90 is involved in maintaining the cellular integrity. Among various Hsp90 inhibitors used, geldanamycin has a dual action in damaging cellular integrity: roughly half of its effects come from an

accelerated superoxide production, but the other half of increased cellular fragility as a direct consequence of diminished Hsp90 function. Additionally, cell lysis after hypoxia and complement attack was also enhanced by any type of Hsp90 inhibition used, which shows that the maintenance of cellular integrity by Hsp90 is important in physiologically relevant lytic conditions of tumor cells. Lastly, we could identify a novel peptide binding region in Hsp90, which is located in the charged domain immediately after the amino terminus of the protein.

6. Most important novel results

Chaperones were present during the prebiotic evolution and the 90 kDa heat shock protein (Hsp90) is a highly conserved molecular chaperone, which have played an extensive role in various points of prebiotic evolution (*Csermely, 1997; Csermely et al, 2003*). This serves as the first uncovered molecular element behind evolutionary jumps (*Rutherford & Lindquist, 1998*). Hsp90 stabilizes the structure of more than a hundred protein kinases and other important signaling molecules (*Csermely et al, 1998; Pratt & Toft, 2003*) suggesting a key role for this chaperone in cell proliferation and survival. In recent years Hsp90 inhibitors gained more and more attention as promising novel anticancer agents. A less toxic geldanamycin derivative, 17AAG recently entered to phase II clinical trials and several other Hsp90 inhibitors also show a promising progress to be efficient anticancer drugs (*Neckers, 2003; Sreedhar et al, 2003b*). Hsp90 inhibitors act at the N- and C-terminus of Hsp90 (*Marcu et al, 2000a; Soti et al, 2002; Soti et al, 2003a*). Although inhibition of Hsp90 may promote tumor cell death either by inhibiting the cell cycle or by promoting apoptosis, the mechanism behind this phenomenon needs to be further explored. Hence I measured the effect of Hsp90 inhibitors for the partial lysis of the Jurkat T lymphoid cell line. We were also interested, if the middle, charged linker region of Hsp90 is an active component in substrate binding or not. My major findings are the following:

1. Hsp90 inhibitors enhance the lysis of Jurkat cells by mild detergent treatment, by hypoxia or by the complement system (*Sreedhar et al, 2003a*).
2. Hsp90 inhibitors do not affect the lysis of bacteria, while they have a smaller effect on yeast cells, than on T lymphocytes (*Sreedhar et al, 2003a*).
3. The enhancement of the cell lysis has two components: it is partially derived from the increased amount of superoxides after the addition of Hsp90 inhibitors, while the rest of the effect comes from the inhibition of Hsp90 (*Sreedhar et al, 2003a*).
4. Hsp90 inhibitors (especially geldanamycin) induce superoxide production, uneven lipid peroxidation of the plasma membrane and a change in membrane fluidity (*Sreedhar et al, 2003a and unpublished data*).
5. Hsp90 can be successfully inhibited by the specific hammerhead ribozyme we constructed (*Sreedhar et al, 2003a*).

6. Analyzing the binding of neuropeptide Y (NPY) to Hsp90 I showed that the binding of NPY to Hsp90 is grossly dependent on the ionic strength of the buffer, which gave a further evidence for the electrostatic nature of NPY/Hsp90 interactions, further supporting the role of the charged linker region in NPY binding (*Hayasaka et al, 2003*).

My results uncovered an important role of Hsp90 in the maintenance of the cellular integrity, showed a novel element of the antitumor mechanism of Hsp90 drugs and helped to establish a functional role of the charged, linker region of Hsp90 by giving additional support for the identification of this site as the third peptide binding site of Hsp90. Moreover, my studies gave the first example for the successful use of anti-Hsp90 ribozymes for the inhibition of Hsp90 function.

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8. Publications

8.1. Publications directly related to the thesis

1. A. S. Sreedhar, K. Mihaly, B. Pato, T. Schnaider, A. Stetak, K. Kis-Petik, J. Fidy, T.Simonics, A. Maraz & P. Csermely. (2003) Hsp90 inhibition accelerates cell lysis: anti-Hsp90 ribozyme reveals a complex mechanism of Hsp90 inhibitors involving both superoxide- and Hsp90-dependent events. ***J. Biol. Chem.*** 278: 35231-35240. IF: 6.696
2. H. Ishiwatari, M. Maruya, A. S. Sreedhar, T. K. Nemoto, P. Csermely, & I. Yahara (2003) Interaction of neuropeptide Y and Hsp90 through a novel peptide-binding region. ***Biochemistry*** 42: 12972-12980. IF: 4.064
3. P. Csermely, C. Soti, E. Kalmar, E. Papp, B. Pato, A. Vermes & A. S. Sreedhar (2003) Molecular chaperones, evolution and in medicine. ***J. Mol. Struct.*** **(THEOCHEM)** (*in press*). IF: 1.014
4. A. S. Sreedhar, Nardai, G, & Csermely, P (2003) Enhancement of complement-induced cell lysis. A novel mechanism for the anticancer effects of Hsp90 inhibitors. ***Immunol. Lett.*** (*in press*). IF: 2.0
5. A. S. Sreedhar & P. Csermely (2003) Novel roles of Hsp90 inhibitors and Hsp90 in: Redox regulation and cytoarchitecture. ***Recent Res. Devl. Life Sci.*** 1:153-171. ISBN: 81-271-0024-2
6. A. S. Sreedhar, C. Soti & P. Csermely (2003) Hsp90 inhibitors: a new strategy to inhibit protein kinases. ***Biochim. Biophys. Acta*** (*in press*).

8.2. Publications indirectly related to the thesis

1. A. S. Sreedhar & P. Csermely (2003) Heat shock proteins in the regulation of apoptosis: new strategies in tumor therapy. A comprehensive review. ***Pharmacol. & Ther.*** *(in press).* IF: 5.630
2. C. Soti, A. S. Sreedhar & P. Csermely (2003) Apoptosis, necrosis and cellular senescence: chaperone occupancy as a potential switch. ***Aging Cell*** *2*: 39-45.
3. A. S. Sreedhar & U. K. Srinivas (2002) Activation of stress response by ionomycin in rat hepatoma cells. ***J. Cell. Biochem***. 86: 154-161. IF: 2.53
4. A. S. Sreedhar, B. V. V. Pardhasaradhi, A. Khar & U. K. Srinivas (2002) Effect of C-terminal deletion of p53 on heat-induced CD95 expression and apoptosis in a rat histiocytoma. ***Oncogene*** 21: 4042-4049. IF: 5.979

5. A. S. Sreedhar, B. V. V. Pardhasaradhi, A. Khar & U. K. Srinivas (2002) A cross talk between cell signalling and cellular redox state during heat induced apoptosis in a rat histiocytoma. ***Free Radic. Biol. Med.*** 32: 221-227. IF: 5.533

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