REVIEW ARTICLE

The wonderous chaperones: A highlight on therapeutics of cancer and potentially malignant disorders

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ABSTRACT

Diverse environmental and physiological factors are known to induce the transcription of a set of genes encoding special protective molecules known as "molecular chaperones" within our cells. Literature abounds in evidence regarding the varied roles; these "guides" can effectively perform in our system. Highly conserved through evolution, from the prokaryotes to the eukaryotes, these make perfect study tools for verifying their role in both the pathogenesis as well as the therapeutics of varied neurodegenerative, autoimmune and potentially malignant disorders and varied cancer states. We present a concise review of this ever dynamic molecule, highlighting the probable role in a potentially malignant disorder, oral lichen planus.

Key words: Cancer therapeutics, cytoprotection, molecular chaperones, heat shock response

INTRODUCTION

"The advent of science and new discoveries has unearthed the wonder lying within us. The very essence of life, the wondrous molecules defending us and laboring hard for every breath we take."

Diverse environmental and physiological factors are known to induce a set of genes encoding special protective molecules known as "molecular chaperones" within our cells. This phenomenon is called as the heat shock response (HSR), which is an ordered genetic response.[1] The HSR was first discovered by Ritossa,[2] who observed a pattern of *Drosophila* salivary gland chromosome puffs which were induced as a response phenomenon to transient exposure to elevated temperatures. Since then, many investigators have proven it to be indeed ubiquitous and highly conserved – in all organisms from prokaryotes to eukaryotes, – an essential defense mechanism, protecting the cells from a wide range of harmful conditions.[3,4]

There are many "stressors" that can presumably activate the transcription of these heat shock genes. The list includes various acute and chronic conditions such as elevated temperatures, heavy metals, small molecule chemical toxicants, infection and oxidative stress. Mutations and environmental influences including inflammation, ischemia, tissue wounding and repair, cancer and neurodegenerative diseases are also associated with the aberrant expression of heat shock proteins (HSPs).[1,5] Once expressed, varied roles are modulated via these molecules [Figure 1].

At the molecular level, the cellular response to stress is demonstrated by the induced synthesis of HSPs, of which molecular chaperones and proteases represent two well-characterized families of proteins. The molecular chaperones chiefly function in protein folding, translocation and refolding of intermediates, whereas the proteases such as the ubiquitin-dependent proteasome, ensure that damaged and short-lived proteins are degraded or destroyed in an effective manner. Under "stressed" conditions the molecular chaperones are directed toward the capture of folding intermediates to prevent misfolding and aggregation and

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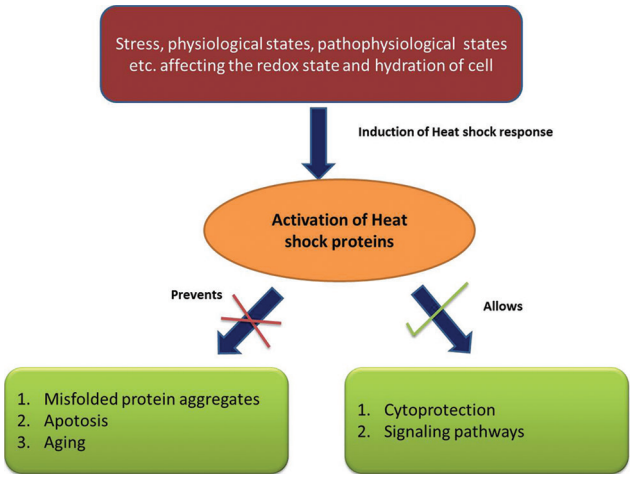

Figure 1: Activation of heat shock proteins and its varied effector functions

to facilitate refolding or degradation.[6-8] HSPs have been classified into six major families according to their molecular sizes: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small HSPs (sHSPs) with sizes ranging from 15 kDa to 30 kDa. High molecular weight HSPs are also known as adenosine triphosphate (ATP)-dependent chaperones. They assist in the folding of newly synthesized or damaged proteins in an ATP-dependent active process. In contrast, sHSPs work in an ATP-independent fashion.[9] Members of each gene family are constitutively expressed, inducibly regulated and/or targeted to different compartments [Table 1].

The molecular analysis of HSP genes in eukaryotes have also identified the heat shock element (HSE). This is a promoter element essential for heat shock inducibility in response to the previously mentioned various conditions of stress and comprises multiple adjacent inverted arrays of the binding site (5_-nGAAn-3_). HSEs are positioned at various distances upstream of transcription initiation; in vertebrates, inducible transcription requires the *de novo* binding of heat shock transcription factors (HSFs) transiently to the HSEs.[10,11] A family of HSF regulate the HSR at the transcriptional level.[12] Of the three human HSF genes, HSF 1, -2 and -4; HSF1 is the best characterized and essential for the HSR. Under normal conditions, HSF1 largely exists as a repressed monomer in the cytoplasm and is thought to be bound, directly or indirectly, by the protein chaperones Hsp90, Hsp70 and Hsp40.

It is a multi-domain stress-activated transcription factor consisting of an amino-terminal helix, winged-loop helix DNA binding domain, three leucine zipper domains (LZ1-3) that form coiled-coil interactions to facilitate HSF1 multimerization, a central regulatory domain that is extensively modified by phosphorylation, acetylation and sumolyation, an additional LZ4 domain and a carboxyl-terminal transcription activation domain.[13] Under varied conditions of stress the HSF1 derepresses, trimerizes and accumulates in the nucleus. HSF1 trimers subsequently bind with high affinity to the previously mentioned HSEs. These consist of inverted repeats of consensus sequence nGAAn. The binding occurs in varied orientations.[14] This then leads to an up-regulation in the expression of HSPs in the cell-HSR. The elevated levels of protective and adaptive response, also known as induced thermotolerance, ensures that the cell responds rapidly to repeated sub-acute challenges by diverse conditions of cell stress.[15] This lead us to propose that the induction of the HSR may have broad therapeutic benefits in the treatment of various types of tissue trauma and disease.

Regulation of heat shock transcription factors

Apart from induction by misfolded protein aggregates, altered intracellular redox status caused by changes in temperature or other stresses have been suggested to be involved in the activation of mammalian HSF1.[16] A role for stress-specific pathways in HSF1 activation has also been suggested.[17,18] The balance of kinase and phosphatase activities acting on HSF1 is of fundamental importance to the regulation of the HSR, as suggested by mathematical modeling.[19] HSF1 is negatively regulated by feedback control through interaction with Hsp70 and Hsp90.[12] In cells expressing high levels of these chaperones, the inducible expression of heat shock genes is affected. Lately, HSF1 as well as other HSFs have also shown to be able to interact or cooperate with signal transducers and activators of transcriptions (STATs) (STAT1 and STAT3) or nuclear factor interleukin-6 family members. It has been concluded that STAT1 can interact with p53 and that both of these factors are able to modulate the effects of HSF1 on HSP expression.[20]

Heat shock proteins as the "coordinating mediators of immunology"

The coordinated response by the innate immunity and the adaptive immunity is essential for efficient immune response. Taking antitumor immunity as an example, the first line of defense is mediated by natural killer cells which are part of the innate immunity.[21,22] These cause lysis of the tumor cells and the cross-presentation of antigens by dendritic cells (DCs) to prime adaptive T-cells. Activated T-cells in turn release cytokines or express cluster of differentiation (CD40) ligand on their cell surface to reciprocally activate DCs. It is suggested that HSPs may play important roles in both innate and adaptive immunity. DCs are activated by a range of microbial molecules, one such being lipopolysaccharide (LPS) which in turn trigger adaptive T- and B-cell immunity.[23]

Studies thus far suggest that HSPs could be such endogenous molecules that activate DCs in manner similar to these microbial antigens.[24] The initial clues came from a study on the immune responses to purified endoplasmic reticular (ER) HSP, gp96,[25] which concluded that the interaction of purified gp96-peptide complexes with antigen presenting cells (APCs), such as macrophages or DCs, leads to binding

Table 1: The HSP family and its varied functions

Family	Location	Function
Hsp100	Cytosol	Role in stress tolerance; helps the resolubilization of heat-inactivated proteins from insoluble aggregates
Hsp90	Cytosol, ER, mitochondria, nucleus	Role in signal transduction (e.g., interaction with steroid hormone receptors, tyrosine kinases, serine/threonine kinases); refolds and maintains proteins *in vitro*; autoregulation of the heat shock response; role in cell cycle and proliferation
Hsp70	Cytosol, nucleus, mitochondria, ER	Roles in lambda phage replication; autoregulation of the heat shock response; interaction with nascent chain polypeptides; functions in interorganellar transport; roles in signal transduction; refolds and maintains denatured proteins *in vitro*; role in cell cycle and proliferation; antiapoptotic activity; potential antigen-presenting molecule in tumor cells
Hsp60	Cytosol, mitochondria	Refolds and prevents aggregation of denatured proteins *in vitro*; may facilitate protein degradation by acting as a cofactor in proteolytic systems; role in the assembly of bacteriophages and Rubisco (an abundant protein in the chloroplast)
Hsp40	Cytosol/nucleus	Essential co-chaperone activity with Hsp70 proteins to enhance rate of adenosine triphosphatease activity and substrate release
Small Hsps	Cytosol	Suppresses aggregation and heat inactivation of proteins *in vitro*; confers thermotolerance through stabilization of microfilaments; antiapoptotic activity

Adapted from: Jolly and Morimoto. Role of the heat shock response and molecular chaperones in oncogenesis and cell death. J Natl Cancer Inst 2000;92:1564-72. HSPs: Heat shock proteins, ER: Endoplasmic reticular

of gp96-peptide complexes to common HSP receptor, CD91, on APCs,[26,27] followed by its internalization, processing of the gp96-chaperoned peptides and their re-presentation by major histocompatibility complex I (MHC I) and MHC II molecules. The MHC-peptide complexes act as "signal one" to stimulate the cognate $CD4^-$ and $CD8^-$ T-cells. Also, the interaction of gp96 with APCs causes activation and maturation of DCs, which secrets pro-inflammatory cytokines and provides costimulatory signals ("signal two") for effective T-cell priming. These findings have been proven correct using cells expressing gp96 on the cell surface.[28] It was found that cell surface expression of gp96 by tumor cells leads to DC maturation and cross priming of tumor-specific T-cells [Figure 2].

Liu has substantiated the role of gp96 (endoplasmin) as an important chaperone for inflammation and cancer. It has been well-established as a mediator in inducing toll-like receptor (TLR) signaling when challenged with pathogen-associated LPS molecules, thus leading to inflammatory responses. Its role in immunology can also be verified by the fact that gp96 is induced 10-folds on B-cell activation.[29]

The immunological features of HSPs have been summarized:

- HSPs chaperone interact with immunologically important molecules such as MHC I,[30] immunoglobulin's,[31] T-cell receptors and TLRs[32]
- HSPs chaperone bind cellular peptides
- Extracellular HSPs serve as cytokines to activate the innate functions of APCs, such as DCs, because of their binding to specific receptors on APCs
- HSPs can deliver their chaperoned peptides from non-APCs to MHC molecules of APCs and
- Depending upon different modes of tissue damage, the release of HSPs may play immunoregulatory roles in *in vivo*.

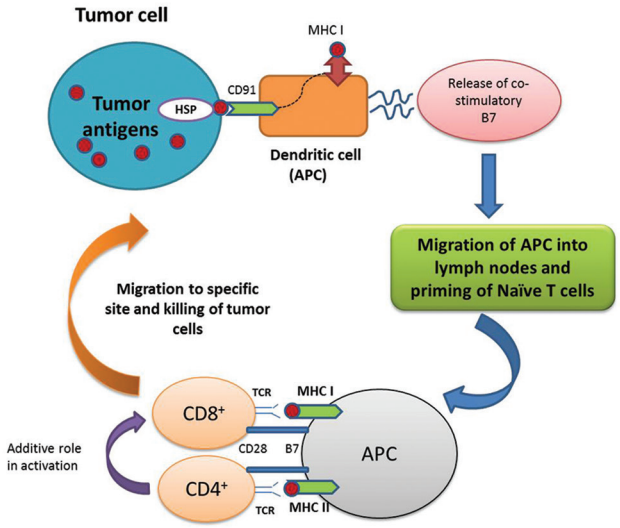

Figure 2: Role of heat shock proteins in cancer: Heat shock proteins (e.g., gp96) stimulating anticancer immunity. The interaction of gp96 with its specific receptors, such as CD91 on antigen presenting cells is followed by cross-presentation of antigens to major histocompatibility complex class I and increase in the release of co-stimulatory molecules such as B7. These dendritic cells subsequently migrate to the draining lymph nodes and prime antigen-specific naive T-cells. Those $CD8^+$ T-cells (helped by $CD4^+$ T-cells) exit from lymph into the tumor sites for lysis and clearance of tumors in an antigen specific manner

Thus, they were hailed by some as the immune system's"swiss army knives".[33]

Role of heat shock proteins in antigen processing and presentation

Formation of stable MHC complexes capable of presenting antigenic peptides to T-cells depends on their proper folding and assembly in the ER, as well as on the availability of peptide ligands. Folding and assembly of both MHC class I and class II molecules is initiated in the ER, whereas the site

of peptide loading depends on the intracellular compartment in which degraded protein fragments are sampled.[34] MHC class I molecules are loaded in the ER with ligands derived from endogenous proteins present in the cytosol (viral, tumor or self-antigens). Peptides from the cytoplasm are transported into the ER by a specialized transport system, termed the transporter associated with antigen processing (TAP). In contrast, MHC class II molecules bind ligands of extracellular origin in the endosomal compartment. To prevent premature loading of the MHC class II molecule in the ER, its binding site is blocked by the invariant chain, which is released in the endosome, so that loading of MHC class II molecules with endosomal peptides becomes possible.[35] Several lines of evidence suggest that HSP plays a role in MHC-antigen processing.[36-39] Folding and assembly of MHC-peptide complexes are promoted by molecular chaperones, which holds true for many other proteins. Members of the Hsp70 family are critically involved in the processing and presentation of antigens.[37,39-42] Binding immunoglobulin protein (BiP) and another endoplasmic chaperone, calnexin, promote the assembly of both MHC class I and class II molecules in the ER.[43,44] Furthermore, for BiP and other chaperones such as gp96 and Hsp70 (ERp72), an interaction with misfolded MHC class II molecules has been demonstrated, resulting in their retention in the ER.[45]

Srivastava *et al.* have provided substantial evidence that peptide transport from the proteasome to the ER and subsequent peptide loading of MHC class I molecules in the ER depend on a battery of HSP including cytosolic and endoplasmic members of the Hsp70 and Hsp90 families.[46,47] Recent studies have revealed that gp96 in the ER acts as a peptide acceptor, receiving peptides of cytosolic origin after their transport through the ER membrane by TAP molecules.[48] Subsequently, gp96-peptide complexes bind to MHC and the peptides are then translocated from gp96 to MHC class I molecules in an ATP-dependent manner.[47] Due to its proteolytic activity, gp96 may also participate in further trimming of MHC class I peptides in the ER.[49,50]

The immunological roles of HSPs have come to light primarily because of their involvement in antitumor immunity and the ensuing implications for antigen presentation and re-presentation. According to Li *et al*., HSP polypeptides interact with macrophages, DCs, T-cells and platelets through known and yet to be discovered receptors. HSP/APC interaction leads to the secretion of cytokines and chemokines and to the maturation and migration of DCs, possibly as a result of the translocation of nuclear factor-kappa beta into the nucleus. They are effective in antigen presentation via the MHC I and MHC II pathway.[30]

Recently, Javid *et al.* have shown similarities in the peptide binding between HSP70 and MHC I molecules and detailed on the role of Hsp70 and Hsp90 in antigen processing and presentation in an ATP-dependent manner.[51]

Autoimmune diseases: Heat shock proteins role in breaking immune tolerance: A hypothesis

The beauty of the immune system lies in its ability to mount effective immune responses against pathogens, while remaining nonresponsive to more abundant and normal self-antigens.

For T-lymphocytes, the vast majority of potentially self-reactive cells are eliminated during development in the thymus by what is called as negative selection.[52,53] When self-reactive T-cells do migrate into the periphery, multiple mechanisms play pivotal role to prevent these cells from inappropriate activation, including antigen sequestration, clonal exhaustion, anergy and antigen-specific suppression or regulation.[54] The prevalent hypothesis regarding antigen-driven peripheral tolerance is that antigen (signal 1) alone without the presence of costimulatory molecules (signal 2) leads to antigen-specific unresponsiveness or anergy.[55,56] The default pathway for immunological response to tumor thus might be tolerance attributable to lack of signal 2. This tolerance has been shown to be overcome by transfecting tumor cells with costimulatory molecules,[57,58] introducing proinflammatory cytokines/chemokines to the tumors,[59,60] administration of systemic cytokines or signal 2 agonist[61,62] and other means to activate/modulate the function of DCs.[63,64] Since HSPs are capable of activating DCs to up-regulate signal 2, in addition to delivering signal 1 through cross-presentation of HSP-chaperoned peptides, it is justified to hypothesize that HSPs can break peripheral tolerance against tumor associated antigens.

Under the condition of stress or "danger," HSPs are not only increased in expression level (for the purpose of cytoprotection and antigen presentation) but could also undergo dynamic redistribution to gain access to the extracellular environment. Their cell surface expression or secretion might possibly lead to sending an "ON" signal to activate the immune system and thus break down peripheral tolerance. Liu *et al*.[65] and previously Zügel and Kaufmann[66] have beautifully illustrated the cytokine role of these molecules.

Heat shock proteins in disease

Neurodegenerative disorders like Alzheimers, Huntington disease, spinocerebellar ataxias, Parkinson's disease, etc., have been linked to the aberrant expression of HSPs.[67] Various cell imaging experiments have shown an increase in the level of Hsp70 in relation to the huntingtin aggregates.[68] This suggests that these chaperone interactions may reflect the efforts of Hsp70 to direct the unfolding and dissociation of substrates from the aggregate and dampen its damaging effects.[69]

Relation to aging

The HSR has recently been implicated in the regulation of longevity in *Caenorhabditis elegans*. RNA interference experiments show that a decrease in sHSPs and other HSPs leads to a decrease in longevity.[70,71] Therefore, in addition to the prevention of diseases of aging, increased levels of HSPs may lead to increase in life span.

Heat shock proteins and cancer

Tumor cells typically express higher levels of HSPs compared with nontransformed cells, leading to the suggestion that the aberrant expression of chaperones is associated with the tumorigenic state.[72] An intriguing proposition is that tumor cells are dependent on elevated levels of HSPs, perhaps is a generalized mechanism to suppress cumulative mutations that would otherwise result in the expression of deleterious proteins. The chronic upregulation of HSPs could also promote cancer by the anti-apoptotic functions demonstrated by all chaperones. Hsp70, Hsp40, Hsp27 and Hsp90 act at multiple points in apoptosis, including inhibition of c-Jun NH2-terminal kinase activation, prevention of cytochrome C release, regulation of the apoptosome, prevention of lysosomal membrane permeabilization and prevention of caspase activation.[73] Therefore, compounds that downregulate the HSR and chaperone levels, when given in combination with chemotherapy, may prove beneficial for cancer treatment.

Therapeutically active small molecules that regulate HSF1 or modulate chaperone activities could benefit diseases that have in common alterations in protein conformation that cause an imbalance in protein homeostasis. The classes of small molecules that modulate the HSR are represented by a diverse set of chemically unrelated compounds consistent with the various environmental and physiological signaling pathways that induce the HSR [Table 2].[74-84]

Heat shock proteins as effective cancer vaccine

Tumor-derived HSPs have been shown to be effective cancer vaccines not only for prophylaxis against cancers but also for the treatment of existing cancers in many preclinical tumor models.[25,85] These have prompted systemic clinical testing of tumor-derived HSPs for the treatment of human malignancy.[86,87] The current effort has been focused mainly on gp96 and Hsp70. Unlike traditional cancer drugs, HSP-peptide vaccine is individually based and tailored toward an individual tumor of an individual patient.[86] This is based on two reasons: (a) HSPs chaperone is antigenic fingerprint of cells from which they are isolated and (b) tumor-protective antigens are individually distinct. As early as in the 1940s, it was appreciated that tumors were antigenically distinct from one another, most likely because of the subsequent realization of the differences in peptide pools among different tumors, as a result of random mutations of DNAs in the transformed cell. Depending on the tumor types, grade, differentiation stage or genetic background, peptide pools that are associated with HSPs should also be individually unique. Therefore, to customize the tumor vaccine for the patient, tumor antigens (HSP-peptide complexes) should be effectively derived from autologous tumors of this patient and not from those of someone else. More than 300 patients have been treated with HSP vaccines thus far.[86] The diseases include lymphoma, renal cell carcinoma, melanoma, colorectal cancer, gastric cancer, pancreatic cancer, breast cancer and others.

On monitoring clinically and immunologically, no significant toxicities including the generation of autoantibodies have been reported.

The ability of human melanoma-derived Hsp70 to stimulate autologous melanoma-specific T-cells for producing interferon-gamma were demonstrated using peripheral monocytes pulsed with Hsp70 as targets.[87,88] This makes us ponder further on the prospect of using this modality for next phase clinical trials in advanced melanoma.

Table 2: Activators and inhibitors of the HSR

Affecting molecule	Subgroups	Target	Study phase	Reference number
Activators	Protein synthesis inhibitor	HSPs: Mol wgt=110,000, 87,000, and 70,000	Preclinical: One dimensional geelectrophoresis studies	[74]
	Proteasome inhibitors	HSF-1, Hsp70	Preclinical: Phosphorylation studies	[75]
	Serine protease inhibitor	HSF1	Preclinical: Enzyme-linked immunosorbent assay	[76]
	Hsp90 inhibitors	Hsp90	Phase I, II, III clinical trial	[77]
	Inflammatory mediators	HSF1	Preclinical: Electro mobility shift analysis	[78,79]
Co-inducers	Sodium salicylate	68-kDa HSP	Preclinical: Two-dimensional gel electrophoresis	[80,81]
	Indomethacin	HSF1	Preclinical: Gel mobility shift analysis	[82]
Inhibitors	Flavonoids	HSF-HSE complex	Preclinical: Gel electrophoresis study	[83]
	Benzylidene lactam compounds	Hsp90	Preclinical: Immunoblot analysis	[84]

HSF: Heat shock transcription factor, HSPs: Heat shock proteins, SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, HSE: Heat shock element, HSR: Heat shock response

A detailed account of these findings have been reviewed by Jolly and Morimoto.[89]

Heat shock proteins 70 in oral lichen planus: Role in pathogenesis and proposal of a therapeutic model…

Oral lichen planus (OLP) has been the focus of study for its proposed potential malignant nature. Sugerman *et al*. drew comparisons in HSP staining among OLP, dysplastic OLP, normal oral mucosa and nonspecific oral ulceration.[90] The expression of HSP was prominently noticed in 94% of their cases. Hsp70 expression was found throughout the full thickness of the epithelium among 26 of the 30 samples of clinically and histologically confirmed cases of OLP. These were quantitatively and qualitatively analyzed by myself and colleagues in a previous study.[91] This finding was justified since these molecules are essential protein folding tools in the cellular machinery.[91-93] The normal, nondiseased mucosa of the oral cavity shows a faint expression of HSPs in the epithelium. This has been previously confirmed in literature by various studies conducted by Bramanti *et al*.[94] and Seoane *et al*.[95] The expression of HSPs was found to be de-regulated in OLP.

The basal cell zone in this particular premalignant condition is an apparent target for destruction by the sub epithelial T-lymphocytic population ($CD8^+$).[96,97] The raised index of the HSPs here re-asserts the effect of "stress of dying". HSPs probably represent the antigenic proteins that may potentially be involved in both the initiation and the persistence of the autoimmune lymphocytic response of lichen planus.[91,94,97]

In contrast, Chaiyarit *et al*. confirmed the expression of Hsp60 in the basal layer of OLP, but found no significant difference in the expression of Hsp70 between the OLP and oral fibroma groups.[98] Bramanti *et al*.[94] and Seoane *et al*.[95] reported that Hsp70 expression in OLP, when compared to the normal mucosa were slight and inconclusive.

There is literature pertaining to anticancer research that suggests the usage of inhibitors of the HSPs as a novel tool in cancer therapy. The benzoquinoid ansamycin antibiotics, first isolated from the actinomycete *Streptomyces hygroscopicus* var. geldanus var. *nova*, include geldanamycin and its semi-synthetic derivatives, 17-allylamino-17-demethoxygeldanamycin(17-AAG) and water-soluble 17- demethylam inoethylamino -17 -demethoxygeldanamycin. These inhibitors work by interacting specifically with a single molecule, Hsp90; cause destabilization and eventual degradation of multiple Hsp90 client proteins. The first-in-class Hsp90 inhibitor, 17-AAG is currently in phase II clinical trials. About 20 interventional studies are in clinical phase I and II trials, delivered either orally or via IV route. However, direct tumor monitoring either by biopsy or noninvasive methods is critical to optimal clinical efficacy.[99,100]

New alternatives and synthetic analogs based on 17-AAG (17-amino-17 demethoxygeldanamycin, in phase III clinical trial) backbone have been developed which have overcome *in vivo* inactivity, are safer and easier to produce.[101]

Lately, as there is evolving evidence that HSPs are present in the extracellular environment and identification of HSP and antibodies directed against it in normal individuals has shown that reactivity to these does not necessarily reflect adverse, pro-inflammatory responses and that the promotion of reactivity to self-HSPs can downregulate pathogenic processes, all suggesting a potential role for HSPs as therapeutic agents, rather than as therapeutic targets.[102,103] The potential therapeutic value of HSPs purified from appropriate tissues lies in their capacity to induce pro-inflammatory responses at low concentrations and induce regulatory immunity at high doses. The key lies in delivering the appropriate peptide.[104]

Recent studies

Over the past few years, the research in the field of HSR has intensified. Evaluation to establish the relationship between Hsp90 and Hsp70 was performed by Nakamoto *et al*.[105] A mutual supportive function, helping each other in protein refolding was found between the two chaperones.

Messmer *et al*. reestablished the *in vivo* immunogenic role of HSPs.[106] Dysregulation in the intracellular expression of these molecules has been linked to functional and pathological aggregate formations.[107]

Strong pathological correlations[108] and association between autoimmunity and new related autoimmune diseases has been resurfacing in more recent literature.[109,110] Extensive database has accumulated to justify the role of these molecules in carcinogenesis and tumor advancement.[111,112]

CONCLUSION

Surprising immunological features have been linked with HSPs. Their functioning in the cells has both a role in normal as well as pathological states. In the current context, understanding the implications of these wonderful molecules is an ardent task, a challenge to be taken up in full swing. Using this technology for immunotherapy should involve designing well-planned customized therapies. More clinical studies should be conducted to be sure how to use these for battling against diseases.

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Conflicts of interest

There are no conflicts of interest.

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