REVIEW

Heat shock proteins as emerging therapeutic targets

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Chaperones (stress proteins) are essential proteins to help the formation and maintenance of the proper conformation of other proteins and to promote cell survival after a large variety of environmental stresses. Therefore, normal chaperone function is a key factor for endogenous stress adaptation of several tissues. However, altered chaperone function has been associated with the development of several diseases; therefore, modulators of chaperone activities became a new and emerging field of drug development. Inhibition of the 90 kDa heat shock protein (Hsp)90 recently emerged as a very promising tool to combat various forms of cancer. On the other hand, the induction of the 70 kDa Hsp70 has been proved to be an efficient help in the recovery from a large number of diseases, such as, for example, ischemic heart disease, diabetes and neurodegeneration. Development of membrane-interacting drugs to modify specific membrane domains, thereby modulating heat shock response, may be of considerable therapeutic benefit as well. In this review, we give an overview of the therapeutic approaches and list some of the key questions of drug development in this novel and promising therapeutic approach.

Molecular chaperones

Molecular chaperones (1) protect other proteins against aggregation, (2) solubilize initial, loose protein aggregates, (3) assist in folding of nascent proteins or in refolding of damaged proteins, (4) target severely damaged proteins to degradation and (5) in case of excessive damage, sequester damaged proteins to larger aggregates. Chaperones are ubiquitous, highly conserved proteins, which utilize a cycle of ATP-driven conformational changes to refold their targets, and which probably played a major role in the molecular evolution of modern enzymes (Hartl, 1996; Csermely, 1997; 1999; Thirumalai & Lorimer, 2001). Due to the significant overlap in their functions, the major classes of molecular chaperones are best classified by their molecular weights, thus, for example, the abbreviation Hsp90 refers to the 90 kDa heat shock protein (Hsp).

Cellular stress leads to the expression of Hsp's. Stress can be any sudden change in the cellular environment, to which the cell is not prepared to respond, such as heat shock. However, almost all types of cellular stress induce Hsp's. Owing to the generality of this phenomenon, Hsp's are often called stress proteins. The rationale behind this phenomenon is that after stress there is an increased need for the chaperone function of Hsp's, which triggers their induction. This need is caused by the increased amount of damaged proteins, by the inhibition of their elimination *via* the proteasome as well as by the damage of the chaperones themselves. Hsp induction might help to renature chaperones and, therefore, Hsp induction might lead to a 'cascading amplification' of available chaperone activity.

Hsp synthesis is induced by the activation of the heat shock factor (HSF)-1. In resting cells several chaperones, most importantly Hsp90, were shown to bind to HSF-1 and keep it in an inactive form. During stress, these repressing chaperones become occupied by misfolded proteins, which results in the dissociation of the cytoplasmic chaperone/HSF-1 complex. Dissociation of HSF-1 from Hsp90 uncovers the nuclear localization signal of this transcription factor and allows its translocation to the cell nucleus. During this process, the trimerization and phosphorylation of HSF-1 occurs (Morimoto, 2002). Though the exact sequence of these events has not been clearly established, recent studies uncovered the polo-like kinase 1 as an important actor in the phosphorylation and consequent nuclear translocation of HSF-1 at the Ser-419 residue (Kim *et al.*, 2005). However, other studies (Guettouche *et al.*, 2005) found Ser-326 but not Ser-419 as an important site of activation-related phosphorylation of HSF-1. Part of the nuclear HSF-1 is assembled to heat shock granules, which may modify the chromatin structure (Jolly *et al.*, 2004). Binding of HSF-1 to the heat shock elements of the heat shock-inducible genes unlocks the RNA polymerase, which becomes arrested ('pauses') in most of these genes after transcribing the initial segment of the mRNA in the absence of HSF-1. HSF-1 is released from the DNA by a nuclear complex of Hsp90, which is probably followed by its retrotranslocation to the cytoplasm.

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The details of the nuclear translocation of Hsp90 during stress, their complex formation in the nucleus and consequent recruitment to the DNA-bound HSF-1 are not entirely known. All elements of the HSF-1 activation and downregulation cascade, such as additional proteins of the cytoplasmic or nuclear HSF-1/Hsp90 complexes, for example, the Ral-binding protein 1 and tubulin in the cytoplasmic HSF-1/Hsp90 complex (Hu & Mivechi, 2003) or p23 in the nuclear HSF-1/Hsp90 complex (Guo *et al.*, 2001), are of great interest as potential drug targets (Figure 1).

Hsp's never work alone. They are always forming a complex with each other and recruit various smaller proteins, called cochaperones, which regulate their ATP-ase cycle, therefore the speed of Hsp-assisted refolding. A central chaperone complex of the cytoplasm is assembled around Hsp90 and is called foldosome (Pratt & Toft, 2003).

Therapeutic potential of Hsp90 inhibition

Inhibition of Hsp90 as an efficient tool for anticancer therapies

Hsp90 is one of the most abundant proteins of eukaryotic cells, comprising 1–2% of total proteins under nonstress conditions. It is evolutionarily conserved among species, and is essential for cell survival. Hsp90 exerts its chaperone activity together with a number of cochaperones, playing an important role in the folding of at least 200 specific proteins of various signaling pathways, and in the refolding of denatured proteins after stress (Csermely *et al.*, 1998; Buchner, 1999; Pratt & Toft, 2003).

Hsp90 is an ATP-dependent chaperone. The N-terminal domain of Hsp90 contains a rather unique ATP-binding site, the Bergerat-fold, characteristic of only some bacterial gyrases, topoisomerases and histidine-kinases besides Hsp90 (Prodromou *et al.*, 1997; Stebbins *et al.*, 1997). The unique ATP-

binding site allowed the development of specific Hsp90 inhibitors. Recently, it was shown that Hsp90 contains a second nucleotide-binding site at its C-terminal domain, which may open new possibilities for the inhibition of this chaperone (Marcu *et al.*, 2000; Garnier *et al.*, 2002; Soti *et al.*, 2002).

Hsp90 interacts with and stabilizes a growing list of various kinases including several key members of malignant transformation, such as the ErbB2, Src, Abl or Met tyrosine kinases, or the Raf, Akt and cyclin-dependent serine kinases. Besides these, Hsp90 is necessary for the maturation of several transcription factors, like the nuclear hormone receptors and the hypoxia-inducible factor-1. Additionally, Hsp90 binding has been shown to contribute to the accumulation of mutant forms of the tumor suppressor transcription factor p53. Hsp90 is associated with nitric oxide synthases, the antiapoptotic protein, Apaf-1, etc. (Csermely *et al.*, 1998; Buchner, 1999; Pratt & Toft, 2003; Zhao *et al.*, 2005).

The above examples show that a large number of proteins need the help of molecular chaperones to maintain their activation-competent conformation. 'Conventional' inhibitors interact with their target, directly inhibiting its function. However, chaperone-based inhibitors do not interact with the effector proteins, but inhibit the ability of the associated chaperone(s) to maintain their activation-competent conformation. As a result, the client proteins became degraded by the proteasome (Schulte *et al.*, 1997). In contrast to most direct inhibitors, which are often fairly specific for a given protein, chaperone-based inhibitors diminish the level of many protein targets in parallel (Sreedhar *et al.*, 2004b; Neckers & Neckers, 2005). Thus, chaperone inhibitors behave as typical multi-target drugs, which were suggested to be more efficient than highly selective single-target drugs in many applications (Csermely *et al.*, 2005).

The most important Hsp90 inhibitors are geldanamycin (Whitesell *et al.*, 1994), its less toxic analog, 17-allylamino-17-demethoxy-geldanamycin (17AAG) (Schulte & Neckers, 1998)
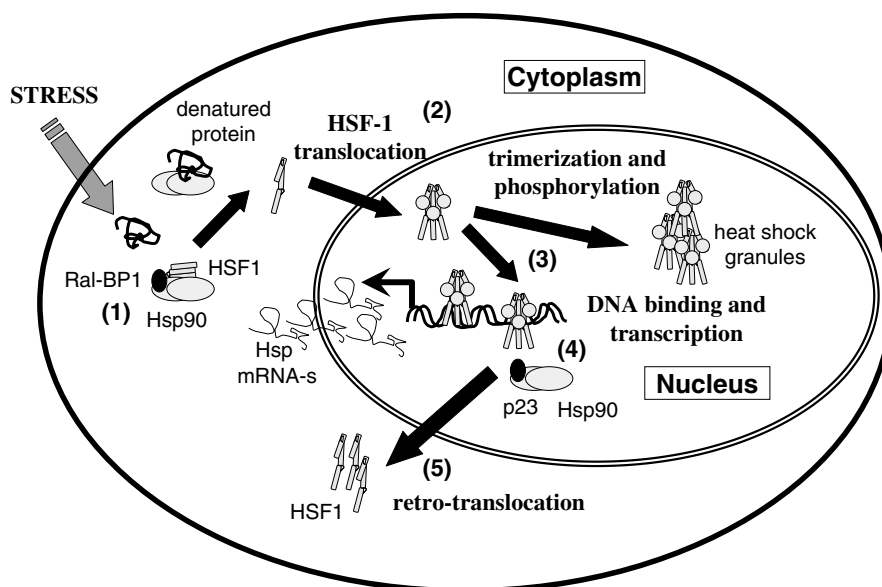

Figure 1 Elements of HSF-1 activation: potential drug targets. The figure contains the major elements of the activation of HSF-1, the major transcription factor leading to the induction of Hsp synthesis. The potential drug targets are the following: (1) cytoplasmic complex of HSF-1 and Hsp90; (2) HSF-1 translocation to the nucleus; (3) intranuclear distribution of HSF-1; (4) nuclear complex of HSF-1 and Hsp90; (5) retrotranslocation of HSF-1 to the cytoplasm. RalBP-1, Ral-binding protein-1; p23, cochaperone of Hsp90.

as well as radicicol, and its more stable oxime derivatives (Soga et al., 1998; Agatsuma et al., 2002; Ikuina et al., 2003), which have a higher affinity for Hsp90 than geldanamycin (Roe et al., 1999). Recently, new geldanamycin analogs (Hargreaves et al., 2003) as well as a third class of tumor-specific Hsp90 inhibitors, the purine-scaffold inhibitors, were developed (Chiosis et al., 2002; Vilenchik et al., 2004), and there are ongoing efforts to synthesize even more Hsp90-interacting drug candidates (Banerji et al., 2003; Maloney et al., 2003; Workman, 2004; Dymock et al., 2005; Kreusch et al., 2005) (Table 1). Hsp90 inhibition was achieved in an unusual way, using the antitumor agent histone deacetylase inhibitors. These compounds, such as Trichostatin-A, induce the acetylation and consequent inhibition of Hsp90 (Yu et al., 2002).

The tumor specificity of Hsp90 inhibitors is helped by an unusual mechanism (Figure 2; Kamal et al., 2003). Hsp90 is largely in a latent, uncomplexed state in normal cells. However, Hsp90 becomes activated, forming a large complex with various cochaperones in tumor cells. 17AAG binds to the tumor-specific, complex form of Hsp90 with a 100-fold higher affinity than to the latent form (Kamal et al., 2003), raising the possibility that active Hsp90 behaves as a tumor-selective catalyst to convert geldanamycin derivatives to their active conformation (Lee et al., 2004). The increased affinity of Hsp90 inhibitors towards tumor-specific Hsp90 is also true for purine-based inhibitors (Vilenchik et al., 2004).

Inhibition of Hsp90 induces apoptosis of various tumor cells (Sreedhar & Csermely, 2004). Hsp90 inhibition also leads to a defect in a number of proliferative signals, including the Akt-dependent survival pathway (Munster et al., 2001; 2002; Basso et al., 2002). Hsp90 inhibition may also sensitize tumor cells against various attacks by helping their lysis under hypoxia, complement attack or mild detergent treatment (Sreedhar et al., 2003; 2004a).

Importantly, there are additional drugs which interact with Hsp90, such as the widely used chemotherapeutic agents, cisplatin (Itoh et al., 1999), taxol (Byrd et al., 1999), as well as the antibactericid novobiocin (Marcu et al., 2000). Cisplatin was recently shown to bind to a novel nucleotide-binding site of Hsp90 at its C-terminus (Soti et al., 2002), while novobiocin binds to several domains of Hsp90 (Marcu et al., 2000; Soti et al., 2002). The C-terminal nucleotide-binding pocket has a unique nucleotide-binding specificity (Soti et al., 2003), as well as a differential effect on Hsp90 client proteins (Soti et al., 2002), which gives hopes that selective inhibitors against this segment of Hsp90 can be developed, showing novel properties in various anticancer protocols. This can be an important approach all the more, since recently a new Hsp90 isoform, Hsp90N, has been reported, which lacks the N-terminal domain; thus, the tumor cells which accumulate this chaperone are resistant against any conventional Hsp90 inhibitors (Grammatikakis et al., 2002).

Hsp90 inhibitors enter clinical trials

Though the very first Hsp90 inhibitor geldanamycin showed clear antitumor effects, it encountered difficulties in clinical trials due to its high hepatotoxicity in some of the human tumor models (Supko et al., 1995). Thus, a search for new classes of Hsp90 inhibitors with lower toxicity began, and was successful in developing the analog 17AAG. 17AAG possesses all the Hsp90-related characteristics of geldanamycin (Schulte & Neckers, 1998) with lower toxicity (Brunton et al., 1998; Chiosis et al., 2003; Workman, 2003) and completed five Phase I clinical trials and enters to phase II trials soon (Banerji et al., 2005; Neckers & Neckers, 2005). Both geldanamycin and 17AAG can be metabolized by NADH quinone oxidoreductase 1 (DT-diaphorase), which is known to potentiate antitumor activity by stabilizing the tumor suppressor p53. NADH quinone oxidoreductase 1 may be a major factor in conferring on 17AAG as well as its parent compound the advantage that they specifically accumulate in tumor cells (Chiosis et al., 2003; Workman, 2003), which also contributes to the explanation why Hsp90 inhibitors are not so generally

Table 1 Hsp modulator compounds (a few examples)

| Compound	Modulation	Targets	Potential indications	References
Carbenoxonole	Inducer	Hsp70	Ulcer	Nagayama et al. (2001)
2-Cyclopenten-1-one	Inducer	HSF-1	Viral infections	Rossi et al. (1996)
Dexamethasone	Inducer	HSF-1	Ischemic heart disease, inflammation	Sun et al. (2000)
Geranyl-geranyl acetone	Inducer	Hsp70	Cerebral and cardiac ischemia	Ooie et al. (2001), Yasuda et al. (2005)
Paeoniflorin	Inducer	Hsp27, Hsp40, Hsp70	Not identified yet	Yan et al. (2004)
Proteasome inhibitors	Inducer	Hsp70	Neuro-degeneration	Kim et al. (2004)
Stannous chloride	Inducer	Hsp70	Tissue transplantation	House et al. (2001)
Glycyrrhizin	Co-inducer	HSF-1	Not identified yet	Yan et al. (2004)
Bimoclomol, arimoclomol, BRX-220	Co-inducer	HSF-1, membranes	ALS, diabetic neurophaty, pancreatitis, ischemic heart disease	Vigh et al. (1997), Jednakovits et al. (2000), Kurthy et al. (2002), Rakonczay Jr. et al. (2002), Kieran et al. (2004)
Geldanamycin, 17AAG	Inhibitor	Hsp90	Cancer	Whitesell et al. (1994), Neckers & Neckers (2005)
Radicicol, cyclo-proparadicicol	Inhibitor	Hsp90	Cancer	Soga et al. (1998), Yang et al. (2004)
PU3, PU24F-Cl (purin-based molecules)	Inhibitor	Hsp90	Cancer	Chiosis et al. (2001)
Dihydroxy-phenylpyrazole	Inhibitor	Hsp90	Cancer	Kreusch et al. (2005)
Trichostatin-A (histone deacetylase inhibitors)	Inhibitor	Hsp90	Cancer	Yu et al. (2002)
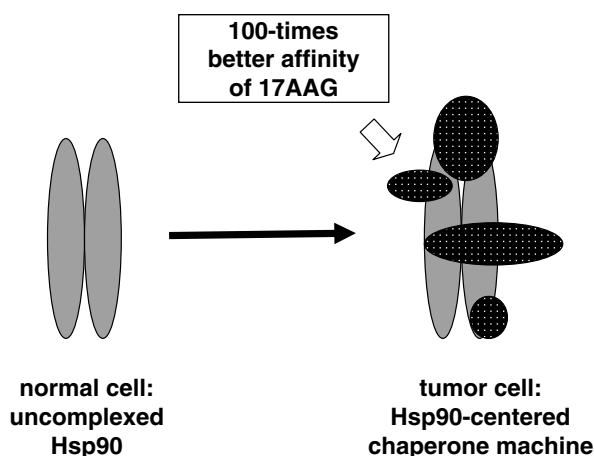
Figure 2 The tumor specificity of 17AAG, a geldanamycin analog inhibitor of Hsp90. 17AAG has a 100-times higher affinity towards the tumor-specific Hsp90 complexed by a large number of cochaperones than to the Hsp90 dimer, which is the predominant form of this chaperone in normal cells (Kamal et al., 2003; Lee et al., 2004).

toxic to patients as one would expect from the pleiotropic roles of Hsp90 inhibited by them besides their higher affinity to the tumor-specific, complexed form of Hsp90 mentioned before (Kamal et al., 2003; Vilenchik et al., 2004).

Combination therapies, applying low doses of Hsp90 inhibitors together with conventional chemotherapeutic agents, seem to be an effective way to target various cancers. For example, in the case of Bcr–Abl-expressing leukemias, a low dose of geldanamycin is sufficient to sensitize these cells to apoptosis in the presence of ineffective concentrations of doxorubicin (Blagosklonny et al., 2001). 17AAG in combination with taxol showed enhanced cytotoxic effects on taxol-resistant ErbB2-overexpressing breast cancer cells (Munster et al., 2001; Sausville, 2001). Another approach is to combine 17AAG with angiogenesis inhibitors, which was proven to be highly successful in breast tumors (de Candia et al., 2003). As an alternative strategy the synthesis of geldanamycin hybrids, such as the adduct with steroids (Kuduk et al., 1999; 2000) as well as with inhibitors of the PI-3-kinase related survival pathway (Chiosis et al., 2001), conferred further selectivity and efficiency for these drugs besides their tumor-specific accumulation. Besides the success of 17AAG, other Hsp90 inhibitors also entered phase I trials, which shows the high therapeutic potential of these drugs (Neckers & Neckers, 2005).

Hsp90 inhibitors as Hsp inducers?

Hsp synthesis is induced by the activation of HSF-1. Hsp90 dissociates during HSF-1 activation due to a competitive binding of misfolded proteins to this chaperone (Morimoto, 2002). Hsp90 inhibitors may also cause the transcriptional activation of HSF-1 by disrupting Hsp/HSF-1 complexes. Indeed, geldanamycin and 17AAG were shown to activate HSF-1 (Kim et al., 1999b; Bagatell et al., 2000) and the expression of Hsp40, Hsp70 and Hsp90 (Sittler et al., 2001). Thus, the inhibition of Hsp90, paradoxically, leads to an increase in their overall amount as well, which should be taken into account as a potential disadvantage, when clinical

applications of these drugs are designed. Indeed, both geldanamycin and 17AAG were shown to antagonize the action of cisplatin in human colon adenocarcinoma cells (Vasilevskaya et al., 2003).

Inhibition or activation of Hsp90 is cardioprotective?

Griffin et al. (2004) has recently demonstrated that radicicol, which activates Hsp expression by binding to Hsp90, induces Hsp expression in neonatal rat cardiomyocytes, and this increase in Hsp's confers cardioprotection to these cardio-myocytes. However, they found that radicicol induction of the Hsp's and cardioprotection are dependent on the inhibition of Hsp90 in cardiomyocytes, and concluded that inhibitors of the function of Hsp90's in the cell may represent potential cardioprotective agents. In contrast, another recent study shows that in vivo gene transfer of Hsp90 in the myocardium leads to a protection of the ischemic myocardium in pigs via a direct stimulation of eNOS by Hsp90 (Kupatt et al., 2004). It is well known that NO is cardioprotective; however, increased NO synthesis with concomitant superoxide synthesis leads to the formation of the cytotoxic peroxynitrite in certain cardiovascular disorders (Ferdinandy & Schulz, 2003). Therefore, further studies are necessary to evaluate the possible role of Hsp90 inhibitors in the therapy of ischemic heart disease.

Therapeutic potential of upregulation of Hsp's

While the inhibitors of Hsp90 are of potential therapeutic interest primarily in cancer therapy, upregulation of other Hsp's, especially that of Hsp70, has been shown to be of great therapeutic potential in a variety of diseases.

Hsp-based immunotherapies of cancers and infections

The ability of Hsp's to interact with a wide range of proteins and peptides, a property that is shared by major histocompatibility complex molecules, has made the Hsp's to be used in new immunotherapies of cancers and infections. Increased Hsp60, Hsp70 and Hsp72 may lead to tumor cell sensitization for immune attacks by two mechanisms: tumor cells may express Hsp's on their surface, which leads to their enhanced recognition by the natural killer cells of the native immune system (Multhoff et al., 1997; Multhoff, 2002), as well as a specific antitumor immunity may be induced by Hsp-related antitumor vaccination (Chu et al., 2000; Baker-LePain et al., 2003; Shigapova et al., 2005). Hsp induction may help these processes and may be overcome by the limitations of aging-(Wick & Grubeck-Loebenstein, 1997; Pawelec et al., 2002) and chaperone overload-induced (Csermely, 2001) immuno-suppression. As these topics have been extensively reviewed recently (Ranford & Henderson, 2002; Srivastava, 2002; Manjili et al., 2004; Oki & Younes, 2004), no further details are given in our present review.

Tissue protection by induction and 'coinduction' of Hsp70 and other Hsp's

From the now classical observations of Currie et al. (1988), we know that heat-shock response has a significant role in

cardioprotection. Similarly, Hsp72 induction helps the survival of neurons after stroke (Yenari et al., 1998), as well as improves the efficiency of tissue transplantation (Perdrizet et al., 1993). Hsp70 induction eases the deleterious consequences of chronic diseases, such as diabetes, as revealed by the application of the Hsp coinducer compounds bimoclomol and BRX-220 (Nanasi & Jednakovits, 2001; Kurthy et al., 2002). Conditions like Alzheimer's, Parkinson's, Huntington's or prion disease, where the accumulation of misfolded proteins is the major cause of neurodegeneration (Warrick et al., 1999; Carmichael et al., 2000; Sittler et al., 2001), as well as conditions such as trauma, where neuroregeneration becomes necessary (Kalmar et al., 2002; 2003), also have beneficial effects from overexpression of Hsp40 and Hsp70.

There are various approaches to induce Hsp's using pharmacological interventions without any traditional forms of stress, such as heat shock. Proteasome inhibitors upregulate Hsp synthesis by increasing the amount of misfolded proteins, which compete for Hsp's with HSF-1 (Kim et al., 1999a; 2004). Some of the protein kinase inhibitors were also shown to induce Hsp27 induction (Kawamura et al., 1999). Stannous chloride has been shown as a nontoxic, efficient inducer of Hsp70, improving the success rate of tissue transplantations (House et al., 2001). Geranyl-geranyl acetone, a nontoxic Hsp70 inducer, has been shown to protect neurons against cerebral ischemia (Yasuda et al., 2005). Similarly, the antiulcer drug carbenoxolone has also been shown to be an inducer of Hsp70 (Nagayama et al., 2001) (Table 1).

Most of the above methods introduce a certain level of stress to the cells and thus provoke Hsp synthesis. However, in most of the diseases it seems to be more efficient if the administered drug does not induce Hsp's, and just helps the natural Hsp induction provoked by the natural stimuli on the cell. This help in Hsp induction was termed as chaperone 'coinduction' (Vigh et al., 1997). Chaperone coinducers act like 'smart drugs' by a selective interaction with only those cells which are in danger, and may provide an important novel therapy in a number of acute and chronic diseases.

The best-known chaperone coinducer is aspirin, which enhances Hsp70 synthesis (Jurivich et al., 1992). Recently, certain herbal extracts were also shown to possess Hsp coinducer activity (Yan et al., 2004). Another family of drug candidates exemplified by Bimoclomol helps the induction of Hsp70 synthesis by both perturbing various membrane structures and helping the release of putative lipid-signaling molecules, as well as by the prolongation of the binding of HSF-1 to the heat shock elements on the DNA (Vigh et al., 1997; Hargitai et al., 2003; Torok et al., 2003; Kieran et al., 2004). Bimoclomol binds to HSF-1 with a low affinity (Figure 3), which may contribute to its effect to prolong HSF-1 binding to DNA. Chaperone coinducers also stabilize membranes, which may be of special importance to prevent apoptotic events related to the decomposition of cardiolipin and the consecutive instabilization of the mitochondrial membrane (Torok et al., 2003). Recently, a successful application of the chaperone coinducer arimoclomol has been shown to delay the onset of amyotrophic lateral sclerosis (Kieran et al., 2004). Another chaperone coinducer, BRX-220, has been shown to protect against CCK-induced acute pancreatitis (Fujiwara et al., 2004) (Table 1).
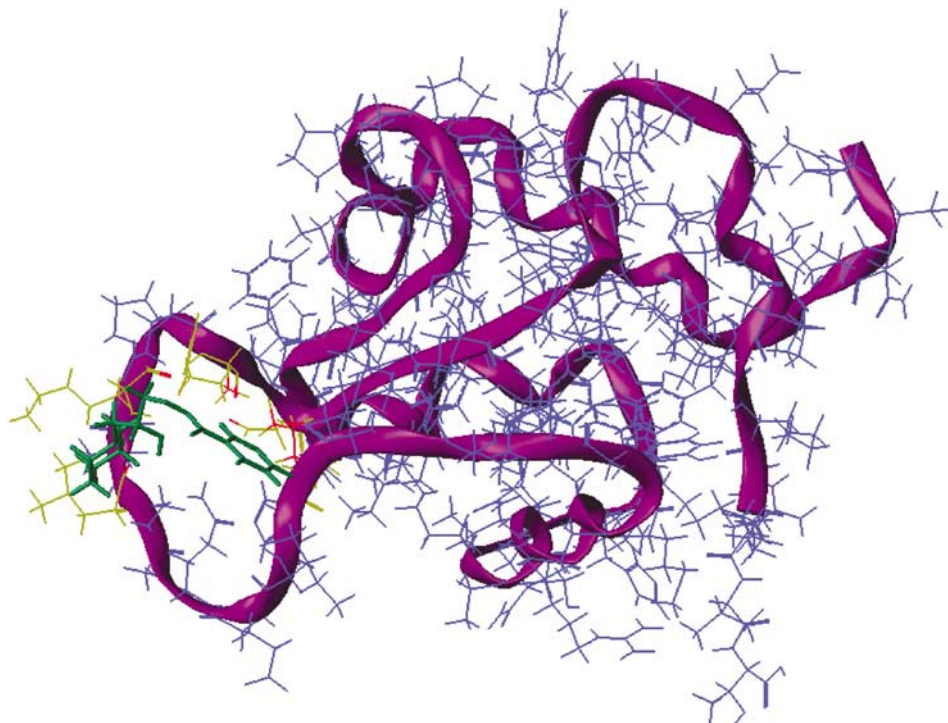
Figure 3 Bimoclomol binds to the HSF-1 with a low affinity (Hargitai et al., 2003). Fitting of /R/-bimoclomol to the 3D model of the DNA-binding domain of human HSF-1. The SiteID, FlexX and the FlexiDock modules of the Sybyl package were used sequentially to identify the loop domain as the active center (yellow–red amino acids) of the DNA-binding domain of human HSF-1 and dock bimoclomol (green) into this site. The calculated binding energy for the /R/-bimoclomol was $-26\,\mathrm{kcal\,mol^{-1}}$. The backbone of the polypeptide is shown (purple).

Diminished heat stress response in hyperlipidemia and aging: potential use of Hsp inducers as anti-ischemic agents?

It is well known that aging leads to a diminished heat shock response in several tissues, thereby resulting in the deterioration of stress adaptation (Demirel *et al.*, 2003) (for a review, see Soti & Csermely, 2003). We have recently reported that experimental hyperlipidemia also leads to diminished heat-stress response, as measured by Hsp70 expression in the ischemic heart of rats (Csont *et al.*, 2002). It is of great interest that both aging and hyperlipidemia have been shown to decrease the cardioprotective effects of the ischemic preconditioning, that is, the endogenous adaptive response of the heart to ischemic stress (for reviews, see Ferdinandy *et al.*, 1998; Baxter & Ferdinandy, 2001; Ferdinandy, 2003). The role of Hsp's in the mechanism of preconditioning has been extensively reviewed elsewhere (Latchman, 2001; Snoeckx *et al.*, 2001). It has been shown that the cardioprotective effect of preconditioning is linked to the functions of Hsp70, Hsp27, and alphaB-crystalline. Heat stress or Hsp70 gene transfection into rat hearts has been shown to protect the ischemic myocardium (Arnaud *et al.*, 2001; Jayakumar *et al.*, 2001). Therefore, it is plausible to speculate that the loss of the protective effect of preconditioning in these disease states is related – at least in part – to diminished heat stress response. We have recently shown using DNA-microarray assay that hyperlipidemia alters the expression of several chaperones in the rat myocardium, including upregulation of Hsp86, metallothionein and glutathione-*S*-transferase, as well as downregulation of proteasome component C9, ubiquitin-like protein FUBI, Hsp105, calreticulin and chaperonin subunit 5 epsilon (Puskas *et al.*, 2004).

The mechanisms by which hyperlipidemia and aging lead to diminished heat stress response are not known. It is suggested, however, that the altered membrane lipid composition and physical state (fluidity) of membranes are decisive factors in the processes of perception and transduction of stress into a signal that triggers the transcriptional activation of stress protein genes. Nevertheless, Hsp inducers and coinducers may have great therapeutic potential in aging and hyperlipidemia to regain the endogenous adaptation of the heart to ischemic stress.

Cellular membranes act as alternative thermosensors to regulate Hsp expression

Elucidation of primary sensors that perceive various stress stimuli and transducers that carry signals culminating in the expression of a particular Hsp is of great interest for the development of novel Hsp inducers and coinducers. According to the classical model, the common primary signal for induction of Hsp's is an increase in the amount of denatured proteins within the cell during stress and their competition with HSF (Morimoto, 1998) (see above for more refs). Hsp's are present in abnormal levels in a variety of human diseases and aging, but there is no evidence for concomitant modification of the kinetics of accumulation of denatured proteins that could justify the changes observed in the expression of Hsp's (Vigh *et al.*, 1998; Vigh & Maresca, 2002). For example, curcumin, a well-known dietary pigment with potent anti-inflammatory, antioxidant and anticancer effects, causes heat shock response in transformed cell lines such as leukemia, breast, colon, etc.; however, it has no effect on nontransformed cell lines (Khar *et al.*, 2001). Since no differences in the pattern of protein denaturation have been reported between normal and transformed cell lines, these data imply that the protein denaturation is not (or not only) the mechanism by which Hsp's are upregulated. There is also evidence for differential expression patterns of different Hsp's in the same cell type in response to the same stimulus (Heads *et al.*, 1995). For example, herbimycin-A provokes Hsp70 synthesis and protects against a subsequent lethal heat stress; however, it does not activate the transcription of other Hsp genes such as hsp90, hsp60, hsp27 and grp78 in cultured neonatal cardiomyocytes (Morris *et al.*, 1996). Monophosphoryl lipid A, a nontoxic derivative of endotoxin with potent anti-ischemic effect, induced a remarkable accumulation of Hsp70, but did not change the expression of Hsp27, Hsp32 and Hsp90 in adult rat cardiac myocytes (Nayeem *et al.*, 1997). Selective stimulation of small Hsp's, Hsp27, and alfaB-crystalline but not Hsp70 was shown by anisomycin following heat stress in C6 glioma cells (Kato *et al.*, 1999), by sphingosine1-phospate in osteoblasts (Kozawa *et al.*, 1999), by hydroxyurea in B16 murine melanoma cells (Eskenazi *et al.*, 1998), by short-chain fatty acids in the rat intestinal epithelial cells (Ren *et al.*, 2001) or by thrombin in aortic smooth muscle A10 cells (Hirade *et al.*, 2002), respectively. These observations indicate that Hsp expression in response to various stressors is regulated by differential control mechanisms rather than by uniform mechanisms.

On the basis of experimental evidence, it is proposed that, besides protein denaturation, specific membrane domains may act as alternative sensors or 'cellular thermometers'. Stress-induced membrane perturbations are converted into signal(s) leading to activation of heat-shock genes (Carratu *et al.*, 1996; Horvath *et al.*, 1998; Vigh *et al.*, 1998; Vigh & Maresca, 2002; Shigapova *et al.*, 2005). Changes in membrane composition and/or fluidity alter the 'set point' for Hsp expression, with expression initiated at lower temperatures in cells with more fluid membranes (Carratu *et al.*, 1996; Horvath *et al.*, 1998; Chatterjee *et al.*, 2000; Shigapova *et al.*, 2005) (Figure 4).

Cellular membranes as potential targets for Hsp inducers

Although the Hsp coinducing effects of bimoclomol were shown to be mediated *via* HSF-1 as described above, bimoclomol does not affect protein denaturation in the cells (Vigh *et al.*, 1997; Hargitai *et al.*, 2003). It has been shown that bimoclomol and its analogs specifically interact with and significantly increase the fluidity of negatively charged membrane lipids. Accordingly, the Hsp coinducing activity of bimoclomol is highly susceptible to the fatty acid composition and fluidity of membrane of target cells. In addition, bimoclomol is an efficient inhibitor of bilayer-to-nonbilayer lipid-phase transitions. Consequently, while sensitizing the cellular membranes at mild heat shock conditions, the drug protects against irreversible membrane damage at higher temperatures (Torok *et al.*, 2003). Thus, even subtle alterations of the lipid phase of membranes caused by aging or pathophysiological conditions may influence membrane-initiated signaling processes either through fluidity changes or by specific interactions of membrane lipids with receptor proteins localized in the membrane. Taken together, it is highly conceivable that plasma membrane, which is the barrier to the
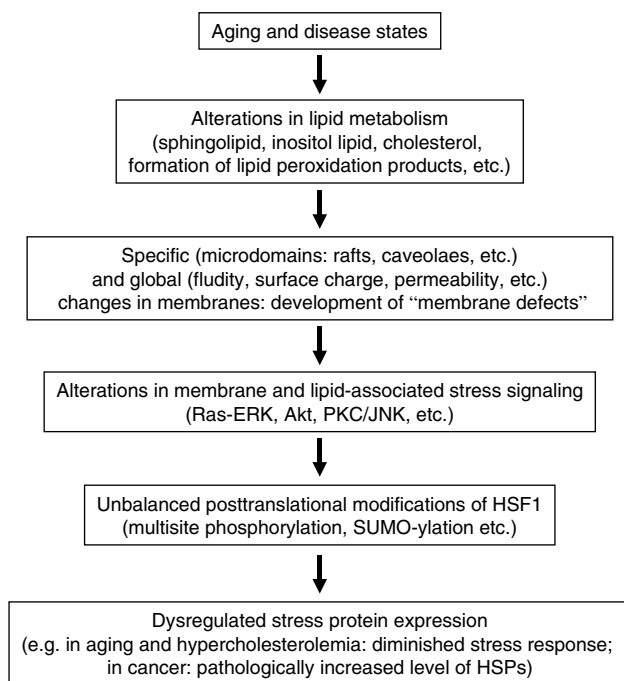
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┌─────────────────────────────┐
│   Aging and disease states  │
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│     Alterations in lipid metabolism      │
│ (sphingolipid, inositol lipid, cholesterol,│
│ formation of lipid peroxidation products, etc.)│
└─────────────────────────────────────────┘
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│  Specific (microdomains: rafts, caveolaes, etc.)│
│ and global (fluidity, surface charge, permeability, etc.)│
│ changes in membranes: development of "membrane defects"│
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│ Alterations in membrane and lipid-associated stress signaling│
│      (Ras-ERK, Akt, PKC/JNK, etc.)            │
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│ Unbalanced posttranslational modifications of HSF1│
│   (multisite phosphorylation, SUMO-ylation etc.)│
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┌─────────────────────────────────────────────┐
│       Dysregulated stress protein expression  │
│ (e.g. in aging and hypercholesterolemia: diminished stress response;│
│ in cancer: pathologically increased level of HSPs)│
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Figure 4 Cascade of events from membrane defects to dysregulated stress protein response.

external environment and well suited for sensing stress, acts also as an important regulatory interface. In line of this concept, recently it was speculated that the reduced HSF-1 and Hsp levels in diabetes are the result of reduced membrane fluidity (Hooper & Hooper, 2005). In fact, as a result of glycation, oxidative stress and insulin deficiency, diabetes is associated with less fluid membranes in human mononuclear leucocytes and platelets (Tong *et al.*, 1995; Srivastava, 2002). Since heat treatment itself causes membrane hyperfluidization, it is not surprising that the daily hot water immersions in patents with type II diabetes improve glycemic control and reduce neuropathic symptoms (Hooper, 1999).

Perturbation of the organization and phase state of acidic glycerophospholipids, the major determinants of the activity of membrane proteins (van Klompenburg *et al.*, 1997), by nontoxic drugs like bimoclomol may influence the structure/activity of membrane-bound proteins without direct drug–protein interaction. Protein function influenced by membrane fluidity and/or microheterogeneity has been suggested for phospholipase A_2, which is stimulated by heat shock, resulting in arachidonic acid release (Honger *et al.*, 1996; Samples *et al.*, 1999). Arachidonic acid stimulates HSF–DNA binding, increases phosphorylation of HSF-1 and upregulates transcription of the *Hsp70* gene in HeLa cells (Jurivich *et al.*, 1994). Elevated activity of membrane-bound phospholipases and the resultant release of free fatty acids and diacylglycerols could also enhance the membrane association and activation of protein kinase C (PKC) isoforms responsible for the phosphorylation of HSFs. In agreement with these data, administration of a PKC activator phorbol ester compound in combination with heat shock markedly enhanced Hsp70 expression in K562 cells (Holmberg *et al.*, 1997). Several other pharmacological modulators of kinase/phosphatase activities can alter the different regulatory steps of the heat shock response. Highlighting the complexity of this point, over-

expression of inducible Hsp70 downregulated the basal activities of protein kinase A, various free and membrane-associated PKC isoforms and the MAP kinase pathways including c-Jun N-terminal kinase and p38 stress-activated protein kinase (p38 SAPK) (Kiang *et al.*, 2002). Through the activation of the glycosylation of membrane sterols, cholesterol glucoside is rapidly accumulating in cells from mold to humans by exposure to environmental stress, and cholesterol glucoside production is followed by the activation of certain PKCs and induction of Hsp's (Kunimoto *et al.*, 2002). Cholesterol glucoside accelerated the binding of HSF-1 to HSE and upregulated Hsp70 synthesis in human fibroblast (Kunimoto *et al.*, 2002). Orally administered cholesterol glucoside apparently showed antiulcer activity in rats *via* HSF activation and Hsp70 induction (Kunimoto *et al.*, 2003). A bimoclomol-related compound, BRX-235, was shown to induce phosphorylation of p38 SAPK, implying that the molecule acted upstream of p38 SAPK (Denes *et al.*, 2002; Kabakov *et al.*, 2005). HSF-1 appears to have several additional layers of regulation in the cell, including Ras/ERK1/GSK3/14-3-3 pathway (Hamaguchi *et al.*, 2003; Wang *et al.*, 2003), Akt-induced inhibition of GSK-3β (Bijur & Jope, 2000), small G-protein signalling such as Ras (Engelberg *et al.*, 1994; Murakoshi *et al.*, 2004) and oxidative stress-induced membrane translocation of Rac1 (Xu *et al.*, 2000; Han *et al.*, 2001), all potential targets for Hsp modulator development. Noteworthy that simvastatin, the known hydroxymethyl-glutaryl-CoA reductase inhibitor antihyperlipidemic drug, blocked the oxidative stress-induced membrane translocation of Rac1 (Negre-Aminou *et al.*, 2002).

It is highly conceivable that the above findings are linked to those hypothetic signal transduction pathways which transmit the heat stress signal from membranes to DNA to induce expression of Hsp's. However, a lipid-selective association of a subpopulation of Hsp's with membranes, leading to increased molecular order, may in turn lead to downregulation of the heat shock gene expression (Torok *et al.*, 1997; 2001; Tsvetkova *et al.*, 2002). Such a 'crosstalk' between the primary stress sensor in the membranes and Hsp's suggests a feedback mechanism in the regulation of heat-shock genes, explaining the known temporality of induction of Hsp's. These findings show that knowledge on pathways of stress signaling will provide several molecular targets for further development of Hsp modulators.

Conclusions and future perspectives

Chaperones play a major role in the mechanism of endogenous stress adaptation of several tissues. However, altered chaperone function has been associated with the development of several pathologies; therefore, chaperone modulators became a new and emerging field of drug development. Inhibitors of Hsp90 recently emerged as a very promising tool to combat various forms of cancer. On the other hand, activation of chaperone induction proved to be an efficient tool for the recovery from a large number of diseases, such as, for example, ischemic heart disease, diabetes and neurodegeneration. Development of several Hsp modulators has already reached clinical phases. Due to the promising results, specific chaperone modulators could be one of the future blockbuster drugs on the market for several different therapeutic indications.

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